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5-HT$_{1F}$ Receptor Agonist Induces Mitochondrial Biogenesis and Promotes Recovery from Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) is characterized by vascular disruption leading to ischemia, decreased oxygen delivery and loss of mitochondrial homeostasis. This mitochondrial dysfunction results in loss of cellular functions, calcium overload and oxidative stress. Pharmacological induction of mitochondrial biogenesis (MB) may be an effective approach to treat SCI. LY344864, a 5-hydroxytryptamine 1F (5-HT_{1F}) receptor agonist, is a potent inducer of MB in multiple organ systems. To assess the efficacy of LY344864-induced MB on recovery post-SCI, female mice were subjected to moderate force-controlled impactor-induced contusion SCI followed by daily LY344864 administration for 21 days. Decreased mitochondrial DNA and protein content was present in the injury site 3d post-SCI. LY344864 treatment beginning 1hr after injury attenuated these decreases, indicating MB. Additionally, injured mice treated with LY344864 displayed decreased Evan's Blue dye accumulation in the spinal cord compared to vehicle-treated mice 7d after injury, suggesting restoration of vascular integrity. LY344864 also increased locomotor capability, with treated mice reaching a Basso-Mouse Scale (BMS) score of 3.4 by 21d, while vehicle-treated mice exhibited a score of 1.9. Importantly, knockout of the 5-HT_{1F} receptor blocked LY344864-induced recovery. Remarkably, a similar degree of locomotor restoration was observed when treatment initiation was delayed until 8hr after injury. Furthermore, cross-sectional analysis of the spinal cord 21d after injury revealed decreased lesion volume with delayed LY344864 treatment initiation, emphasizing the potential clinical applicability of this therapeutic approach. These data provide evidence that induction of MB via 5-HT_{1F} receptor agonism may be a promising strategy for the treatment of SCI.

Significance Statement

Treatment with LY344864 induces mitochondrial biogenesis in both the naive and injured mouse spinal cord. In addition, treatment with LY344864 beginning after impactor-induced contusion SCI improves mitochondrial homeostasis, BSCB integrity and locomotor function within 7 days. Importantly, similar locomotor results are observed whether treatment is initiated at 1 hour after injury or 8 hours after injury. These data indicate the potential for pharmacological induction of mitochondrial biogenesis through a 5-HT_{1F} agonist as a novel therapeutic approach for SCI.

Keywords: Spinal Cord Injury; Mitochondrial Biogenesis; Locomotor Recovery; 5-HT_{1F} receptor

Highlights (3-5)

- 5-HT_{1F} receptor agonism beginning 1hr post-SCI restored vascular integrity.
- 5-HT_{1F} receptor agonism beginning 1hr post-SCI induces mitochondrial biogenesis and improves locomotor recovery.
- Knockout of the 5-HT_{1F} receptor blocked agonist-induced recovery.
- 5-HT_{1F} receptor agonism is equally effective whether treatment is initiated 1 or 8hr after injury.

Introduction

Traumatic spinal cord injury (SCI) is a debilitating disorder with no meaningful pharmacological therapy. There are approximately 18,000 new cases of SCI documented each year in the United States alone. With an individual cost-of-care estimated at $3 million, SCI places a tremendous burden on patients, caregivers and the healthcare system (Devivo, 2012; Fitzharris et al., 2014). As such, continued research into the development of therapeutics for individuals suffering from SCI remains a necessity.

SCI is comprised of the primary injury, or immediate mechanical damage, followed by secondary injury, beginning within seconds and, depending on the severity of the trauma, potentially lasting years (Oyinbo, 2011). Primary injury results in extensive vascular damage, including vasoconstriction and ischemia (Hu et al., 2015a; Hu et al., 2016), leading to insufficient oxygen delivery and subsequent mitochondrial dysfunction (Hu et al., 2016; Rasbach et al., 2010). Given that neuronal cells are highly reliant on ATP-driven processes (Castro et al., 1997; Tian et al., 2016), failure to maintain adequate energy production exacerbates secondary injury, resulting in further cell death and dysfunction (Castro et al., 1997; Scholpa et al., 2017; Scholpa et al., 2018).

Therapeutics aimed at mitigating secondary injury have the potential to limit injury spread and promote the opportunity for recovery (Oyinbo, 2011). Mitochondrial dysfunction post-SCI is essential to the propagation of secondary injury, and evidence suggests that restoration of mitochondrial homeostasis shortly after injury may improve neuronal survival and promote functional recovery (Rabchevsky et al., 2011; Scholpa and Schnellmann, 2017; Sullivan et al., 2007). Multiple studies have targeted mitochondria as a therapeutic strategy following SCI, specifically focusing on consequences of mitochondrial dysfunction such as increased oxidative damage or altered mitochondrial dynamics (Hall, 2011; McEwen et al., 2011; Monaco et al., 2013; Patel et al., 2010; Teng et al., 2004). We propose that pharmacological induction of mitochondrial biogenesis (MB) is a more comprehensive approach to restore mitochondrial function and promote recovery post-SCI.

MB is a dynamic process of generating new, functional mitochondria that involves a complex network of transcriptional pathways governed by the “master regulator,” peroxisome proliferator-activated receptor y coactivator-1α (PGC-1α) (Rasbach et al., 2010; Scholpa and Schnellmann, 2017; Wills et al., 2012). PGC-1α expression is rapidly decreased in the spinal cord following

contusion injury *in vivo* (Hu et al., 2015b; Scholpa et al., 2018), suggesting impaired MB. Interestingly, augmentation of PGC-1α expression post-SCI has been shown to not only improve mitochondrial homeostasis, but also reduce lesion volume and improve functional recovery (Hu et al., 2016; Scholpa et al., 2018).

Neuronal 5-hydroxytryptamine (serotonin, 5-HT) receptors are involved in generating and regulating locomotor activity (Ghosh and Pearse, 2014). Following SCI, there is a disruption in descending serotonergic projections in spinal motor areas implicated in locomotor dysfunction (Ghosh and Pearse, 2014). Treatment with exogenous serotonin or a 5-HT analog has been shown to promote locomotor recovery following SCI (Ghosh and Pearse, 2014). A caveat of this approach, however, is the activation of many classes of 5-HT receptors (Ghosh and Pearse, 2014). Through our drug discovery program to ascertain inducers of MB (Beeson et al., 2010), we identified the 5-HT_{1F} receptor as a mediator of MB (Beeson et al., 2010; Rasbach et al., 2010). This receptor, while not fully characterized, is found in the spinal cord and various brain regions (Castro et al., 1997; Tian et al., 2016). In addition, high levels of the 5-HT_{1F} receptor were detected in human vasculature (Nilsson et al., 1999). While agonism of the 5-HT_{1F} receptor is known to decrease migraines (Mitsikostas and Tfelt-Hansen, 2012; Vila-Pueyo, 2018) the full extent of its role in the CNS, particularly following injury, has not yet been determined.

We previously showed that treatment with the specific 5-HT_{1F} agonist LY344864 increases MB in multiple organ systems, including the CNS (Gibbs et al., 2018b; Scholpa et al., 2017). Furthermore, treatment with LY344864 in a mouse model of Parkinson's disease increased MB, attenuated neuronal loss and improved behavioral endpoints (Scholpa et al., 2017). Given these data and the detrimental impact of mitochondrial dysfunction post-SCI, the goal of this study was to assess the therapeutic efficacy of LY344864-induced MB on recovery after SCI.

Methods

Animal handling and care

All mice were purchased from Jackson Laboratories and female mice (8-10 weeks of age) were used in all experiments. Male and female 5-HT_{1F} receptor knock-out (KO) mice (B6N(Cg)-*Htr1f*$^{tm1.1(KOMP)Vlcg}$/J, stock no. 024269) were used to generate an in-house breeding colony. For experiments using the KO strain, age-matched females of the suggested wild-type (WT) control line (C57bl/6NJ) were purchased. In all other experiments, female C57bl/6J mice were used. All animals were housed in groups of 3-5 in temperature-controlled conditions under a light/dark photocycle with unrestricted food and water supplied ad libitum.

LY344864 was obtained from Tocris Bioscience (Ellisville, MO) and dissolved in saline/0.5% DMSO. To determine the MB effect of LY344864 in the spinal cord under physiological conditions, naïve mice were treated with 2.0 mg/kg LY344864, i.p. once daily for 21 days followed by euthanasia via isoflurane overdose and subsequent isolation of the T9-13 region of the spinal cord.

In the SCI model, mice were randomized into sham and SCI groups. Animals were anesthetized with 10 mg/kg ketamine and 6 mg/kg xylazine via i.p injection and continuously monitored for spontaneous breathing. Mice underwent a complete single-level laminectomy at the 10^{th}-12^{th} thoracic vertebrae (T10-12). The vertebral column was clamped and stabilized at the upper thoracic and lumbar levels, and a controlled contusion with a force of 80 kilodynes was administered using the Infinite Horizon IH-0400 impactor (Lexington, KY) with the dura intact. Sham mice received laminectomy only. Manual bladder expression was performed twice daily until functional recovery. Injured mice were further randomized into LY344864- or vehicle-treated

groups and treated intraperitoneally with either LY344864 or vehicle control beginning 1hr or 8hr after injury and continuing daily until euthanasia. A dose of 2 mg/kg LY344864 utilized in our previous studies (Gibbs et al., 2018a; Scholpa et al., 2017) was continued in these SCI experiments. Groups were euthanized 3d, 7d or 21d post-SCI via isoflurane overdose and spinal cords were isolated for analysis. All studies were approved by the University of Arizona in accordance with the guidelines set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

BMS Assessment

Locomotor capability was assessed using the ten-point (0-9) Basso Mouse Scale (BMS) (Basso et al., 2006) by two trained observers blinded to experimental groups. Each mouse was observed for 3 min, with bladder expression taking place prior to assessment. Animals were observed 24hr after surgery and every other day thereafter until euthanasia. Sham animals maintained full locomotor capabilities with a BMS score of 9 throughout the experiment.

RNA Isolation and qPCR

The injury site of the spinal cord was collected and total RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's protocol. cDNA was synthesized using the iScript cDNA Synthesis Kit and qPCR performed using 500 ng of cDNA template and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Fold changes were calculated using the ΔΔCt method and normalized to the nuclear encoded gene β-actin.

DNA Isolation and qPCR

The injury site of the spinal cord was collected and total RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's protocol. The complementary DNA

(cDNA) was synthesized using the iScript cDNA Synthesis Kit and quantitative polymerase chain reaction (qPCR) performed using 500 ng of cDNA template and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Fold changes were calculated using the ΔΔCt method.

DNA was isolated from the injury and peri-injury sites using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) with 5 ng used for qPCR of relative mitochondrial DNA (mtDNA) content. ND1, a mitochondrial gene, was measured and normalized to the nuclear encoded gene β-actin.

To determine presence of the 5-HT_{1F} receptor gene, genomic DNA was amplified using Promega 2X PCR Master Mix (Promega, Madison, WI) in accordance with manufacturer's protocols. Amplified DNA was separated on a 2.5% agarose gel and visualized by ethidium bromide fluorescence.

Protein Isolation and Immunoblot Analysis

Protein was extracted from the injury site of the spinal cord using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4) with protease inhibitor cocktail (1:100), 1 mM sodium fluoride and 1 mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO). Samples were agitated for 2hr at 4º C and then centrifuged at 14,000 x g for 20 min and the supernatant collected. Protein was quantified using a bicinchoninic acid assay, and 10-12 µg of protein was separated via electrophoresis using 4-15% SDS-PAGE gels, then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% milk in TBST and incubated overnight with primary antibodies with constant agitation at 4ºC. Membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and visualized using chemiluminescence (Thermo Scientific, Waltham, MA) on a GE ImageQuant LAS4000 (GE Life Sciences, Pittsburg, PA). Optical density was determined using

ImageJ software. Primary antibodies used were as follows: Nrf2 ((1:500), Santa Cruz Biotechnology, Dallas, TX) PGC-1α (1:1000), TFAM (1:1000), NDUFS1 (1:1000), NDUFB8 (1:1000), ATP Syn β (1:1000), and α-tubulin ((1:1000), Abcam, Cambridge, UK).

Evan's Blue Extravasation

Integrity of the blood-spinal cord barrier (BSCB) was assessed using Evans Blue (EB) dye extravasation (Fang et al., 2015). Evans Blue dye (2%, 0.08 ml) was administered i.v. and allowed to circulate for 30 min. Mice were then transcardially perfused with saline and a 6mm segment of spinal cord centered at the epicenter was collected. EB dye was extracted in formamide at room temperature overnight and optical density values of the prepared samples were measured at 620 nm with a microplate reader. EB content was calculated as ng dye/mg tissue using a standard curve.

Lesion Volume Analysis

For histopathological analysis, spinal cord tissues were processed as described previously (Patel et al., 2017). Briefly, mice were transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde (PFA). A 1 cm segment of the spinal cord centered on the injury epicenter was removed and post-fixed in PFA for 2hr, then washed in 0.2 M phosphate buffer overnight at 4ºC. Tissues were then cryoprotected in 20% sucrose with 0.1% sodium azide at 4ºC until the spinal cords sank (≥3 days). Spinal cords were trimmed to 6 mm segments centered on the injury sight and frozen in OCT at -80ºC. The entire 6 mm was cryosectioned into 10 µm coronal sections and every section collected.

Eriochrome cyanine (EC) staining for myelin was used to distinguish damaged and spared tissue (Patel et al., 2017). Slides were warmed for 60 min at 37ºC, then hydrated in dH_2O, submerged in acetone for 2 min, and rehydrated in dH_2O. Slides were exposed to serial dilutions of decreasing

concentrations of ethanol and incubated in EC solution for 30 min. Selective myelin staining was obtained by differentiation in 0.3% ammonium hydroxide for 30 sec. Slides were then exposed to serial dilutions of increasing ethanol concentrations. Analyses were performed in a blinded fashion, with respect to treatment group, using an Olympus microscope (Tokyo, Japan) and ImageJ software. Lesion and spared tissue areas were quantified across 2 mm of spinal cord centered on the epicenter at 100 μm intervals using the Cavalieri method (Patel et al., 2017), totaling 21 sections per animal.

Statistical Analysis

Tissue isolated from a single animal or a single animal's behavior represented n=1. Behavioral assays were n=8 sham mice and n=10 injured mice per group and data were analyzed using Two-way ANOVA with repeated measures followed by Tukey's post-hoc test. Differences in mtDNA, individual protein expression and total EB content between all three groups (Sham, SCI + Vehicle, SCI + LY344864) was analyzed using One-way ANOVA. Two-way ANOVA was used to analyze tissue histopathology across the spinal cord. Total histopathological assessments were analyzed using two-tailed student's t-test. In all cases, GraphPad Prism software (La Jolla, CA) was used and a $p<0.05$ was considered indicative of a statistically significant difference between mean values.

Results

Effect of LY344864 on MB in the naïve spinal cord

Naïve mice were treated with 2.0 mg/kg LY344864 or vehicle i.p. daily for 21 days. Following treatment, the T9-12 portion of the spinal cord was collected and analyzed for mitochondrial endpoints. LY344864-treated mice displayed a 1.4-fold increase in mtDNA content (**Figure 1.A**)

and 1.3-fold increase in PGC-1α mRNA expression (**Figure 1.B**) in the spinal cord compared to vehicle-treated mice. It should be noted that 1.3-1.4-fold increases are physiologically relevant (Funk and Schnellmann, 2013; Garrett et al., 2014; Whitaker et al., 2016).

Effect of LY344864 on mtDNA and mitochondrial protein expression post-SCI

Mice were subjected to SCI followed by daily administration of LY344864 or vehicle i.p. beginning 1hr after injury. By 3d post-SCI, mtDNA content was reduced approximately 45% in the injury and peri-injury sites compared to sham controls (**Figure 2.A**). LY344864 administration attenuated this decrease in the injury site and increased mtDNA content to greater than that of sham controls in the peri-injury site.

Immunoblot analysis revealed decreased protein expression of PGC-1α, nuclear respiratory factor 2 (Nrf2), a transcription factor which controls mitochondrial gene regulation, and electron transport chain (ETC) subunits ATP synthase β (ATP Syn β) and NADH:ubiquinone oxidoreductase core subunit 8 (NDUFB8) in the injury site of SCI mice (**Figure 2.B**), further indicating mitochondrial dysfunction. LY344864 treatment not only attenuated these decreases, but fully restored Nrf2 and ATP Syn β to that of sham levels. Furthermore, LY344864 increased the expression of mitochondrial transcription factor A (TFAM) 1.5-fold compared to sham. These data provide evidence of LY344864 –induced MB in the injury site 3d post SCI

Effect of LY344864 on Evans Blue (EB) dye accumulation post-SCI

EB dye accumulation was increased in injured mice compared to sham controls beginning 1d after injury and persisting until 3d post-SCI, regardless of treatment. While the degree of EB extravasation was comparable among injured mice up to 3d, by day 7, LY344864-treated injured mice displayed decreased dye accumulation compared to vehicle-treated mice (**Figure 3.A**).

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Expression of the intracellular scaffolding protein zonula occluden protein-1 (ZO-1) and claudin-5, a tight junction protein, were decreased by more than 75% compared to sham at 3d post-SCI (**Figure 3.B**). While decreased expression persisted 7d after injury in vehicle-treated mice, ZO-1 and claudin-5 were comparable to sham levels in LY344864-treated injured mice (**Figure 3.C**).

Effect of LY344864 on locomotor recovery post-SCI in control and 5-HT$_{1F}$ knockout mice

The presence of the 5-HT$_{1F}$ receptor in the spinal cord of female wild-type (WT) mice, and absence in KO mice, was confirmed using PCR amplification of a 163 bp target sequence for the 5-HT$_{1F}$ receptor, with β-actin (69 bp) used as a reference gene (Supplemental Figure S.1). WT (**Figure 4.A**) and KO (**Figure 4.B)** mice were subjected to SCI followed by daily LY344864 treatment beginning 1hr after injury. Locomotor capability was assessed using the Basso-Mouse scale (BMS) beginning 24hr after SCI and continuing every alternate day (Basso et al., 2006). As expected, all injured mice displayed paralysis 24hr after injury and shams maintained normal function throughout the experiment.

Vehicle-treated mice of both genotypes displayed improved locomotor function 7d post-SCI and reached a maximum BMS score of approximately 2 (ankle movement) by 21d. Injured WT mice treated with LY344864 exhibited recovery by 5d and an increased BMS score compared to vehicle-treated mice by 7d post-SCI (1.4 v 0.5), reaching a score of 3.5 by 21d, indicative of dorsal and occasional plantar stepping (**Figure 4.A**). Conversely, LY344864 had no effect on locomotor recovery in the injured KO mice (**Figure 4.B**). In WT and KO mice, analysis revealed a significant effect of treatment/injury and days post-injury, as well as a significant interaction.

To determine the effect of delaying treatment, daily LY344864 administration was initiated 8hr after SCI and locomotor recovery assessed (**Figure 4.C**). Similar to that observed when treatment

began 1hr post-SCI, LY344864-treated mice displayed an increased BMS score compared to both day 1 and vehicle-treated mice by 5d after injury, reaching a BMS score of 3.7 by 21d post-SCI. Again, vehicle-treated mice exhibited recovery compared to day 1 by 7d and a maximum BMS score of approximately 2. Analysis again revealed a significant effect of treatment/injury and days post-injury, as well as a significant interaction.

Effect of LY344864 on histopathology of spinal cord post-SCI

Spinal cord histopathology was assessed following SCI and delayed treatment initiation 21d after injury using eriochrome cyanine staining for myelin (**Figure 5.A**) Analysis of 2 mm of spinal cord centered on the epicenter revealed decreased total lesion volume (**Figure 5.B**) and increased tissue sparing (**Figure 5.C**) in LY344864-treated mice compared to vehicle-treated mice. Cross-sectional analysis also indicated decreased lesion tissue in LY344864-treated mice (**Figure 5.D**), particularly at the epicenter. Main effects of treatment and spinal level were observed.

Discussion

Given that neuronal cells are highly reliant on ATP-driven processes (Castro et al., 1997; Tian et al., 2016), failure to generate and maintain adequate energy production exacerbates the pathology of secondary injury in SCI and results in further cell dysfunction and death (Castro et al., 1997; Scholpa and Schnellmann, 2017; Scholpa et al., 2018). Mitochondria in the CNS primarily serve to generate energy, but can also play a role in the homeostasis and degeneration of neurons (Dubinsky, 2005). Furthermore, pathological conditions can develop even in the presence of minimal mitochondrial dysfunction (Dubinsky, 2005). Evidence suggests that not only does mitochondrial dysfunction post-SCI contribute to the propagation of secondary injury, but that

restoration of mitochondrial homeostasis promptly following injury may improve neuronal survival and promote functional recovery (Rabchevsky et al., 2011; Scholpa and Schnellmann, 2017; Sullivan et al., 2007). By generating new, functional mitochondrial via pharmacological induction of MB, we sought to mitigate the detrimental impact of mitochondrial dysfunction and assess recovery after SCI.

We previously reported that LY344864-induced agonism of the 5-HT_{1F} receptor in renal proximal tubule cells lead to the activation of the Gβγ-Akt-eNOs-sGC-PGC-1α pathway, ultimately resulting in induction of MB (Gibbs et al., 2018b). Additionally, treatment with LY344864 in a mouse model of Parkinson's disease increased MB, attenuated neuronal loss and improved behavioral endpoints (Scholpa et al., 2017). While we have shown that the 5-HT_{1F} receptor is a potent inducer of MB peripherally and centrally (Garrett et al., 2014; Rasbach et al., 2010; Scholpa et al., 2017; Whitaker et al., 2016), this receptor remains understudied in the spinal cord. Here, we show that 5-HT_{1F} receptor agonism induces MB in the naïve and injured spinal cord. Following SCI, we observed a rapid decrease in the expression of mitochondrial proteins at the injury site, corresponding with previous reports of mitochondrial dysfunction after injury (Dubinsky, 2005; Scholpa et al., 2018). A dose of 2 mg/kg LY344864 utilized in our previous induced MB in several organ systems (Gibbs et al., 2018a; Scholpa et al., 2017). As such, this dose was continued in these SCI experiments. Interestingly, LY344864 treatment attenuated these decreases as early as 3d post-SCI, notably that of PGC-1α, indicating induction of MB and improved mitochondrial homeostasis.

Vascular disruption is a well-established consequence of SCI (Cohen et al., 2009; Patel et al., 2009). The BSCB exists at the capillary level and controls the flux of molecules entering the spinal cord. Studies revealed that restoring BSCB integrity prevents necrosis and apoptosis of neuronal

cells after SCI, consequently improving functional endpoints. (Wang et al., 2018). Additional studies report maximal BSCB disruption at 24 h post-SCI (Figley et al., 2014). Similarly, we observed permeability increases of the BSCB at 24 h, persisting for 7 days after injury. LY344864-treated mice displayed reduced dye accumulation at 7d post-SCI, suggesting accelerated recovery of BSCB integrity. Additionally, increased BSCB integrity has been correlated with improved locomotor recovery (Cohen et al., 2009). Functions of the BSCB rely on an intricate network of tight junction proteins including ZO-1 and claudin-5 (Kumar et al., 2017). Following SCI, we report decreased expression of both ZO-1 and claudin-5, consistent with previous reports (Kumar et al., 2018; Zhang et al., 2016; Zhou et al., 2017). Interestingly, LY344864 treatment attenuated these decreases by 7d post-SCI. This recovery of tight junction protein expression corroborates our observed decrease in permeability of the BSCB in LY344864-treated animals at 7d post-SCI. Though we suspect LY344864 treatment induces MB within the endothelial cells composing the BSCB, further work is required to elucidate the pathway by which MB induction facilitates restoration of the BSCB after SCI.

Treatment with LY344864 1h post-SCI in WT animals resulted in improved locomotor capability by 7d post-SCI, with mice reaching a BMS score of 3.4 by day 21, corresponding to consistent dorsal to occasional plantar stepping. Vehicle-treated mice, regardless of genotype, depicted a score of only 2 (ankle movement) by day 21. The similar degree of recovery observed in both the vehicle-treated 5-HT_{1F} KO and WT mice suggests that the lack of the 5-HT_{1F} receptor does not affect injury progression or inhibit basal improvement after SCI. Nonetheless, LY344864 had no locomotor effects in the KO mice, indicating that the improved functional recovery observed in WT mice is dependent on the 5-HT_{1F} receptor. In addition, the loss of LY34464 efficacy in the

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KO mice provides evidence that LY344864 specifically activates its receptor to produce the observed biological effects.

Remarkably, delaying treatment initiation until 8h after injury also resulted in improved functional recovery, indicating a potential therapeutic window for 5-HT_{1F} receptor agonism of at least 8h post-SCI. It has been reported that activation of the 5-HT_{1F} receptor inhibits postsynaptic potentials that trigger spasms after SCI (Murray et al., 2011), and that mitigating serotonergic disruption after SCI can promote locomotor recovery (Ghosh and Pearse, 2014). Therefore, in addition to the MB-related effects, LY344864 treatment after SCI could also attenuate serotonergic disruption or decrease muscle spasms, further enhancing the improved functional behavior observed. In addition to functional improvement, injured mice subjected to the 8h delay of LY344864 treatment initiation demonstrated decreased lesion volume, particularly at the injury epicenter, which has also been correlated with improved locomotor capability (Patel et al., 2010).

Endogenous serotonin is known to play a stimulatory role in locomotor activity (Slawinska et al., 2014). As such, we cannot preclude the possibility that the locomotor effects observed with LY344864 treatment are in part due to actions on serotonergic systems. The 5-HT_{1F} receptor has yet to be fully characterized. However, given that uninjured KO animals retained full locomotor capabilities and injured KO animals displayed comparable locomotor activity to that of WT vehicle mice, it is unlikely that the 5-HT_{1F} receptor plays a pivotal role in basal locomotor function in this model. In addition, previous research demonstrates that LY344864-induced MB promotes functional recovery in organ systems not directly reliant on the serotonergic system (e.g. renal function) (Gibbs et al., 2018a; Gibbs et al., 2018b).

Though we know treatment with 2 mg/kg LY344864 induces MB in both male and female mice across several different tissues (Gibbs et al., 2018a; Scholpa et al., 2017), we have not investigated

the effects of LY344864 in male mice following SCI. Future studies will explore potential sex-related differences in response to 5-HT_{1F} receptor agonism following SCI."

This is the first report indicating that 5-HT_{1F} receptor activation stimulates MB in SCI and promotes tissue, locomotor activity, and vascular integrity recovery. Importantly, the 5-HT_{1F} receptor agonist lasmiditan has recently been approved by the FDA for the treatment of migraines and could be repurposed, for the treatment of SCI. Finally, the equal efficacy of LY344864 when administered 1 and 8h after SCI suggests this approach is clinically relevant for the treatment of SCI.

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Authorship Contributions

Participated in research design: Simmons, Scholpa and Schnellmann

Conducted experiments: Simmons, Scholpa and Cleveland

Performed data analysis: Simmons, Scholpa and Cleveland

Wrote or contributed to the writing of the manuscript: Simmons, Scholpa and Schnellmann

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Footnotes[1]

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Figure Legends

Figure 1. Effect of LY344864 on MB in the naïve spinal cord. Naïve mice were exposed to LY344864 (2 mg/kg i.p) or vehicle daily for 21 d. The spinal cord was extracted and analyzed for mtDNA content (A) and PGC-1α mRNA expression (B). Data represent 4 mice per group and are expressed as mean ± SEM (*$p<0.05$ by Student's t-test*).

Figure 2. Effect of LY344864 on mtDNA and protein expression 3 d post-SCI. Mice were subjected to moderate SCI using an 80 Kdyn force-controlled impactor induced contusion model followed by daily administration of vehicle or LY344864 (2 mg/kg, i.p) beginning 1 h post-injury and continuing for 3 d. The injury and peri-injury sties were extracted and analyzed for mtDNA content (A). The injury site was also analyzed for mitochondrial protein expression (B). Data represent 5 mice per group and are expressed as mean ± SEM *(*$p<0.05$ compared to Sham and #$p<0.05$ compared to SCI + Vehicle by One-way ANOVA followed by Tukey's post-hoc test*).

Figure 3. Effect of LY344864 on spinal cord Evans Blue (EB) dye accumulation. Mice were subjected to moderate SCI using an 80 Kdyn force-controlled impactor induced contusion model followed by daily administration of vehicle or LY344864 (2 mg/kg, i.p) beginning 1 h post-injury. Subsets of mice were euthanized 1 d, 3 d and 7 d post-SCI. Prior to euthanasia, mice were intraocularly injected with EB and the dye was allowed to circulate for 2 h. 1 cm of spinal cord surrounding the injury epicenter was extracted and analyzed for dye accumulation. A subset of mice were transcardially perfused and fixed with 4% PFA and the spinal cords extracted for representative images (A). The injury site was also collected from a subset of mice at 3 d (B) and 7 d (C) post-SCI and analyzed for protein expression. Data represent 5 mice per group and are expressed as mean ± SEM *(*$p<0.05$ compared to Sham and #$p<0.05$ compared to SCI + Vehicle by One-way ANOVA followed by Tukey's post-hoc test*).

Figure 4. Effect of LY344864 on recovery post-SCI in C57BL/6J WT and 5-HT_{1F} KO mice. Mice were subjected to moderate SCI using an 80 Kdyn force-controlled impactor induced contusion model followed by daily administration of vehicle or LY344864 (2 mg/kg, i.p) beginning 1 h post-SCI (A and B). Locomotor function was assessed using the Basso-Mouse scale beginning 24 h after injury and continuing every alternate day. Mice were also subjected to moderate SCI using an 80 Kdyn force-controlled impactor induced contusion model followed by daily administration of vehicle or LY344864 (2 mg/kg, i.p) beginning 8 h post-injury (C). Data are representative of 8 mice per sham group and 10 mice per SCI group and are expressed as mean ± SEM. Significance between sham and injured animals is not represented on graph for simplicity (**p<0.05 compared to SCI + Vehicle and #p<0.05 compared to Day 1 by Two-way ANOVA with repeated measures followed by Tukey's post-hoc test*).

Figure 5. Effect of LY344864 on spinal cord histopathology post-SCI. Mice were subjected to moderate SCI using an 80 Kdyn force-controlled impactor induced contusion model followed by daily administration of vehicle or LY344864 (2 mg/kg, i.p) beginning 8 h post-injury and continuing for 21 d. Spinal cords were extracted and evenly spaced tissue sections stained with Eriochrome cyanine (A) and analyzed for lesion volume (B) and spared tissue (C) across 2 mm of spinal cord centered on the epicenter. Cross-sectional analysis of lesion (D) across 2 mm of spinal cord centered on the epicenter. Data are representative of 5 mice per group and are expressed as mean ± SEM (**p<0.05 compared to SCI + Vehicle by Student's t-test*).

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Tables 1

Target	Sense	Antisense
PGC-1α	5′-AGGAAATCCGAGCGGAGCTGA-3′	5′-GCAAGAAGGCGACACATCGAA-3′
ND1	5′- TAG AAC GCA AAA TCT TAG GG -3′	5′- TGC TAG TGT GAG TGA TAG GG -3′
β-Actin	5′-GGGATGTTTGCTCCAACCAA-3′	5′-GCGCTTTTGACTCAGGATTTAA-3′
5-HT$_{1F}$	5′-GCCGTGATGATGAGTGTGTC-3′	5′-ACTATCCGACTCGCTTGTCT-′3

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Figure 1

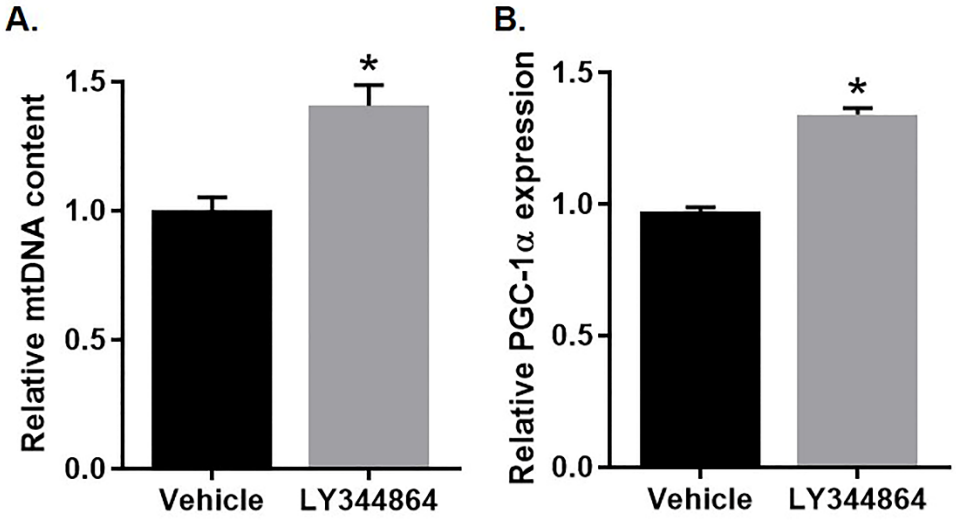

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Figure 2

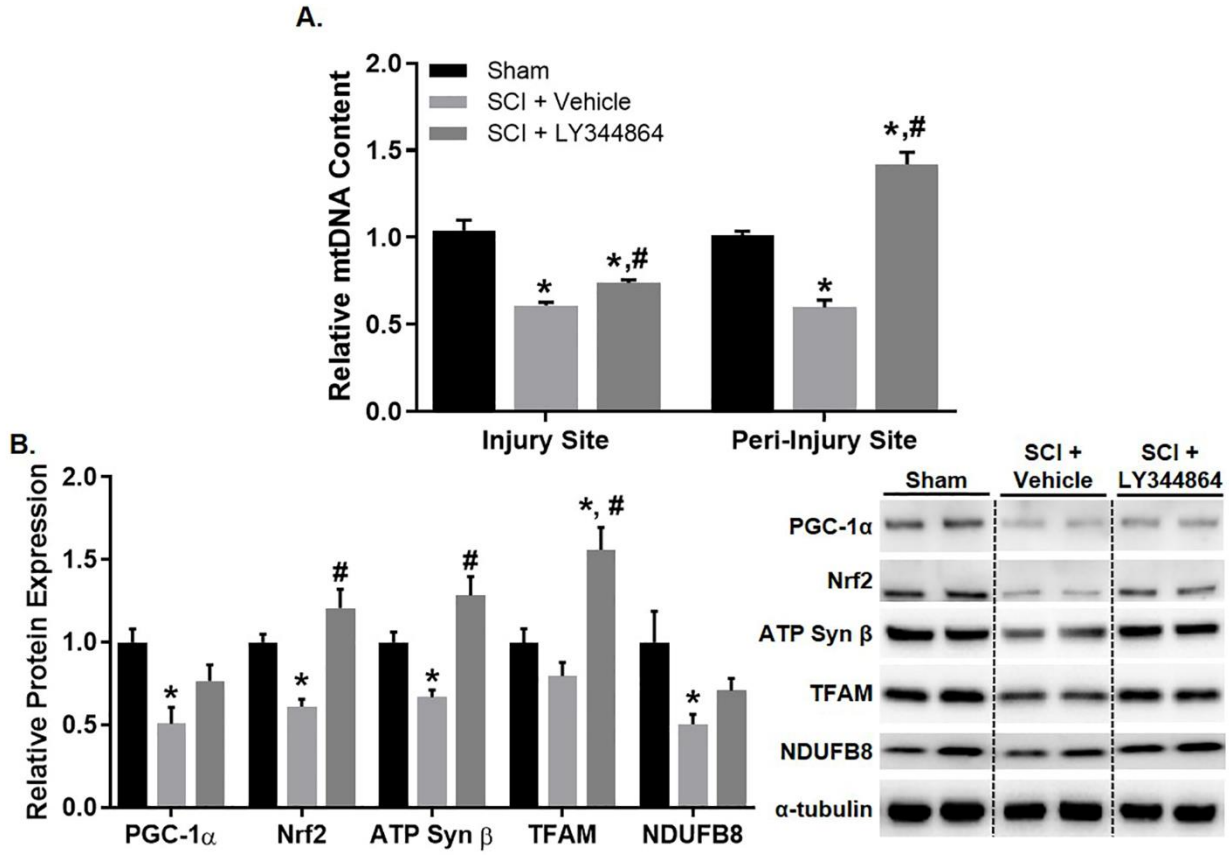

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Figure 3

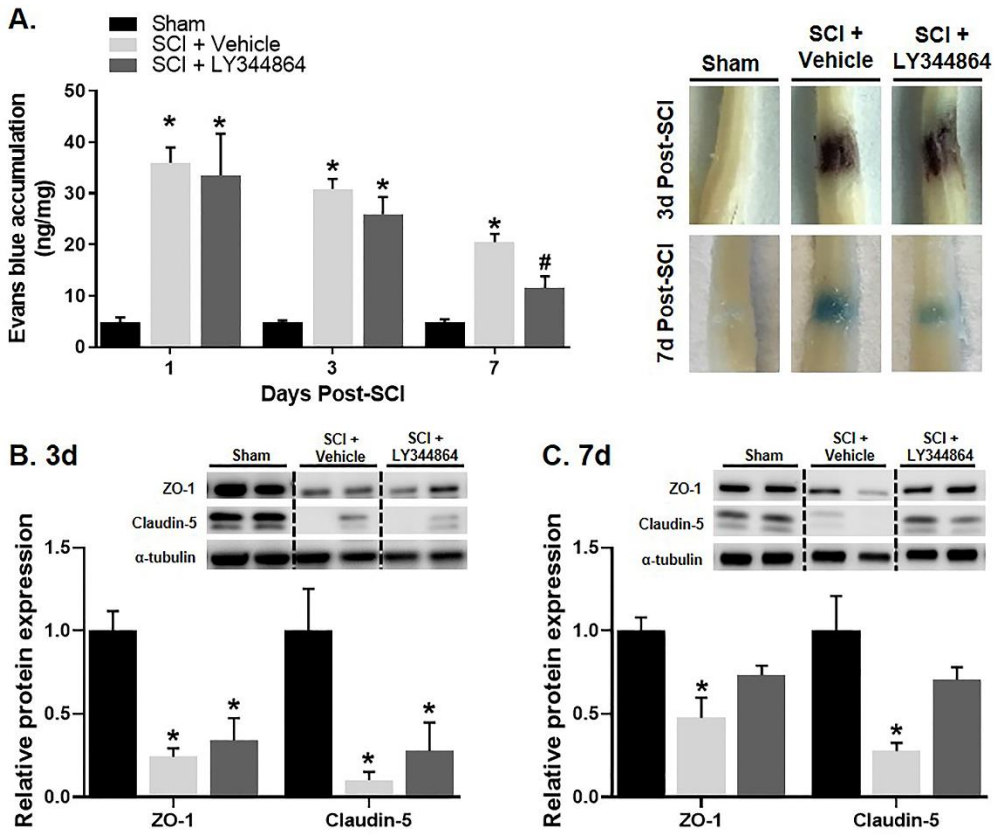

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Figure 4

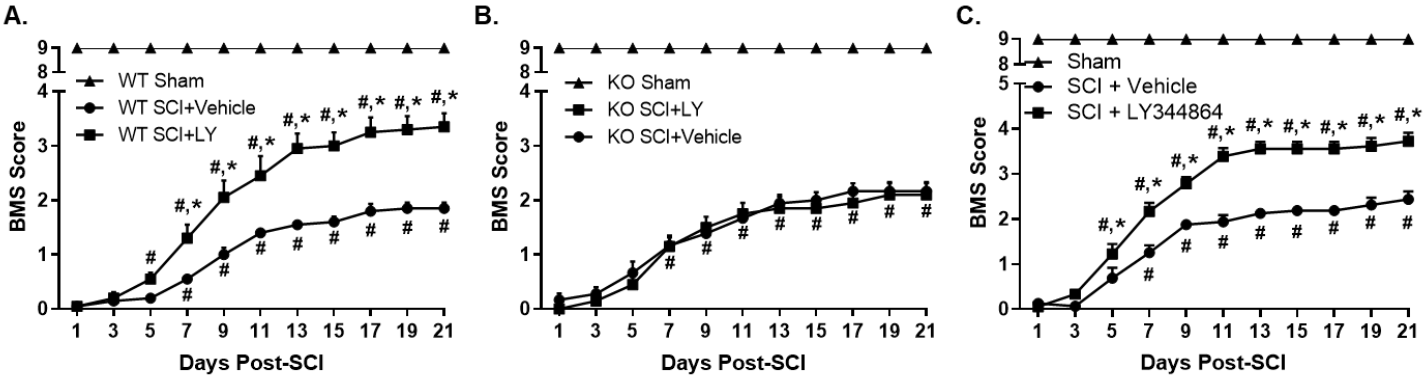

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Figure 5