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Salubrinal reduces oxidative stress, neuroinflammation and impulsive-like behavior in a rodent model of traumatic brain injury

Aric F. Logsdon^{a,b,d,1}, Brandon P. Lucke-Wold^{b,d,1}, Linda Nguyen^a, Rae R. Matsumoto^c, Ryan C. Turner^{b,d}, Charles L. Rosen^{b,d}, and Jason D. Huber^{a,b,d,*}

^aDepartment of Pharmaceutical Sciences, School of Pharmacy, Health Sciences Center, West Virginia University, One Medical Center Drive, Morgantown, WV, United States

^bDepartment of Neurosurgery, School of Medicine, West Virginia University, Morgantown, WV, United States

^cDean's Office, College of Pharmacy, Touro University California, Vallejo, CA, United States

^dCenters for Neuroscience, School of Medicine, West Virginia University, Morgantown, WV, United States

Abstract

Traumatic brain injury (TBI) is the leading cause of trauma related morbidity in the developed world. TBI has been shown to trigger secondary injury cascades including endoplasmic reticulum (ER) stress, oxidative stress, and neuroinflammation. The link between secondary injury cascades and behavioral outcome following TBI is poorly understood warranting further investigation. Using our validated rodent blast TBI model, we examined the interaction of secondary injury cascades following single injury and how these interactions may contribute to impulsive-like behavior after a clinically relevant repetitive TBI paradigm. We targeted these secondary pathways acutely following single injury with the cellular stress modulator, salubrinal (SAL). We examined the neuroprotective effects of SAL administration on significantly reducing ER stress: janus-N-terminal kinase (JNK) phosphorylation and C/EBP homology protein (CHOP), oxidative stress: superoxide and carbonyls, and neuroinflammation: nuclear factor kappa beta (NF κ B) activity, inducible nitric oxide synthase (iNOS) protein expression, and pro-inflammatory cytokines at 24 h post-TBI. We then used the more clinically relevant repeat injury paradigm and observed elevated

*Corresponding author at: Department of Pharmaceutical Sciences, School of Pharmacy, Health Sciences Center, West Virginia University, One Medical Center Drive, Morgantown, WV 26505, United States.

¹Denotes equal contribution.

Conflict of interest

The authors have no conflicts of interest to disclose.

Authors' contributions

AFL conceived the study, designed the experiments, performed blast/injections, dissected out brain regions, executed biochemical analyses, analyzed the data, and wrote the manuscript. BPL conceived the study, designed the experiments, performed blast/injections, helped execute biochemical analyses, analyzed the data, and wrote the manuscript. LN performed the behavioral experiments and helped write the manuscript. RRM provided invaluable biochemical and behavioral knowledge, in addition to helping conceive the study. RCT assisted with blast exposure and helped write the manuscript. CLR provided invaluable clinical knowledge, in addition to helping conceive the study, design experiments, and manuscript preparation. JDH provided invaluable scientific knowledge, in addition to helping conceive the study, design experiments, and manuscript preparation.

NF κ B and iNOS activity. These injury cascades were associated with impulsive-like behavior measured on the elevated plus maze. SAL administration attenuated secondary iNOS activity at 72 h following repetitive TBI, and most importantly prevented impulsive-like behavior. Overall, these results suggest a link between secondary injury cascades and impulsive-like behavior that can be modulated by SAL administration.

Keywords

Traumatic brain injury; Oxidative stress; Endoplasmic reticulum stress; Neuroinflammation; Impulsive-like behavior

1. Introduction

3.2 million Americans are currently living with disabilities from traumatic brain injury (TBI) (Zaloshnja et al., 2008). Impulsivity is one of the most common and potentially dangerous symptoms associated with brain injury (Schwarzbold et al., 2010; Adhikari et al., 2011; Logsdon et al., 2014; Michael et al., 2015). Impulsivity is a key finding in patients diagnosed with chronic traumatic encephalopathy (CTE) and presents early in disease progression (Banks et al., 2014; Rebetz et al., 2015). Currently, no therapies for impulsivity are available for patients diagnosed with CTE. The underlying mechanisms linking neurotrauma to subacute neuropsychiatric symptoms are still poorly understood (Lucke-Wold et al., 2014a).

The concept of an interrelationship between cellular stress and lasting degenerative changes remains to be elucidated. Endoplasmic reticulum (ER) stress and oxidative stress have emerged as contributors to neurodegeneration and behavioral dysfunction. ER stress has been shown to play a significant role in acute and chronic disease pathology following TBI (Zhang et al., 2012; Abdul-Muneer et al., 2014; Begum et al., 2014; Lucke-Wold et al., 2015a). We recently showed that markers of ER stress were increased in the brains of athletes diagnosed with CTE, and rodents exposed to repetitive blast injury (Lucke-Wold et al., 2016).

Janus-N-terminal kinase (JNK) is a common downstream component of ER stress (Urano et al., 2000), which is activated following TBI (Otani et al., 2002; Szmydynger-Chodobska et al., 2010). JNK activity can influence nuclear factor kappa beta (NF κ B) translocation to the nucleus, which upregulates pro-inflammatory mediators (Ruan et al., 2015). It is well known that neural injury accelerates the release of pro-inflammatory cytokines which can signal lasting neuronal cell stress (Hong et al., 2016). If the cellular stress response is severe, or sustained, the neuron will undergo apoptosis (Nakagawa and Yuan, 2000), causing extensive gliosis and neuroinflammation (Harvey et al., 2015). We propose that TBI induces NOX4-mediated oxidative stress and JNK-mediated ER stress, which subsequently contributes to neuroinflammation through NF κ B activation.

Simultaneous to ER stress activation, oxidative stress occurs and generates free radicals, which play a role in cell death and disease pathology following TBI (Toklu and Tumer, 2015). Free radicals damage cellular membranes, increase carbonyl formation, and can

contribute to cell death and neurobehavioral dysfunction (Ferguson et al., 2010). We previously showed NOX4-mediated oxidative stress increased neuronal apoptosis following traumatic brain injury (Lucke-Wold et al., 2015b). In addition, Wu and colleagues used an *In vitro* model of endothelial injury to causally link NADPH-oxidase (Nox4)-mediated oxidative stress to Janus-N-terminal kinase (JNK)-mediated ER stress (Wu et al., 2014b). The group also silenced JNK-mediated ER stress and observed an attenuation of nuclear translocation NF κ B (Wu et al., 2014b). These findings suggest NOX-mediated oxidative stress and JNK-mediated ER stress to be linked to NF κ B activation.

In conjunction with these cell stress responses, chronic neuroinflammation has emerged as a possible contributory factor to behavior change (Faden et al., 2015). Preclinical models have shown that TBI is associated with a significant inflammatory burden (Kumar et al., 2014). Furthermore, it has been shown that neuroinflammation can persist years after injury in the brains of retired athletes (Coughlin et al., 2014). Recent clinical evidence ties neuroinflammation to neurobehavioral symptoms (Cho et al., 2013; Wu et al., 2014a). We propose that acute modulation of cellular stress after TBI will positively influence the extracellular inflammatory milieu leading to improved behavioral outcomes.

Salubrinal (SAL) is a modulator of cellular stress known to inhibit protein phosphatase 1, and attenuate global translation (Boyce et al., 2005). Reducing the ER workload promotes proteostasis and cell survival (Hotamisligil, 2010; Tsaytler et al., 2011; Walter and Ron, 2011). SAL has been shown to be neuroprotective in models of protein toxicity (Colla et al., 2012; Huang et al., 2012), stroke (Nakka et al., 2010), excitotoxicity (Sokka et al., 2007), and TBI (Rubovitch et al., 2015). In our model of TBI, we have previously shown SAL to reduce ER-mediated apoptosis and to ameliorate impulsive-like behavior (Logsdon et al., 2014). In the present study, we investigated the effects of SAL on reducing neuroinflammation, and impulsive-like behavior following a more clinically relevant repetitive TBI paradigm (Fig. 1).

2. Results

2.1. SAL attenuated markers of ER stress after single blast

ER stress is a common secondary cascade implicated in subacute injury expansion following TBI (Farook et al., 2013; Begum et al., 2014). Our previous study showed that markers of the acute phase ER stress were upregulated following sTBI (Logsdon et al., 2014). In this study, we investigated additional markers of ER stress, JNK phosphorylation and CHOP activation, which have been associated with NF κ B activity (Deng et al., 2004; Tsai et al., 2012).

Fig. 2(A) indicates a significant difference between experimental groups in JNK activity after sTBI ($F_{(2,9)}=9.04$; $P<0.01$). A significant increase in the ratio between JNK phosphorylation and total JNK expression was observed at 24 h after sTBI compared to control rats ($q=5.79$; $P<0.01$). SAL administration significantly attenuated JNK phosphorylation when compared to vehicle-treated sTBI rats ($q=4.31$; $P<0.05$).

Fig. 2(B) indicates a significant difference between experimental groups in CHOP activation after sTBI ($F_{(2,9)}=8.769$; $P<0.01$). A significant increase in CHOP expression at 24 h was observed in sTBI rats as compared to control rats ($q=5.86$; $P<0.01$). SAL administration significantly attenuated CHOP activation when compared to vehicle-treated sTBI rats ($q=3.673$; $P<0.05$). SAL successfully reduced markers of ER stress when administered acutely after injury.

2.2. SAL reduced markers of oxidative stress after single blast

ER stress activation has been proposed to directly increase oxidative stress particularly in the striatum (Malhotra and Kaufman, 2007). ROS generation is a consequence of TBI mainly through membrane damage and subsequent NOX4 system activation (Zhang et al., 2012; Loane et al., 2013). Activation of the NOX4 system predominantly creates superoxide (Brennan et al., 2009; Lucke-Wold et al., 2015b). A previous report indicated that both NOX4 and superoxide were acutely elevated after TBI (Ansari et al., 2014).

Fig. 3(A) shows a significant difference in carbonyl levels between groups after sTBI ($F_{(2,9)}=10.21$; $P<0.01$). Carbonyl formation is an end product of oxidative stress damage. A significant increase in carbonyl levels was measured in sTBI rats as compared to control rats ($q=6.26$; $P<0.01$). SAL administration significantly reduced carbonyl levels ($q=4.23$; $P<0.05$) when compared to vehicle-treated sTBI rats.

Fig. 3(B) shows a significant difference in superoxide levels between experimental groups after sTBI ($F_{(2,9)}=7.68$; $P<0.05$). A significant increase in superoxide levels was measured in sTBI rats when compared to control rats ($q=4.92$, $P<0.05$). SAL administration significantly reduced superoxide levels as compared to vehicle-treated sTBI rats ($q=4.68$, $P<0.01$). Fig. 3(C) shows no difference in total ROS levels ($F_{(2,9)}=0.16$; $P>0.05$).

Fig. 3(D) shows a significant difference in corrected total cell fluorescence for NOX4 in the striatum at 24 h post-sTBI ($F_{(2,27)}=3.76$; $P<0.05$). A significant increase in NOX4 fluorescence was observed in sTBI rats compared to control rats ($q=5.46$, $P<0.001$). SAL administration significantly reduced NOX4 fluorescence when compared to vehicle-treated sTBI rats ($q=5.61$, $P<0.01$). These results suggest that SAL attenuates NOX4-mediated oxidative stress by specifically reducing super-oxide in the striatum.

2.3. SAL attenuated markers of neuroinflammation after single blast

ER stress and oxidative stress have both been associated with increased neuroinflammation after TBI (Deslauriers et al., 2011; Bellezza et al., 2014; Hayashi, 2015). Integral to the process of neuroinflammation is activation and nuclear translocation of nuclear factor kappa B (NF κ B) (Bracchi-Ricard et al., 2013). NF κ B and inducible nitric oxide synthase (iNOS) are known to promote pro-inflammatory cytokines, such as, tumor necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1 β) (Hu et al., 2014). These inflammatory cytokines can signal changes that damage neuronal cell membranes and intracellular organelles (Abdullah and Bayraktutan, 2014).

Fig. 4(A) shows a significant difference in NF κ B p65 expression between experimental groups at 24 h after sTBI ($F_{(2,9)}=4.67$; $P<0.05$). NF κ B p65 expression was significantly

increased in sTBI rats compared to control rats ($q=4.101$; $P<0.05$). SAL administration significantly reduced NF κ B p65 expression when compared to vehicle-treated sTBI rats ($q=3.24$; $P<0.05$).

Fig. 4(B) shows a significant difference in iNOS expression at 24 h after sTBI ($F_{(2,9)}=6.81$; $P<0.05$). A significant increase in iNOS expression was measured in sTBI rats compared to control rats ($q=4.92$; $P<0.05$). SAL administration significantly reduced iNOS expression when compared to vehicle-treated sTBI rats ($q=3.97$; $P<0.05$).

Fig. 4(C) shows a significant difference in IL-1 β mRNA abundance at 24 h post-sTBI amongst the treatment groups ($F_{(2,9)}=5.62$; $P<0.05$). A significant increase in IL-1 β mRNA abundance was measured in sTBI rats compared to control rats ($q=4.13$; $P<0.05$). SAL administration significantly reduced IL-1 β mRNA abundance when compared to vehicle-treated sTBI rats ($q=4.08$; $P<0.05$).

Fig. 4(D) shows a significant difference amongst the treatment groups in TNF α mRNA abundance at 24 h post-sTBI ($F_{(2,9)}=5.54$; $P<0.05$). A significant increase in TNF α mRNA abundance was measured in sTBI rats compared to control rats ($q=4.49$; $P<0.05$). SAL administration significantly reduced TNF α mRNA abundance when compared to vehicle-treated sTBI rats ($q=3.47$; $P<0.05$). These results show that SAL significantly reduced neuroinflammatory markers following blast injury.

2.4. SAL attenuated markers of neurodegeneration after single blast

Gliosis and degenerative changes are initial signs of persistent neurodegeneration (Damjanac et al., 2007). We previously showed that ER stress is closely tied to neurodegenerative disease following neurotrauma (Lucke-Wold et al., 2016), and that TBI can produce gliosis and degenerative changes acutely post-injury (Turner et al., 2012).

Fig. 5(A) shows a significant difference in corrected total cell fluorescence for GFAP in the lateral orbitofrontal cortex at 72 h post-sTBI ($F_{(2,29)}=35.23$; $P<0.001$). A significant increase in GFAP fluorescence was observed in sTBI rats compared to control rats ($q=10.07$, $P<0.001$). SAL administration significantly reduced GFAP fluorescence when compared to vehicle-treated sTBI rats ($q=7.58$, $P<0.001$).

Fig. 5(B) shows a significant difference in corrected total cell fluorescence for FJB in the lateral orbitofrontal cortex at 72 h post-sTBI ($F_{(2,29)}=7.43$; $P<0.01$). A significant increase in FJB fluorescence was observed in sTBI rats compared to control rats ($q=5.18$, $P<0.05$). SAL administration significantly reduced FJB fluorescence when compared to vehicle-treated sTBI rats ($q=3.32$; $P<0.05$). These results demonstrate that SAL significantly reduces gliosis and neurodegeneration.

2.5. SAL reduces neuroinflammation after repetitive blast

Under persistent conditions, iNOS, can contribute to neurodegeneration and behavioral symptoms (Jayakumar et al., 2014). Hsieh and colleagues discovered that iNOS activation is the mechanism by which ER stress causes the formation of ROS and is a direct tie to neuroinflammation (Hsieh et al., 2007).

Fig. 6(A) shows a significant difference in NF κ B p65 expression between experimental groups at two weeks after rTBI ($F_{(2,12)}=14.04$; $P<0.05$). NF κ B p65 expression was significantly increased in rTBI rats compared to control rats ($q=6.05$; $P<0.05$). NF κ B p65 expression was also increased in rTBI+SAL rats when compared to control rats ($q=6.86$; $P<0.05$).

Fig. 6(B) shows a significant difference in iNOS expression between experimental groups at two weeks after rTBI ($F_{(2,12)}=6.25$; $P<0.05$). A significant increase in iNOS expression was measured in rTBI rats compared to control rats ($q=4.84$; $P<0.05$). SAL administration significantly reduced iNOS expression when compared to vehicle-treated rTBI rats ($q=3.49$; $P<0.05$).

Fig. 6(C) shows IHC colocalization (yellow) of ER stress marker CHOP (green) with neuroinflammation marker iNOS (red) in the lateral orbitofrontal cortex. Overlap Pearson's coefficient revealed a small correlation in vehicle controls ($r=0.17$), a large correlation in rTBI+Vehicle ($r=0.65$), and a small correlation in rTBI+SAL ($r=0.22$). These results suggest neuroinflammation and ER stress are increased in the same cells after repetitive blast exposure.

2.6. SAL ameliorated impulsive-like behavior after repetitive blast

Neuropsychiatric symptoms, such as impulsive-like behavior, commonly burden people who have suffered from multiple mild concussions, such as athletes and soldiers (Omalu et al., 2011; Bailes et al., 2013). Impulsive-like behavior becomes evident in rodents with damage to the lateral orbitofrontal cortex (Mar et al., 2011; Bidzan et al., 2012; Johnson et al., 2013).

We have previously observed impulsive-like behavior among rats exposed to a single blast from our TBI model (Logsdon et al., 2014). Using this more clinically relevant repetitive injury paradigm, we looked at impulsive-like behavior by measuring the time spent in the open arms of the EPM after repeat blast exposure.

Fig. 7(A) shows a significant difference in open arm time at two weeks post-rTBI amongst the treatment groups ($F_{(2,24)}=4.72$; $P<0.05$). A significant increase was measured in the time that the rTBI rats spent in the open arms of the EPM compared to control rats ($q=4.09$; $P<0.05$). Interestingly, SAL administration significantly attenuated the open arm time when compared to vehicle-treated rTBI rats ($q=3.31$; $P<0.05$).

Fig. 7(B) shows no difference in the total distance the rats travelled between the experimental groups ($F_{(2,24)}=2.54$; $P<0.05$). Track plots from AnymazeTM were overlaid for each subject to provide a clear visual representation of the rats' behavior during the EPM trials (Fig. 7(C)–(E)). SAL had a protective effect on reducing impulsive-like behavior.

2.7. Proposed mechanism of blast injury

Fig. 8 shows a proposed detailed schematic of the injury cascade following blast injury. We propose blast TBI causes vascular disruption and blood extravasation into the brain parenchyma that can damage plasma membranes and trigger secondary injury cascades (Uranga et al., 2009; Liu et al., 2013; Nisenbaum et al., 2014). The secondary injury

cascades contribute to neurodegeneration and ultimately lead to neurobehavioral dysfunction.

3. Discussion

Blast TBI cause primary injury due, in part, to acceleration-deceleration forces to the rat's head (Goldstein et al., 2012). The current study shows an initiation of secondary injury cascades including ER stress, oxidative stress, and neuroinflammation following primary injury. These acute injury cascades lead to additional glial reactivity and neurodegenerative changes. We also show that the cellular stress responses occurred concurrent with neuroinflammation after blast TBI. Neuroinflammation is a common secondary effect of brain injury demonstrated in a variety of rodent TBI models (Abdul-Muneer et al., 2013, 2014; Cho et al., 2013; Hu et al., 2014; Roth et al., 2014), and has been linked clinically to single and repetitive head injuries (Aungst et al., 2014; Webster et al., 2015). While previous studies have suggested an association between ER stress and neuroinflammation, what remains unknown is whether regulation of these responses would affect neurodegeneration and improve neurobehavioral outcome (Fenn et al., 2015). Additionally, ER stress activation has been proposed to directly increase oxidative stress through activation of iNOS particularly in the striatum (Malhotra and Kaufman, 2007). Using the ER stress modulator SAL, we show a potential link between these pathways. We also offer compelling evidence that targeting acute cellular stress cascades can prevent impulsive-like behavior after repeat injury as measured by time spent in the open arms of the EPM.

An important secondary injury cascade often increased following TBI and intimately linked to ER stress is oxidative stress (Cho et al., 2013). We report an increase in oxidative stress damage in the striatum following blast exposure, as evidenced by increased carbonyl and NOX4-mediated superoxide levels. Super-oxide production is a consequence of TBI mainly through activation of the NOX system (Brennan et al., 2009; Zhang et al., 2012; Loane et al., 2013). A previous report indicated that NOX4 and superoxide levels were elevated at 6 h after blast TBI and persisted for 72 h (Ansari et al., 2014). Additionally, NOX4 mediated oxidative stress perpetuates damage to neuronal membranes leading to cell death (Zhang et al., 2012). A late-onset symptom of CTE is motor dysfunction similar to Parkinson's disease that indicates cell death and damage to the striatum (Stern et al., 2011). In a future study we will investigate late-onset motor disturbance associated with the striatal damage reported herein.

Persistent damage to the cell from oxidative stress can trigger a measurable increase in ER stress associated JNK phosphorylation (Quan et al., 2015), and subsequent NFkB activity, which affects neuroinflammation. An increase in both pJNK and NFkB p65 expression were demonstrated in this study. JNK can signal NFkB translocation to the nucleus, which may upregulate pro-inflammatory mediators such as iNOS, TNF α , and IL-1 β (Hu et al., 2014; Ruan et al., 2015). In line with this ensuing injury cascade following blast, we found increased levels of pro-inflammatory markers: NFkB, iNOS, TNF α , and IL-1 β . These pro-inflammatory markers are thought to cause a sudden oxidative burst that overwhelms antioxidant defense cascades leading to further damage of surrounding brain tissue (Liao et al., 2013). We show repetitive blast TBI causes a subacute increase in iNOS fluorescence,

suggesting a persistent inflammatory response. Consequently, while neuroinflammation is a necessary response to brain injury (Finnie, 2013), unabated, persistent neuroinflammation can lead to irreversible neurodegeneration and poor injury outcome.

Furthermore, we recently showed that ER stress is an important secondary injury response activated after blast TBI (Logsdon et al., 2014), and in human CTE (Lucke-Wold et al., 2016). ER stress can also lead to cognitive dysfunction following TBI as we previously demonstrated (Dash et al., 2015; Lucke-Wold et al., 2015a). In this paper, we show ER stress markers CHOP and pJNK fluorescence to be increased following single injury, and co-localized with iNOS following repeat injury. Similar to oxidative stress, the ER stress response has significant cross talk with neuroinflammatory pathways through JNK signaling (Prell et al., 2014). A recent study suggests crosstalk between JNK phosphorylation and NF κ B activity after inflammatory challenge with an ER stressor (Ruan et al., 2015).

Importantly, we show, for the first time, that SAL administration post-injury attenuates iNOS protein expression and JNK phosphorylation. This is in agreement with a recent study where SAL administration reduced NF κ B and microglia activation in a model of Alzheimer's disease (Huang et al., 2012). SAL also improved chronic motor performance in a model of spinal cord injury (Ohri et al., 2013). In a future study, we will investigate if SAL improves motor performance long-term following TBI. Further investigation is needed to see if acute modulation of cellular stress pathways will lead to sustained behavioral improvements. Our group and others showed that docosahexaenoic acid, another less-specific ER stress modulator, improved cognitive performance following TBI (Begum et al., 2013; Lucke-Wold et al., 2015a). Based on the results of this current study and the supporting background information from prior studies, we propose a novel mechanism of action for the beneficial properties of SAL through downstream regulation of NF κ B activity. In support of this claim, we demonstrated that SAL administration after blast attenuated pJNK, NF κ B, iNOS, TNF α , and IL-1 β expression. SAL also decreased markers of glial reactivity after single blast exposure, suggesting a protective effect at the blood-brain barrier as we previously reported (Logsdon et al., 2014). Following repetitive blast, SAL furthermore was shown to reduce iNOS and ER stress markers.

Abnormal behavioral symptoms can be a manifestation of persistent neuroinflammation, and are of particular clinical relevance for patients suffering multiple mild-head injuries (Sominsky et al., 2015). We showed that in the more clinically relevant repetitive injury scenario, rats exposed to injury displayed impulsive-like behavior as measured on the EPM 72 h following the last blast. Interestingly, SAL administration reduced impulsive-like behavior when administered after each blast exposure. The data suggest that SAL may mitigate subacute markers of neuroinflammation and thereby reduce impulsive-like behavior after repetitive brain trauma.

These findings have broad reaching implications regarding the importance of targeting cellular stress acutely post-injury in order to reduce neurodegenerative changes and ameliorate neurobehavioral dysfunction. We showed that ER stress modulation after blast injury decreases acute superoxide formation in the striatum, attenuates neuroinflammatory

markers in the frontal cortex, and ameliorates impulsive-like behavior in rats. In doing so, we map a unique interconnection between intracellular stress cascades and neuroinflammation. Further pre-clinical studies are warranted to determine the role of ER stress, oxidative stress, and neuroinflammation at subacute time points following brain injury and how these pathways contribute to neurobehavioral dysfunction.

4. Conclusions

In summary, cellular stress and neuroinflammation are intrinsically interconnected and play an important role in injury progression following TBI. We show that blast exposure induced markers of oxidative stress, which is known to contribute to the exacerbation of ER stress and neuroinflammation. Furthermore, we show that ER stress may be linked to neuroinflammation through the JNK-mediated NF κ B pathway. Surprisingly, SAL reduced markers of ER stress and oxidative stress; thereby, reducing markers of neuroinflammation post-blast. The likely mechanism is reduction of JNK phosphorylation and iNOS activity. Most importantly, SAL ameliorated impulsive-like behavior when administered after each repetitive blast exposure. Secondary injury modulation with key multi-target pharmaceuticals offers a promising approach to reduce the long-term neuropsychiatric symptoms associated with head injuries.

5. Experimental procedure

5.1. Animals

Fifty-one (51) male Sprague-Dawley rats (Hilltop Lab Animals) at 2–3 months of age were used in this study. The West Virginia University Animal Care and Use Committee approved all procedures involving rats. Rats were acclimated for 1 week prior to use and housed under 12 h light/dark conditions with food and water available ad libitum. Animal experiments were performed according to the principles of the *Guide for the Care and Use of Laboratory Animals*.

5.2. Salubrial

SAL (Tocris Biosciences) was dissolved in 0.5% DMSO and delivered via intraperitoneal (i.p.) injection at a dose of 1 mg/kg (Sokka et al., 2007; Liu et al., 2014).

5.3. Blast overpressure exposure

Blast exposure was induced as previously described (Turner et al., 2013). Blast pressure with a 0.005" membrane (15 psi incident wave; 50 psi reflected wave) was determined, from our previous work, to exhibit neural injury markers with no mortality (Turner et al., 2013; Logsdon et al., 2014; Lucke-Wold et al., 2014b). Following blast exposure, rats were returned to a holding cage equipped with a homeothermic heating pad. Once basic reflexes were restored, rats were returned to their home cage.

5.4. Experimental groups

A detailed timeline shows the course of injury and treatment for each group of rats (Fig. 1). Rats were randomly assigned to one of three treatment groups. Group 1 rats served as sham

controls (anesthetized with 4% isoflurane). Following anesthesia, rats were injected with vehicle (0.5% DMSO; i.p.) 5 min after being placed on blast table. Group 2 rats served as our experimental control group, in which anesthetized rats were oriented similarly to sham rats and subsequently exposed to blast. Rats were injected with vehicle 5 min after each blast exposure. Group 3 served as our experimental group. Rats received blast exposure as group 2, but SAL (1 mg/kg; i.p.) was administered 5 min after each blast exposure (Rubovitch et al., 2015).

There was two blast TBI subgroupings: a single blast TBI (sTBI) and a repeated blast TBI (rTBI). Rats in the rTBI group received a blast every other day over two weeks, for a total of six blast injuries. This repetitive schedule was determined by our previous work that showed markers of ER stress activation (Lucke-Wold et al., 2015a), and from other TBI models that showed markers of neuroinflammation and neurodegeneration (Mouzon et al., 2012; Mouzon et al., 2014).

Twenty-four (24) sTBI rats were randomly divided into the three treatment groups and euthanized for biochemical analysis at 24 h post-blast ($n=4$ rats per group), and immunohistochemistry (IHC) at 72 h post-blast ($n=4$ rats per group). Twenty-seven (27) rTBI rats were randomly divided into the three treatment groups and were subject to behavioral testing 72 h after the repetitive blast schedule ($n=9$ rats per group). Four rTBI rats from each experimental group were euthanized for IHC after behavioral testing was complete.

5.5. Tissue preparation

Rats used for biochemical analysis were euthanized and their brains were rapidly removed in an ice-cold HaltTM protease/phosphatase inhibitor cocktail mix (Thermo Scientific). The striatum was dissected out from each hemisphere, flash frozen in liquid nitrogen, and stored at -80°C for later measurement of carbonyl and reactive oxygen species (ROS) levels. The frontal cortex was dissected out from each hemisphere, flash frozen in liquid nitrogen and stored at -80°C for later measurement of protein expression and mRNA abundance. Rats used for IHC were anesthetized with 4% isoflurane and perfused transcardially with ice-cold saline. Following perfusion, brains were removed, flash frozen in isopentane (-60°C) and stored at -80°C for IHC preparation.

5.6. Carbonyl measurement

For carbonyl measurement, striatal tissue samples from sTBI rats ($n=4$ rats per group) were assayed using an OxyBlot Protein Oxidation Detection kit (Millipore). Striatal tissues from each treatment group were sonicated in 6% SDS. Control samples were mixed with derivation control solution and experimental samples were mixed with 2,4-dinitrophenylhydrazine. Samples were incubated for 15 min followed by addition of neutralization solution. Samples were loaded (20 μl per well) and electrophoresed on a 10% acrylamide gel and immunoblotted as outlined in the Western blot methods below.

5.7. ROS measurement

For ROS detection, striatal tissue from sTBI rats were homogenized and cells were isolated by incubation in collagenase at 2 mg/ml for 30 min. Cells were separated by enzyme digestion and manual disruption by repeated pipetting. Cells were strained through a 70 μ m nylon cell strainer followed by centrifugation at 400g for 5 min. Pellets were resuspended to a concentration of 1.0×10^6 cells/ml in DMEM.

Total ROS and superoxide levels were detected using a Total ROS/Superoxide Detection kit (Enzo Life Sciences) according to manufacturer's instructions. In brief, 100 μ l of suspended cells were added to each well of a dark-sided 96 well plate with a clear bottom. Cells were incubated overnight at 37 °C in DMEM. The following day, media was removed and 100 μ l of ROS/Superoxide Detection Solution was added to each well and incubated in the dark for 1 h. Detection of total ROS (green) and superoxide (red/yellow) fluorescence was detected at an excitation wavelength of 488/520 nm and an emission wavelength of 550/610 nm, respectively. Data were collected using Gen5 software (BioTek). Concentrations were determined based on a known standard curve.

5.8. Western blot

Protein samples from sTBI and rTBI rats were prepared in 1% SDS and Western blot analysis was performed as previously described (Lucke-Wold et al., 2014b). Primary antibodies (and dilution factors) were rabbit anti-pJNK monoclonal antibody (mAB) (Thr183/Tyr185) (1:500), rabbit anti-binding immunoglobulin protein (BiP) mAB (1:1000), rabbit anti-JNK mAB (1:1000) (Cell Signaling); mouse anti-NF κ B p65 polyclonal antibody (pAB) (1:200), rabbit anti-iNOS pAB (1:200) (Santa Cruz). A rabbit anti- β -actin mAB (1:10,000) (Cell Signaling) was used as an endogenous control to normalize protein loading. Secondary antibodies were IRDye[®] 800CW (goat anti-rabbit) and IRDye[®] 680RD (goat anti-mouse) (LI-COR Biosciences). Images were collected using the Odyssey Classic Infrared Imaging System (LI-COR Biosciences). Images were converted to gray scale, and detected bands were quantified using Image Studio Lite Software (LI-COR Biosciences). Bands were normalized to β -actin values to measure relative intensity.

5.9. Quantitative real-time polymerase chain reaction

RNA samples from sTBI and rTBI rats were prepared in TRIzol[®] reagent (Life Technologies) and confirmed for quality (1.8–2.1 absorbance ratio). Reverse transcription was conducted and real-time PCR analyses were performed on cDNA using the following oligonucleotide primer sets: TNF α (Rn01525859_g1), IL-1 β (Rn00580432_m1), and 18s rRNA (Hs99999901_s1; endogenous control) (Life Technologies). Changes in mRNA abundance were determined by the Ct method with a threshold cycle value of 0.2 normalized to 18s rRNA.

5.10. Histology

Whole brains from sTBI and rTBI rats were mounted on a Leica CM3050S cryostat (Leica Microsystems) set to –20 °C. Coronal sections of the frontal cortex were sliced at a thickness of 20 μ m and mounted onto glass slides for IHC staining as previously described (Lucke-Wold et al., 2015a). Briefly, brain slices were circumscribed, and incubated

overnight with primary antibodies: iNOS (Santa Cruz), glial fibrillary acidic protein (GFAP), C/EBP homology protein (CHOP), BiP, and pJNK (Cell Signaling). The next day, an Alexa Flour® secondary antibody (Invitrogen) was applied to slides for 3 h, and coverslip mounted with Vectashield® 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector). If staining for colocalization, a second set of primary and secondary antibodies were applied prior to fixing the coverslip. Fluorochrome B (FJB) (Millipore), and ROS (Enzo Life Sciences) staining was performed in accordance with manufacturer's instructions.

All immunohistochemistry was performed as described previously (Lucke-Wold et al., 2014b, 2015b). Briefly, images were acquired from the lateral orbitofrontal cortex (15 slides per animal). Fluorescent imaging was performed using a Zeiss Axio Observer Z1. For fluorescent staining, 10 distinct cells with clear morphology were randomly selected per slide, outlined, and measured with ImageJ software (NIH) by an observer blinded to experimental group. Density was adjusted per mean area to give corrected total cell fluorescence normalized to background. Co-localization quantification with the *Just Another Co-localization plugin* for ImageJ was used to determine overlap coefficient or Pearson's coefficient (Bolte and Cordelières, 2006). Overlap coefficient was calculated using $k2 = k1 * k2$ with values adjusted to threshold (Lucke-Wold et al., 2015a).

5.11. Elevated plus maze

Impulsive-like behavior was assessed in rTBI rats using the Elevated plus maze (EPM). Increased time spent in the open arms was considered a sign of impulsive-like behavior in the rodent (Mosienko et al., 2012; Johnson et al., 2013). The EPM was set at a height of 60 cm above the floor. The two open arms intersected perpendicular to the two closed arms. Each arm was 50 cm × 10 cm. The closed arms were encased by black siding 30 cm tall. Each rat was placed in the middle of the EPM facing an open arm and tracking was performed for 5 min using AnyMaze software (Stoelting), which pinpointed the location of the rat's head and body continuously throughout the testing trial.

5.12. Statistical analysis

Data were analyzed using a one-way analysis of variance (AN-OVA) followed by Newman-Keul's *post hoc* tests for between groups comparison. For colocalization studies, Pearson's coefficient was obtained for control sections. Overlap coefficient was obtained for experimental groups to determine the extent of same-cell protein expression. Sample sizes were determined using a power analysis with an α of 0.05, a β of 0.2, and a sample effect of 0.4 for behavioral data and 0.3 for all other data (DSS Research Power Analysis). $P < 0.05$ was considered significant.

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Abbreviations

TBI	traumatic brain injury
sTBI	single blast-induced traumatic brain injury
rTBI	repeated blast-induced traumatic brain injury
ER	endoplasmic reticulum
SAL	salubrial
GFAP	Glial fibrillary acidic protein
FJB	Fluorojade B
JNK	c-jun N-terminal kinase
NFκB	nuclear factor kappa beta
iNOS	intrinsic nitric oxide synthase
BiP	binding immunoglobulin protein
CHOP	C/EBP homology protein
TNFα	tumor necrosis factor alpha
IL-1β	interleukin 1 beta
NOX	NADPH-oxidase

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Fig. 1.

Experimental design. Detailed experimental timeline showing: sTBI (single blast) animals at top; rTBI (repetitive blast) animals at bottom. SAL (Salubrinal); ROS (Reactive oxygen species); WB (Western blot); PCR (Polymerase chain reaction); IHC (Immunohistochemistry).

Fig. 2. SAL reduced ER stress markers after single blast. We measured a significant increase in JNK activity (pJNK/tJNK) at 24 h post-sTBI (** P <0.01 vs Vehicle); SAL significantly mitigated JNK activity (# P <0.05 vs sTBI+Vehicle). (A). We measured a significant increase in CHOP protein expression at 24 h post-sTBI (** P <0.01 vs Vehicle); SAL significantly mitigated CHOP expression (# P <0.05 vs sTBI+Vehicle) (B). One-way ANOVA, Newman-Keul's *post hoc*. Mean±S.E.M. n =4.

Fig. 3. SAL reduced markers of oxidative stress after single blast. A significant increase in protein carbonyl levels was measured at 24 h post-sTBI (** $P < 0.01$ vs Vehicle); SAL treatment significantly attenuated carbonyl levels (# $P < 0.01$ vs sTBI) (A). A significant increase in superoxide levels was measured at 24 h post-sTBI (* $P < 0.05$ vs Vehicle); SAL treatment significantly attenuated superoxide levels (## $P < 0.01$ vs sTBI) (B). No significant differences were observed in total ROS levels ($P < 0.05$) (C). We observed a significant increase in NOX4 (green) fluorescence at 24 h post-sTBI (*** $P < 0.001$ vs Vehicle); SAL significantly reduced NOX4 fluorescence (## $P < 0.01$) ($n = 4$). NOX4 images include nuclear counterstain DAPI (blue) (D). Images are from the striatum. Images are displayed at 20 $\times$; insets at 63 $\times$. (Scale bars = 30 μ m). One-way ANOVA, Newman-Keul's *post hoc*. Mean $\pm$ S.E.M. $n = 4$.

Fig. 4.

SAL mitigated markers of neuroinflammation after single blast. We measured a significant increase in NFκB p65 expression at 24 h post-sTBI (* $P < 0.05$ vs Vehicle); SAL significantly mitigated NFκB p65 expression (# $P < 0.05$ vs sTBI+Vehicle) (A). We revealed a significant increase in iNOS protein expression at 24 h post-sTBI (* $P < 0.05$ vs vehicle); SAL significantly mitigated iNOS expression (# $P < 0.05$ vs sTBI+Vehicle) (B). We measured a significant increase in IL-1β mRNA abundance at 24 h post-sTBI (* $P < 0.05$ vs Vehicle); SAL significantly mitigated IL-1β abundance (# $P < 0.05$ vs sTBI +Vehicle) (C). We measured a significant increase in TNFα mRNA abundance at 24 h post-sTBI (* $P < 0.05$ vs Vehicle); SAL significantly mitigated TNFα abundance (# $P < 0.05$) (D). One-way ANOVA, Newman-Keul's *post hoc*. Mean±S.E.M. $n=4$.

Fig. 5.

SAL attenuated markers of neurodegeneration after single blast. We observed a significant increase in GFAP (green) fluorescence at 72 h post-sTBI ($***P<0.001$ vs Vehicle); SAL significantly reduced GFAP fluorescence ($###P<0.001$) ($n=4$). GFAP images include nuclear counterstain DAPI (blue) (A). We observed a significant increase in FJB (green) fluorescence at 72 h post-sTBI ($*P<0.05$ vs Vehicle); SAL significantly reduced FJB fluorescence ($\#P<0.05$) (B). One-way ANOVA, Newman-Keul's *post hoc*. Mean $\pm$ S.E.M. $n=4$. Images are from the lateral orbitofrontal cortex. Images are displayed at 20 $\times$; insets at 63 $\times$. (Scale bars =30 μ m).

Fig. 6.

SAL reduces neuroinflammation after repetitive blast. We measured a significant increase in NFκB p65 expression at two weeks post-rTBI (* $P < 0.05$ vs Vehicle); and when SAL was administered post-rTBI (* $P < 0.05$ vs Vehicle) (A). We revealed a significant increase in iNOS protein expression at two weeks post-rTBI (* $P < 0.05$ vs vehicle); SAL significantly mitigated iNOS expression (# $P < 0.05$ vs sTBI+Vehicle) (B). One-way ANOVA, Newman-Keul's *post hoc*. Mean±S.E.M. $n=4$. Colocalization of iNOS (red) merged with CHOP (green) was determined by levels of yellow in each image (Overlap coefficient; r values). All panels display nuclear counterstain DAPI (blue). Arrows demarcate iNOS fluorescence (C). Images are from the lateral orbitofrontal cortex. Images are displayed at 20 ×; Insets displayed at 63 ×. (Scale bars =30 μm).

Fig. 7.

SAL ameliorated impulsive-like behavior after repetitive blast. A significant increase was observed in the time that the rats spent in the open arms of the EPM at 72 h post-rTBI ($*P < 0.05$ vs Vehicle); SAL significantly reduced the time spent ($\#P < 0.05$ vs rTBI+vehicle) (A). No significant differences were observed in the total distance moved among rats in each group ($P < 0.05$) (B). Track plots from Anymaze™ were overlaid for each subject to provide a clear visual representation of the rats' behavior during the EPM trials (C–E). One-way ANOVA, Newman-Keul's *post hoc*. Mean $\pm$ S.E.M. $n = 9$.

Fig. 8.

Blast injury cascade with SAL mechanism of action. Schematic of blast injury cascade and salubrinal mechanism of action. TBI=Traumatic Brain Injury; ER=Endoplasmic Reticulum; CHOP=C/EBP homology protein; NFκB=Nuclear factor kappa beta; JNK=Janus n-terminal kinase; iNOS=inducible Nitric oxide synthase; TNFα=Tumor necrosis factor alpha; IL-1β=Interleukin 1 beta.