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Restoring endoplasmic reticulum homeostasis improves functional recovery after spinal cord injury

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Abstract

The endoplasmic reticulum (ER) stress response (ERSR) is activated to maintain protein homeostasis or induce apoptosis in the ER in response to distinct cellular insults including hypoxia, inflammation, and oxidative damage. Recently, we showed ERSR activation in a mouse model of a contusive spinal cord injury (SCI) and an improved hindlimb locomotor function following SCI when the pro-apoptotic arm of ERSR was genetically inhibited. The objective of the current study was to explore if the pharmacological enhancement of the homeostatic arm of the ERSR pathway can improve the functional outcome after SCI. Salubrinal enhances the homeostatic arm of the ERSR by increasing phosphorylation of eIF2 α . Salubrinal significantly enhanced the levels of phosphorylated eIF2 α protein and modulated the downstream ERSR effectors assessed at the lesion epicenter 6 hours post-SCI. Hindlimb locomotion showed significant improvement in animals treated with salubrinal. Treadmill-based-gait assessment showed a significant increase in maximum speed of coordinated walking and a decrease in rear stance time and stride length in salubrinal-treated animals. This improved functional recovery corresponded with increased white matter sparing and decreased oligodendrocyte apoptosis. In addition, salubrinal protected cultured mouse oligodendrocyte progenitor cells against the ER stress-inducing toxin tunicamycin. These data suggest that boosting the homeostatic arm of the ERSR reduces oligodendrocyte loss after traumatic SCI and supports the contention that pharmacological targeting of the ERSR after CNS trauma is a therapeutically viable approach.

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Keywords

apoptosis; ER stress; hindlimb locomotor recovery; oligodendrocytes; salubrinal; spinal cord injury

Introduction

Despite numerous research endeavors and technological advances, effective clinical therapy after spinal cord injury (SCI) still remains obscure. This unavailability of treatment is due to the complex pathophysiology that ensues after SCI and includes inflammation, hypoxia, excitotoxicity, disruption of blood brain barrier, ischemia and demyelination (Rowland et al., 2008). Therefore, studies are required to explore additional molecular mechanisms for identification of cellular targets for therapeutic intervention. Recent reports demonstrated activation of the endoplasmic reticulum (ER) stress response (ERSR) after a moderate contusive SCI in rats (Penas et al., 2007) and mice (Ohri et al., 2011) and improved functional recovery in hindlimb locomotion of mice deficient in the ERSR pro-apoptotic effector, C/EBP (CCAAT enhancer binding protein) homologous protein (CHOP) (Saraswat Ohri et al., 2011).

The ERSR, mediated through three distinct pathways, is an evolutionary conserved cellular mechanism that is activated in response to insults that disrupt cellular homeostasis (Schroder, 2008; Tabas and Ron, 2011). The protein RNA (PKR)-like kinase (PERK) phosphorylates the α -subunit of elongation initiation factor 2 α (eIF2 α) and inhibits global protein synthesis. Activated inositol-requiring protein-1 α (IRE-1 α) splices X-box-binding protein 1 (XBP1) mRNA (Yoshida et al., 2001) which then activates ERSR specific transcription. The third pathway of the ERSR comprises the proteolytic processing of activating transcription factor-6 (ATF6) (Haze et al., 1999). The main objective of the ERSR is restoration of ER function and, in consequence, cytoprotection. However, the ERSR may also activate apoptosis if ER function cannot be timely restored (Tabas and Ron, 2011). CHOP is one of the major effectors of the apoptotic arm of the ERSR (Ron and Habener, 1992). Involvement of ERSR-activated cell death has been reported in ischemic stroke (Lange et al., 2008), multiple sclerosis (Lin et al., 2006), and Alzheimer's disease (Milhavet et al., 2002).

Our previous study showing improved functional recovery post-SCI with genetic ablation of CHOP indicated a major role of ERSR-mediated apoptosis in the pathogenesis of SCI. That functional recovery correlated with an increase in white matter sparing and transcript levels of myelin basic protein (MBP) and claudin 11 (Saraswat Ohri et al., 2011) suggesting oligodendrocyte protection. However, the role of the homeostatic component of ERSR in SCI pathogenesis has not been addressed. In addition, a possibility to improve the outcome of SCI by pharmacological modulation of the ERSR has also yet to be evaluated.

Salubrinal is a small molecule inhibitor of the protein phosphatase 1 (PP1) complex that reverses PERK-mediated phosphorylation of eIF2 α . As a result, salubrinal enhances the PERK-eIF2 α signaling during ERSR while protecting a wide range of cells against ER stress-induced cell death (Boyce et al., 2005). Salubrinal reduced kainic acid-induced ER stress and neuronal death *in vivo* and *in vitro* (Sokka et al., 2007) and ameliorated hypomyelination and oligodendrocyte loss in cultured hippocampal slices exposed to IFN- γ (Lin et al., 2008). Here, we demonstrate that salubrinal attenuates ER stress-induced apoptosis of oligodendrocyte precursor cells (OPCs) *in vitro* and oligodendrocytes *in vivo* and improves functional recovery of hindlimb locomotion in a mouse model of traumatic SCI.

Materials and Methods

Animals

Procedures were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, 1996) and the University of Louisville Institutional Animal Care and Use Committee. C57Bl/6 female mice (6–8 weeks) were obtained from Harlan (Indianapolis, IN). Growth arrest and DNA-damage protein 34 knockout (GADD34^{-/-}) mice on a 100% C57Bl/6 background were procured from MMRRC (Cat. No – 30266, Chapel Hill, NC) and bred in house.

Isolation of mouse OPCs from cortex

Mouse cortices were dissected from whole brains of wild type and GADD34^{-/-} postnatal day 5–7 pups (Dincman et al., 2012). Briefly, tissue was dissociated using the Neural Tissue Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. OPC-A media was prepared by adding 2.1 g/L NaHCO₃ (Sigma-Aldrich, St. Louis, MO) to DMEM-F12 without HEPES powder (Invitrogen, Carlsbad, CA) and supplemented with N2 supplement (1%), B27 supplement (2%), Penicillin/Streptomycin (1%, all from Invitrogen), BSA (0.01%, Sigma), 40 ng/ml FGF2 (Millipore, Billerica, MA), and 20 ng/ml PDGF α (Sigma). Average yield was $8-10 \times 10^6$ cells/brain with a viability of 85–95%. OPCs were enriched using magnetic cell sorting (MACS) with rat anti-mouse IgM magnetic beads (10% in MACS Buffer). Between 9,000–15,000 cells/cm² cells were plated on a PDL/laminin-coated 10 cm tissue culture dish, and incubated at 37°C, 5% CO₂.

Spinal cord injury and injections

Mice were anesthetized by an intraperitoneal injection of 400 mg/kg body weight Avertin (2,2,2-tribromoethanol in 0.02 ml of 1.25% 2-methyl-2-butanol in saline, Sigma). A laminectomy was done at the T9 vertebrae and moderate contusion injuries (50 kdyn force/400–800 μ m displacement) were performed using the IH impactor (Scheff et al., 2003; Infinite Horizons Inc., Lexington, KY) as described previously (Benton et al., 2008; Han et al., 2010; Saraswat Ohri et al., 2011). Experimental controls included sham animals with the T9 laminectomy only. Salubrinol (1 or 5 mg/kg; Sigma) or vehicle was administered through the jugular vein immediately after surgery and then through tail vein (1x/day) for three days. Animals were given 1 ml of sterile saline and 0.1 ml of gentamycin subcutaneously post-surgery and 3rd and 5th day post-surgery, and 0.1 ml of bupronorphine subcutaneously on the day of surgery and for the next 2 days.

RNA Extraction, Reverse Transcriptase PCR

Total RNA was extracted from treated OPCs and spinal cord tissue of sham, vehicle- and salubrinol-contused wild type (n=4/group) mice from the injury epicenter (4 mm) using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA was quantified by UV spectroscopy and RNA integrity confirmed on an ethidium bromide stained formaldehyde agarose gel. cDNA was synthesized with 500 ng of total RNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA) in a 20 μ l reaction volume. As controls, mixtures containing all components except the reverse transcriptase enzyme were prepared and treated similarly. All cDNAs and control reactions were diluted 10x with water before using as a template for quantitative real time (qRT)-PCR.

Quantitative PCR Analysis

qRT-PCR was performed using an ABI 7900HT Real-time PCR instrument as previously described (Saraswat Ohri et al., 2011). Assay on Demand™ primers (Applied Biosystems) were used: ATF4 (Mm00515324_m1), CHOP (Mm01135937_g1), claudin 11 (Mm00500915_m1), neuron-specific enolase (NSE; Mm00468052_m1), GADD34 (Mm00492555_m1), GFAP (Mm00546086_m1), GRP78 (Mm01333323_g1), glutamine synthetase (Mm00725701_S1), microtubule associated protein 2 (Mtap2; Mm00485230_m1), myelin basic protein (MBP; Mm00521980_m1), olig2 (Mm01210556_m1) and XBP1 (Mm00457359_m1).

Western Blot Analyses

Protein lysates were prepared from treated OPCs and 4 mm of spinal cord tissue isolated from vehicle- and salubrinal- treated injury epicenter of contused mice at 6 hour post-injury in protein lysis solution (20 mM Tris, pH-6.8, 137 mM NaCl, 25 mM B-glycerophosphate, 2 mM NaPPi, 2 mM EDTA, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, protease inhibitor, 0.5 mM DTT, 1 mM PMSF), quantified using the BCA Kit (Pierce, Rockland, IL) and processed using standard procedures with the antibodies: ATF4/CREB-2 (Santa Cruz Biotechnologies, Santa Cruz, CA: 1/500), GADD34 (Santa Cruz Biotechnologies: 1/500), GRP78 (Cell Signaling Technologies, Danvers, MA: 1/1000), eIF2α (Biosource, Camarillo, CA: 1/1000), phospho-eIF2α (Cell Signalling Technologies: 1/1000), and GAPDH (Chemicon, Temicon, CA: 1/5000).

Behavioral Assesment

Open field BMS locomoter analyses were performed prior to injury for each animal to determine the baseline scores and weekly following SCI for 7 weeks (Basso et al., 2006). All raters were trained by Dr. Basso and colleagues at the Ohio State University and were blinded to animal groups.

Gait Analysis

The gait of vehicle- and salubrinal-treated animals was analyzed using the Treadscan® Gait Analysis system (Cleversys, Reston, VA) at week 6 post-SCI (Beare et al., 2009) by core facility staff that were blinded to animal groups. Animals were placed on a variable speed treadmill with a clear belt that allows high-resolution digital video (ventral view) of the stepping animals to be captured. Each animal was placed on the treadmill and the speed of the belt increased until the animal could no longer maintain position, and then decreased slowly until stable walking was achieved for a minimum of 5 step cycles (the 'best speed' for each animal). A minimum of 10 step cycles at best speed were analyzed for each animal using the Treadscan® software. Here, we report the forelimb-hindlimb coordination as homologous coupling and the Regularity index, a measure of foot placement order which was calculated by taking the foot fall order from the Treadscan® data output (Hamers et al., 2006; Kuerzi et al., 2010). The coordination of each step cycle (LeBlonde et al., 2003) was calculated and its absolute difference from 0.5 (normal) was determined and averaged across step cycles.

White Matter Sparing

Seven weeks after SCI, vehicle- and salubrinal- treated mice were perfused with 4% paraformaldehyde and spinal cords at the injury epicenter $\pm$ 2 mm were dissected out and stored in 4% paraformaldehyde at 4°C overnight. The cords were cryoprotected in 30% sucrose for at least 3 days at 4°C, blocked and sectioned transversely at 20 μ m and stored at -80°C. Eriochrome cyanine (EC) was used to stain myelin to detect the extent of spared

white matter at the injury (Saraswat Ohri et al., 2011). The percent spared white matter was determined with reference to sham control animals (Magnuson et al., 2005).

Determination of oligodendrocyte apoptosis in vivo

Forty eight hours after moderate contusion injury, vehicle- and salubrinal- treated mice were transcardially perfused with PBS, spinal cords dissected, fresh frozen, blocked and longitudinally sectioned at 20 μ m on a cryostat. Every fifth section was immunostained with cleaved caspase-3 (CC3) and Olig 2 antibody and adjacent sections with GFAP (Saraswat Ohri et al., 2011). Images were acquired with a 10x objective on a Nikon TE300 inverted microscope and stitched with Nikon Elements software during acquisition. To define the regions of interest, the images of the spinal cord sections were overlaid with their adjacent images of GFAP-labeled sections. The injury heterodomain was defined as the area of complete absence of GFAP from the gray matter (Benton et al., 2008). The injury penumbral zone was then defined by expanding the heterodomain region of interest by five hundred microns uniformly both horizontally and vertically (Fassbender et al., 2010). Ratio counts (Coggeshall and Lekan, 1996) were done by a blinded scorer by quantifying co-localized Olig2⁺/CC3⁺ and total Olig2⁺ cells using Elements software. The extent of oligodendrocyte apoptosis was expressed as the percentage of Olig2⁺/CC3⁺ divided by the total number of Olig2⁺ cells.

Tunicamycin treatment, MTT assay

OPCs derived from wild type or GADD34^{-/-} mice were seeded in 96-well plate and treated with salubrinal (0.05–50 μ M) in the presence of tunicamycin (0.025 μ g/ml) for 24 or 72 hrs. OPC survival was assayed by measuring the conversion of tetrazolium, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) to formazan at a wavelength of 570 nm.

Statistical analyses

For functional assessments after injury, a repeated measures analyses of variance (ANOVA) with fixed effects and Bonferroni post-hoc t-test was performed to detect differences in BMS score and subscores between the sham and injury groups over the 7 week testing period. Statistical analysis of qRT-PCR data was performed using independent t-test for means with equal or unequal variances or repeated measures ANOVA (one- way or two-way analysis of variance) followed by post-hoc Tukey HSD test. For all other analyses, independent t-tests for means assuming equal variance were performed.

Results

Salubrinal increases eIF2 α phosphorylation in vivo and modulates the key ERSR markers after SCI

To determine the effects of salubrinal on the PERK-eIF2 α pathway in injured mouse spinal cord, animals were treated with intravenous injections of 1 or 5 mg/kg of the drug immediately after contusive SCI. After 6 hours, increased levels of phosphorylated eIF2 α were observed at the injury epicenter in animals receiving 5 but not 1 mg/kg salubrinal (Fig. 1A, C and data not shown). Levels of total eIF2 α remained similar in both groups (Fig. 1A, C). In contrast, 5 mg/kg salubrinal reduced levels of several ERSR effectors whose expression is induced by ER stress including ATF4, CHOP, GADD34, GRP78 and XBP1 (Fig. 1B, D–E). Importantly, our earlier study did not (Saraswat Ohri et al., 2011) show any detectable pEIF2 α or ATF4 in the sham samples. Collectively, these data indicate the specific activation of the ER stress response following SCI and that salubrinal penetrated the spinal cord tissue *in vivo* and enhanced the PERK-eIF2 α signaling while attenuating levels

of several other components of the ERSR after SCI. In addition, a significant increase in neuron- (NSE and Map 2a, b) and oligodendrocyte-specific (Olig2, Claudin 11 and MBP) transcript levels (Fig. 2A, B) was observed at 72 hours post-injury. In contrast, astrocyte-specific transcripts (GFAP and glutamine synthetase) were unaffected by salubrinal treatment (Fig. 2C). These data indicate that salubrinal exerts a cell-specific response post-SCI.

Administration of salubrinal leads to significant improvement in hindlimb locomotion

Salubrinal-treated animals showed significant improvement in locomotion starting at week 1 and continued through week 7 post-SCI (latest time point examined). The average BMS score for salubrinal-treated animals was 4.6 ($p < 0.05$) at week 1 which increased to 6.9 ($p < 0.001$) at week 7 (Fig. 3A). In contrast, vehicle-treated animals exhibited an average BMS score of 3.8 at week 1 and remained at 5.0 through week 7 (Fig. 3A). All the salubrinal-treated animals had a BMS score of 6.5 and above, indicative of frequent or consistent plantar stepping, which was mostly coordinated with paws parallel at initial contact and rotated at lift off (Basso et al., 2006). In contrast, vehicle-treated animals' BMS score of 4–5 indicates either occasional plantar stepping with no or some coordination and paws rotated at initial contact and lift off. The improvement in functional recovery of salubrinal-treated animals was further analyzed by BMS subscore. The average subscore for vehicle- and salubrinal-treated animals was 2.3 and 4.9 at week 2 ($p < 0.05$) and 3.1 and 6.5 at week 7 ($p < 0.05$), respectively (Fig. 3B). Results showing identical improvement in functional recovery were obtained in an independent replicate of the study ($p < 0.01$; data not shown). These findings are consistent with previous results showing improved recovery in mice with genetic ablation of CHOP after SCI (Saraswat Ohri et al., 2011).

Salubrinal leads to significant improvement in gait parameters

Salubrinal-treated animals showed significant improvement in terminal run speed compared to vehicle-treated animals (11.5 cm/sec vs. 7.9 cm/sec, $p < 0.001$; Fig. 4A). Normal animals have a basal speed over ground that averages 25 cm (unpublished data). Importantly, the rear stance times (Fig. 4B) and stride lengths (Fig. 4C) were decreased in salubrinal-treated animals accounting for their increased speeds. Analysis of the regularity index (RI) as a measure of coordination based on regular step patterns, demonstrated a significant higher RI in salubrinal-treated animals compared to vehicle-treated animals (65% vs. 35%, $p < 0.001$; Fig. 4D). These data suggested that the salubrinal-treated group had fewer missteps interspersed between regular step patterns as defined previously (Hammers et al., 2001, 2006). Furthermore, measurement of the homologous coupling or interlimb coordination (Fig. 4E) showed salubrinal-treated animals to be closer to the normal value of 0.5 (Leblond et al., 2003). Altogether, data from BMS and gait analyses indicate that *in vivo* treatment with salubrinal in mice leads to significantly enhanced functional recovery post-SCI. The results suggest that the treated animals had enhanced balance, coordination and plantar paw placement during stepping that allowed them to maintain a higher stride frequency.

Salubrinal-treated mice show increased white matter preservation post-SCI

To understand possible mechanisms responsible for improved functional recovery in salubrinal-treated animals, the spinal cords were isolated after 7 weeks of behavioral assessment and analyzed for spared white matter. Salubrinal-treated mice showed significantly more spared white matter (29.23%) compared to vehicle-treated mice (21.35%; Fig. 5A–C). qRT-PCR using RNA isolated from injured spinal cords 7 weeks after SCI revealed that salubrinal treatment increased the levels of oligodendrocyte-specific mRNAs including Olig 2 (~15%), MBP (~15%) and Claudin 11 (25%) (Fig. 5D). These data suggest that reduced white matter loss contributed to functional improvement in salubrinal-treated animals.

To determine whether white matter protection was due to reduced death of oligodendrocytes, oligodendrocyte apoptosis was assessed in spinal cords from vehicle- and salubrinal-treated mice 48 hours post-SCI. In salubrinal-treated animals there was a reduction in Olig2-positive cells that co-stained for activated caspase-3. Thus, 28.6% or 21.17% of oligodendrocytes were apoptotic at the injury epicenter of vehicle- or salubrinal-treated mice, respectively ($p < 0.05$). In the penumbra region, salubrinal reduced oligodendrocyte apoptosis from 32.9% to 22.4% ($p < 0.01$) (Fig. 6A, B). In addition, oligodendrocyte protection was evident when their loss was monitored by measuring oligodendrocyte-specific mRNAs. At 72 hour post-SCI, the 60% loss of Olig2 mRNA in vehicle-treated animals was reduced to 50% in the salubrinal group ($p < 0.05$) (Fig. 2A). Similar effects were also observed for claudin 11 and MBP mRNAs (Fig. 2A). Collectively, these data suggest that oligodendrocyte sparing is a major mechanism(s) by which salubrinal improves functional recovery after SCI.

Salubrinal treatment results in enhanced viability of mOPCs in the presence of tunicamycin

To investigate whether salubrinal-dependent protection of oligodendrocytes is cell-autonomous, we tested whether salubrinal protected against experimental ER stress in primary mouse OPCs. Treatment of OPCs with salubrinal suppressed cytotoxicity induced by the ER stress inducing protein glycosylation inhibitor tunicamycin in a dose-dependent manner at 72 hours (Fig. 7A, B). Importantly, salubrinal was nontoxic at concentrations required for maximal cytoprotection. These data are consistent with an earlier study showing salubrinal-mediated protection of oligodendrocytes in the presence of IFN- γ (Lin et al., 2008). To determine the underlying mechanism of salubrinal's protective action on mouse OPCs, we examined its effects on the phosphorylation status of eIF2 α and induction of several effectors of the ERSR. Similar to its effects in whole animals undergoing SCI, salubrinal increased levels of phosphorylated eIF2 α (Fig. 8A) and attenuated ER stress-related inductions of GADD34, GRP78, CHOP, ATF4 and XBP1 mRNAs in tunicamycin-treated mouse OPCs (Fig. 8C–D). It has been shown that inhibition of eIF2 α dephosphorylation by salubrinal is mediated by both the constitutive (CReP) and the stress-induced phosphatases (GADD34; Boyce et al., 2005). Thus, salubrinal should only partially attenuate the cytotoxicity induced by tunicamycin in cells lacking GADD34. Indeed, salubrinal led to only a 10% increased survival of mOPCs derived from GADD34 $^{-/-}$ mice (Fig. 8E) compared to 20–25% survival of wild type-derived mOPCs at 24 hours treatment with tunicamycin. Interestingly, stronger phosphorylation of eIF2 α was observed in mOPCs derived from GADD34 $^{-/-}$ mice. Salubrinal further significantly increased the phosphorylated eIF2 α levels and delayed the translational recovery after ER stress induction in GADD34 $^{-/-}$ mOPCs (Fig. 8A, B). These results demonstrating that salubrinal imparted protection to mOPCs by reducing the burden of ER stress are consistent with its well accepted mechanism of action as an enhancer of homeostatic ERSR signaling between PERK and eIF2 α (Boyce et al., 2005).

Discussion

Oligodendrocyte loss is a major acute pathophysiological consequence after human SCI (Emery et al., 1998; Guest et al., 2005) and in rodent models (Almad et al., 2011). Early pharmacological interventions that could abrogate such loss could have important functional consequences.

Recent studies established the critical role of ER stress in the pathogenesis of SCI (Penas et al., 2007; Saraswat Ohri et al., 2011). Genetically modulating the pro-apoptotic arm of the ERSR, by using mice deficient in CHOP, significantly enhanced hindlimb locomotor recovery and correlated with greater preservation of white matter, thus implicating its direct

functional role after SCI. The current investigation tested the hypothesis as to whether pharmacologically targeting the homeostatic component of the ERSR would be a valid therapeutic approach for functional repair after SCI.

Small molecule compounds that restore ER homeostasis may have a therapeutic potential for the treatment of ER stress related pathologies (Wiseman and Balch, 2005). Salubrinal is an inhibitor of protein Ser/Thr phosphatase (PP1) complex which enhances PERK-mediated phosphorylation of eIF2 α to protect cells against ER stress-induced apoptosis (Boyce et al., 2005). Here, intravenous salubrinal allowed the contused mice to recover from “some coordination” (BMS = 5) to “mostly coordinated, weight bearing and plantar stepping” (BMS score = 7). Our data showing an 8% increase in white matter preservation with a concomitant two-point change in BMS score are consistent with the original description of the BMS (Basso et al., 2006).

Contusive injury in the thoracic spinal cord is predominantly a white matter injury as excitotoxic destruction of neurons in this region does not substantially affect hindlimb locomotor function (Magnuson et al, 1999). When contusive injuries involve the lumbar or cervical enlargement, gray matter loss substantively affects function (Magnuson et al, 2005). The variable that best correlates with hindlimb function after contusive thoracic SCI is spared white matter at the exact lesion epicenter. We contend that the predominant functional effect of salubrinal in this lesion was on oligodendrocyte survival. However, salubrinal also spared thoracic neurons and this may have contributed to the observed functional improvement, especially in the context of trunk stability.

An important aspect of recovery after SCI is forelimb-hindlimb coordination which requires the lumbar and cervical enlargements to communicate with one another across the injury site. Using Treadscan® for a more quantitative assessment of forelimb/hindlimb coordination than that provided by the BMS, we found that salubrinal induced significant improvements in several aspects of gait after SCI, including run speed, stance time, stride length and regularity index, the latter a direct indication of improved forelimb-hindlimb coordination (Hamers et al., 2006; Leblond et al., 2003). That salubrinal was able to partially rescue the forelimb-hindlimb coordination after injury strongly supports the notion that ERSR can be targeted pharmacologically to improve communication across the site of a mid-thoracic contusive SCI.

Present data showed oligodendrocyte sparing as the major mechanism by which salubrinal administration leads to improvement in functional recovery after SCI and is consistent with previous data (Saraswat Ohri et al., 2011). However, induction of ERSR after traumatic SCI occurs in all the major types of CNS resident cells in a temporal manner (Penas et al., 2007; Saraswat Ohri et al., 2011). The sensitivity of oligodendrocytes to ER stress-mediated apoptosis is likely due to their highly developed ER serving the need to produce vast amounts of myelin. SCI-associated dysregulation of intracellular Ca²⁺ homeostasis is one of the triggers of oligodendrocyte ER stress (McTigue et al., 2008). Consistent with this, salubrinal protected cultured oligodendrocytes from excitotoxic death while attenuating ER stress in this system (Ruiz et al., 2010).

The beneficial effects of salubrinal on the outcome of SCI are likely due to restoration of ER homeostasis. Indeed, as in several other models, salubrinal cytoprotection was associated with enhanced PERK-eIF2 α signaling and reduced expression of the ER stress response genes (Sokka et al., 2007; Lin et al., 2008; Saxena et al., 2009). However, other potential mechanisms of action cannot be ruled out. For instance, a recent study showed salubrinal-mediated-inhibition of the NF- κ B pathway as a novel protective mechanism against A β neurotoxicity (Huang et al., 2012). Of note, salubrinal treatment attenuated the pro-apoptotic

arm of the ERSR as indicated by reduced induction of ATF4 and CHOP following SCI or tunicamycin treatment of cultured mOPCs. Similar effects of salubrinal were reported with HK-2 human renal proximal tubular cells *in vitro* (Komoike et al., 2011). Hence, salubrinal appears to reestablish the pro-survival balance between the homeostatic and pro-apoptotic ERSR branches.

Small molecular weight compounds acting on intracellular pathways have emerged as promising cytoprotective agents with the capacity to inhibit cell death and restore function. Although, salubrinal is not approved by the U.S. Food and Drug Administration (FDA) for use in clinical settings, this study strongly supports the contention that ERSR is a valid therapeutic target. Interestingly, guanabenz, a FDA approved α_2 -adrenergic receptor agonist for the treatment of hypertension (Holmes et al., 1983) has recently been shown to enhance the PERK-eIF2 α signaling by similar mode of action as salubrinal (Tsaytler et al., 2011). Preliminary data indicate increased resistance to tunicamycin-induced cell death of mouse and rat OPCs that were treated with guanabenz (data not shown). Thus, pharmacological modulation of ERSR by targeting the PERK-eIF2 α signaling appears as a novel therapeutic opportunity to improve the outcome after SCI and in array of ER stress-related pathologies including neurodegeneration and demyelinating disorders.

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Highlights

- Salubrinal treatment restores ER homeostasis post-SCI.
- Salubrinal leads to improved recovery in hindlimb locomotion after SCI.
- Salubrinal administration results in increased white matter preservation post-SCI.
- Salubrinal treatment leads to decreased oligodendrocyte apoptosis after SCI.
- Targeting of ERSR after CNS trauma is a therapeutically viable approach.

Figure 1.

Salubrinal treatment results in increased phosphorylation of eIF2 α and modulates the key ERSR markers *in vivo* at 6 hours post-injury. A) Western blots show that salubrinal increases phosphorylated eIF2 α levels at the injury epicenter of contused spinal cords. B) Western blots show a decrease in the levels of ATF4, GADD34 and GRP78 at the injury epicenter in salubrinal-treated animals as compared to vehicle-treated group. C, D) Quantification of the Western blots in (A, B) reveal significant differences in protein levels of ERSR markers as indicated. E) Salubrinal leads to significant decreases in transcript levels of various ERSR markers analyzed by qRT-PCR. Transcript levels are expressed as fold changes compared with respective levels in sham controls. Data are the mean $\pm$ SD [n=3 (A, B); n=4 (E), *p<0.05, **p<0.01).

Figure 2.

Salubrinal exerts a cell-specific response at 72 hours post-SCI. Salubrinal treatment results in a significant increase in Olig2, Claudin 11, MBP (A), NSE and Map 2a, b (B) mRNA levels acutely at 72 hours post-injury. There is no difference in GFAP and glutamine synthetase (C) transcript levels. mRNA levels (normalized to Gapdh) are expressed as fold changes compared with levels in sham controls. Data are the mean $\pm$ SD (n=4, *p<0.05, **p<.01).

Figure 3. Administration of salubrinal results in improvement in hindlimb locomotor function. A) Open field BMS locomotor analyses performed weekly for all experimental animals following SCI revealed a significant functional recovery in mice treated with salubrinal (n=8; solid lines) as compared to vehicle-treated animals (n=8; dotted line). B) Analysis of BMS subscore between vehicle- and salubrinal-treated mice show significant group differences at days 14, 35, 42, and 49 post-SCI. Data (A, B) are the mean $\pm$ SD (* p <0.05, ** p <0.01, *** p <0.001; Mixed model ANOVA, post hoc t-test: Bonferroni). There are no subscore data at 3 and 7 day post-SCI as all BMS scores were below 5. Identical significant data were obtained in an independent replicate of this experiment (p <0.01).

Figure 4.

Gait analysis using Treadscan® of vehicle (white bars)- and salubrinal (black bars)-treated mice show significant improvement in maximum speed of coordinated walking (A) and decrease in rear stance times (B) and stride length (C) in the latter group. The mean RI or stepping index was significantly enhanced in salubrinal-treated mice (D). Homologous coupling data indicated salubrinal-treated mice to be less deviated from the normal mice (E). The normal values of homologous coupling of mice are 0.05 (Leblond et al., 2003). RRRL is the ratio of the rear right step cycle when rear left makes initial contact. RLRR is the ratio of the rear left step cycle when rear right makes initial contact. Data are the mean $\pm$ SD (n=8, *p<0.05, ***p<0.001).

Figure 5.

Salubrinal enhances white matter sparing after SCI. Representative histological sections from the injury epicenter stained with EC to identify myelin in (A) vehicle- and (B) salubrinal-treated mice. C) Quantitative analysis of eriochrome cyanine-stained sections from the injury epicenters showed significant increase in spared white matter in salubrinal-treated (black bars, $29.23 \pm 1.99\%$) compared to vehicle-treated mice (white bars, $21.35 \pm 2.01\%$) mice. Data are the mean $\pm$ SD ($n=8$; $*p<0.05$). D) qRT-PCR analysis at 6 weeks post-SCI reveal a significant increase in Olig2, Claudin 11, and MBP mRNA levels at the injury epicenter of salubrinal-treated mice.

Figure 6.

Salubrinal decreases oligodendrocyte apoptosis after SCI. A) Representative images of vehicle- and salubrinal-treated animals (n=4/group) imaged under identical conditions. Nine 20 μ m longitudinal sections (every fifth section through each cord) were immunostained and counted for co-localized Olig2 and cleaved caspase 3 (CC3; yellow) and Olig2 (green) cells at the injury epicenter (E) and penumbral (P) region. Adjacent GFAP stained sections were used to define the injury epicenter. Higher magnification images of the boxes in the upper panels show the co-localized cells (lower panels). Arrowheads identify Olig2⁺ cells and arrows co-localized Olig2⁺ and CC3⁺ cells. B) Quantification of Olig2⁺/CC3⁺ cells showed a significant 7.45% (*p<0.05) and 10.36% (**p<0.01) decrease in apoptotic cells at the injury epicenter and penumbra region, respectively, in salubrinal-treated animals.

Figure 7. Salubrinal enhances mOPCs viability in the presence of tunicamycin (Tm). A) Quantification using a MTT assay show increased dose-dependent survival of mOPCs treated with salubrinal after 72 hour treatment with Tm (0.025 μ g/ml). B) Representative phase contrast images of mOPCs treated with vehicle (left), Tm (middle) and Tm + salubrinal (right) at 72 hour *in vitro*. Magnification bar = 10 μ m.

Figure 8.

A) Western blot analysis show that salubrinal increases level of phosphorylated eIF2α in wild-type mouse OPCs exposed to Tm (upper panel). In *Gadd34*^{-/-} derived-mOPCs, stronger phosphorylation of eIF2α is observed. Salubrinal treatment further increases this phosphorylation and attenuates translational recovery in *GADD34*^{-/-} derived-mOPCs. B) Quantification of the Western blot in (A) reveal significant differences in levels of phosphorylated eIF2α as indicated. C, D) Salubrinal leads to a reduction of *GADD34*, *GRP78*, *CHOP*, *ATF4* and *XBP1* mRNA levels post-24 hour treatment with Tm in wild-type mOPCs. E) Viability of *GADD34*^{-/-}-derived mOPCs exposed to Tm (0.025 μg/ml) for

24 hours with the indicated concentrations of salubrinal, assessed by MTT assay. Data are the mean $\pm$ SD (n=4; *p<0.05, **p<0.01).