Brief oxygen exposure after traumatic brain injury speeds recovery and promotes adaptive chronic endoplasmic reticulum stress responses.

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Abstract

Traumatic brain injury (TBI) is a major public health concern particularly in adolescents who have a higher mortality and incidence of visual pathway injury compared to adult patients. Likewise, we have found disparities between adult and adolescent TBI outcomes in rodents. Most interestingly, adolescents suffer a prolonged apneic period immediately post injury leading to higher mortality; so, we implemented a brief oxygen exposure paradigm to circumvent this increased mortality. Adolescent male mice experienced a closed-head weight-drop TBI then were exposed to 100% O₂ until normal breathing returned or recovered in room air. We followed mice for 7- and 30-days and assessed their optokinetic response; retinal ganglion cell loss; axonal degeneration; glial reactivity; and retinal ER stress protein levels. O₂ reduced adolescent mortality by 40%, improved post-injury visual acuity, and reduced axonal degeneration and gliosis in optic projection regions. ER stress protein expression was altered in injured mice, and mice given O₂ utilized different ER-stress pathways in a time dependent manner. Finally, O₂ exposure may be mediating these ER stress responses through regulation of the redox-sensitive ER folding protein ERO1α, which has been linked to a reduction in the toxic effects of free radicals in other animal models of ER stress.
The association between brain oxygen deprivation (i.e., hypoxia) and traumatic brain injury (TBI) has been known for decades\(^1\) with a reported co-incidence of up to 44\%.\(^2\) Several studies have shown that brain tissue oxygen tension can serve as an independent predictor of TBI outcomes.\(^3, 4\)

Indeed, guidelines for critical care after TBI are largely focused on maintaining brain perfusion and oxygenation after injury.\(^8\) In animal models of TBI, post-injury hypoxia is associated with inflammation,\(^16-18\) worse axonal injury,\(^17-19\) blood-brain-barrier disruption,\(^16, 20\) and functional impairments.\(^16, 18, 21, 22\)

We previously reported that after a closed head weight-drop injury, adolescent mice were at increased risk of death compared to their adult counterparts, with accompanying worse cognitive performance and hippocampal neuron loss.\(^15\) During these studies, we noted that the increased mortality rate in adolescent mice was related to prolonged bouts of apneic/agonal breathing as compared to adult mice. We found that mice that recovered from apnea/agonal breathing did not display any significant amount of post-TBI mortality.

Accordingly, we hypothesized that supplemental oxygen would improve survival, potentially by reducing the duration of agonal breathing immediately after injury. However, we also were concerned with potential side effects of excess oxygen or oxygen toxicity. For example, premature infants given supplemental oxygen to treat chronic lung disease have an increased chance of survival,\(^23\) but they also may develop retinal fibroplasia, known as retinopathy of prematurity, which is caused by oxygen toxicity.\(^24, 25\) Additionally, some retrospective studies have shown that higher brain oxygenation within the first 24 hours after TBI is associated with elevated mortality and worse short-term outcomes.\(^26\) Due to this duality of oxygen treatment, we wondered if a brief oxygen exposure, lasting only through the duration of agonal breathing, would be sufficient to improve survival, without causing oxygen toxicity. We thus hypothesized that a minimal dose of oxygen would increase survival with no effects on other outcome measures.

1 Materials and Methods

1.1 Animals

Experiments were performed in 6-week-old adolescent male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Mice were housed under a 14h:10h light: dark schedule in pressurized individually ventilated cage racks, with 4 mice per cage, and were given ad libitum access to water and standard rodent chow. Animals habituated to the vivarium for one week prior to undergoing traumatic brain injury and subsequent procedures. The University of Cincinnati Institutional Animal Care and Use Committee approved all experimental procedures.

1.2 Traumatic Brain Injury

Closed-head injury was performed by weight drop, as previously described.\(^27, 28\) Briefly, mice were anesthetized using isoflurane (2-3\%), placed in prone position under a metal rod raised above the intact, unshaven scalp. The rod was dropped 1.5 cm, roughly above bregma (Fig 1a,b). After head trauma, mice were placed under an oxygen hood made from a pipet box connected to an oxygen tank and were exposed to 100\% oxygen (O\(_2\)) until normal breathing returned – approximately 1-5 minutes (Fig 1b), or were allowed to recover in room air. Mice were observed for recovery of righting reflex before being returned to their home cages. Injured mice not given oxygen will hereon be referred to as Room Air Mice, while sham mice will be referred to as sham. Sham animals were anesthetized,
weighed, and allowed to recover before being returned to their cages. We performed this protocol in
three cohorts of mice for three behavioral time points and various molecular and histological
measures described below and depicted in an experimental timeline in Figure 1c).

![Experimental Procedure and Timeline](image)

**Figure 1. Experimental Procedure and Timeline.** (A) For closed-head weight-drop injury, anesthetized mice are placed in the prone position on a cork platform (a) and a 400g weight (b) is dropped roughly above bregma as depicted in (B). (C) Brief oxygen exposure was induced by placing mice in a modified pipette box with a tube delivering 100% oxygen until normal breathing returned. (D) 3 cohorts of mice were used for these studies with varying optokinetic testing times and tissue collection times as shown with each of the timelines.

1.3 Behavior

1.3.1 Home cage activity monitoring

Twenty-four hours post injury, animals were weighed and separated into individual cages with
food and water, but without enrichment (e.g., no cotton bedding). Cages were situated into activity
monitors (San Diego Instruments PAS system, N=12; and Lafayette Activity Monitoring System,
N=8) set to record movements for 24–48-hour time blocks. Every 24-48 hours, mice were weighed,
and the systems were reset. This was repeated for 15 days post injury (cohort 2, fig. 1c). Monitors
malfuctioned between days 3-6 after a weekend power outage that reset the system, so
measurements are only present for days 1-2 and 7-15. The number of beam breaks were totaled
during each light or dark phase on each day.

1.3.2 Optokinetic Response (OKR) and Visual Acuity

As described in Hetzer, et al., 29 we measured optokinetic responses and visual acuity. Briefly,
mice were placed on an immobile platform surrounded by a rotating Plexiglas drum. An
exchangeable series of sine-wave gratings were placed around the inside of the drum. For these
experiments, five gratings were used (0.12, 0.26, 0.32, and 0.49 cycles per degree). Once in the
machine, mice were left for a one-minute habituation period before the machine is turned on. The
drum rotates at two revolutions per minute (rpm) for two minutes in either clockwise or
counterclockwise rotation, followed by 30 seconds rest, and two minutes rotation in the opposite
direction. Mice were exposed to one grating per day in random orders. Experimenters were blinded to
conditions during both behavior and scoring. Videos were scored by trained experimenters and the
number of optokinetic responses tallied. Two scorers reviewed each video.

1.4 Histology

1.4.1 Tissue Collection

For immunohistochemical and immunofluorescence (IHC/IF) analyses, mice were euthanized
using Fatal Plus® on days seven or 30 after TBI. Mice were perfused transcardially with 4%
paraformaldehyde in 0.02M phosphate-buffered saline (PBS) solution (pH 7.4). Brains were removed
and post-fixed in 4% paraformaldehyde in 0.02M PBS for 24 hours, rinsed in 0.01M PBS, and
immersed in 30% sucrose solution at 4°C until sectioning. Sucrose-saturated brains were frozen on
dry ice and sectioned at 30μm using a sliding microtome (Leica, Bannockburn, IL). Sections were
stored in cryoprotectant solution (0.01M PBS, polyvinyl-pyrrolidone (Sigma –Cat# PVP-40),
ethylene glycol (Fisher Cat # E178-4), and sucrose (Fisher Cat # S5-3)) at -20°C until staining was
performed. The left eye was also removed after perfusion, post-fixed in 4% PFA for 4 hours,
switched to 30% sucrose, and stored at 4°C until histological staining was performed (see Hetzer, et
al., 2021). For western blotting, the right eye was taken from mice after injection with Fatal Plus® but
before perfusion. After removal, the eye was placed in ice cold 0.01M PBS, and the retina was
removed within 5 minutes, immersed in cold lysis buffer (described below), and frozen on dry ice.
Retinas were stored at -80°C until used for western blotting.

1.4.2 Fluoro-Jade C

Fluoro-Jade C (FJ-C; Histo-Chem, Jackson, AR; CAT# 1FJC), a marker for degenerating
neurons and axons, was used to stain tissue sections according to the manufacturer’s directions
with slight modifications to avoid high background. Sections were incubated in 0.06% potassium
permanganate for 5 minutes, then submerged in 0.0001% FJ-C solution for 5 minutes. After staining,
slides air dried in the dark. Slides were stored without coverslips in a slide box and imaged
immediately to reduce background quenching of positive signals.

1.4.3 Immunofluorescence

Free-floating brain tissue was stained for polyclonal rabbit anti-glial fibrillary acidic protein
primary antibody (GFAP; DAKO, Santa Clara, CA; cat # Z0334; RRID AB_10013382) overnight at
4°C at 1:2000 dilution. On the second day, tissue was incubated with Cy-3 conjugated secondary
antibody (Jackson Immunochemicals, West Grove, PA; cat# 711-165-152, RRID AB_2307443) at
1:500 dilution for 1h at room temperature. Slides were cover slipped using the antifading polyvinyl
alcohol mounting medium gelvatol (Sigma-Aldrich, St. Louis, MO). Increased GFAP
immunoreactivity (i.e., mean florescence intensity; MFI) was measured as an indicator of
astrogliosis.

Whole retinas were flat-mounted on slides coated with Gatenby’s solution and stained
sequentially using the following primary antibodies: brain-specific homeobox/POU domain protein
3A (Brn3a; Millipore; cat #MAB1585; RRID: AB_94166) at 1:1000, then GFAP (1:1000). DAPI
nuclear staining was achieved with Vectashield Antifade Mounting Medium with DAPI (Vector
After Brn3a incubation, retinas were washed in 0.5% TX-100, incubated in anti-mouse biotinylated secondary antibody (1:400; Vector Laboratories; cat # BA-9200; RRID: AB_2336171) for 1.5 hours, incubated in VECTASTAIN Elite ABC Kit (1:800; Vector Laboratories; cat # PK-6100; RRID: AB_2336817) for 1 hour, then incubated in Cy3 streptavidin (1:500; Invitrogen, Grand Island, NY; cat # 434315) for 2 hours at room temperature covered. This was followed by GFAP overnight incubation and subsequent labeling with anti-rabbit Alexa 488 (1:500; Invitrogen; cat # A11034). GFAP staining in retinal flat mounts was inconsistent within and between conditions and thus was not analyzed.

1.5 Image Analysis

Image analysis was performed as described previously. Briefly, 10x and 20x images of left and right regions of interest (ROIs) for GFAP slides were photographed using an Axio Imager Z1 microscope with an Apotome (Leica Microsystems, Buffalo Grove, IL). For measurement of GFAP MFI, all slides were photographed using the same exposure and magnification between treatment conditions. FJ-C brain slides and retinal whole-mounts were imaged on a Nikon C2 Plus Confocal Microscope (Nikon Corporation, Melville, New York). A blinded observer took all pictures. Image analysis and quantification of GFAP MFI was also performed by a blinded investigator using ImageJ software across four non-overlapping 150x150 pixel square sections for both left and right ROIs. FJ-C positive area and Brn3a cell counts were measured using Nikon Elements Analysis software (Nikon, Melville, NY).

1.6 Western Blots

Right retinas were homogenized in freshly prepared lysis buffer (20mM Tris-HCL pH 7.4, 2mM EDTA, 0.5mM EGTA, 1mM DTT, HALT protease/phosphatase inhibitor) using a pellet homogenizer, centrifuged at 3000 rpm for 20 minutes, and supernatant removed for protein concentration analysis using a BCA protein assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific; cat # 23227). Twenty or 30 µg samples were loaded into SDS-PAGE gels and transferred onto either Amersham Hybond-P 0.45 µm PVDF membranes (GE Life Sciences, Pittsburgh, PA; CAT: GE 10600029) or 0.45µm Nitrocellulose membranes (Bio-Rad, Hercules, CA; CAT: 1620145). Membranes were incubated in Fisher No-Stain Total Protein Stain (Thermo Fisher, CAT: A44449) as per manufacturer instructions, blocked in either 5% non-fat milk or 5% BSA (manufacturer dependent) for 1 hour at room temperature, followed by overnight incubation in primary antibodies at 4ºC (see antibody table 1). Membranes were rinsed in TBST then incubated in anti-rabbit HRP for 1.5-2 hours at room temperature. Blots were imaged using an iBright™ Imaging System (Thermo Fisher) and analyzed using Image J. Some blots were stripped following imaging in stripping buffer (β-mercaptoethanol, 20% Sodium Dodecyl Sulfate, and 1M Tris-HCl pH 6.8) for 30 minutes at 50ºC, washed, re-blocked, and exposed to the same immunoblotting steps as above.

1.7 Statistical Analyses

For all analyses, alpha was set a priori to p<0.05. Weight change was analyzed using 2-way ANOVA with repeated measures (injury x day), activity monitoring by 3-way ANOVA with repeated measures (injury x day x light), mortality by student’s t-test, and seizure morbidity by X² using SigmaPlot 14 (Systat Software, San Jose, CA). Remaining analyses were performed, and results graphed, using GraphPad Prism 9 (GraphPad Software, San Diego, California). For OKR data, one-way ANOVAs were analyzed for each spatial frequency but were combined onto one graph for presentation purposes (e.g., figs 3). FJ-C data were analyzed by 2-way ANOVA. The background and exposure differences inherent in GFAP fluorescence intensity analyses, however, were only
conducive to analysis within time points. One-way ANOVA was also used for all western blot data, which was first normalized to total protein, and similarly to OKR data, some proteins were combined on the same graphs for cohesion (e.g., fig. 8-10). Supplementary data analyses are described in supplementary figure legends.

2 Results

1.1 Weight, Morbidity, & Mortality following TBI in adolescent mice

All mice were weighed for the first 7 days after injury, and mice euthanized on day 30 were weighed before euthanasia. There were main effects of injury (F_{2,221}=5.95, p=0.009) and day (F_{8,221}=62.38, p<0.001) and a significant interaction (F_{16,221}=23.28, p<0.001). Mice given O2 weighed significantly less than sham mice throughout the study (p=0.008), while mice not given O2 only weighed significantly less on day 30 (p=0.015; Fig. 2a). Both TBI groups were also significantly less active than sham animals up to day 11 (TBI:Sham p = 0.006; TBI + O2: Sham p = 0.021) with no improvement in mice given O2 (TBI: TBI + O2 p = 0.54). Analysis revealed main effects of Day (F_{10.65}=3.02, p=0.004), Injury (F_{2,65}=5.99, p=0.005), and Light vs dark (F_{1,65}=163.41, p <0.001; Fig 2b).

Oxygen exposure varied among mice (range 1.5-4.25 min, mean=2.95 min, SD=41.5 sec), but had no effect on duration of agonal breathing (p= 0.82; Sup. Fig. 1a) with no correlation between these measures (p=0.89; Sup. Fig. 1b). Oxygen exposure also did not affect righting times (p = 0.16 Sup. Fig. 1c) with no correlation between agonal breathing and righting time Sup. Fig. 1d).

Strikingly, mortality was significantly reduced in mice given O2 from 50.9% (no O2) to 13.3% (X^2=10.47, p = 0.001; Fig. 2c). Despite our initial thinking that oxygen would improve/shorten
agonal breathing times, labored breathing time was not altered in mice given O₂ (t=0.22, p=0.82, M(TBI)=1.3 min, M(+O₂)=1.4min), nor was righting time (t=1.48, p=0.16, M(TBI)=11.2 min, M(+O₂)=11.4min). The duration of oxygen exposure was also not correlated with righting time (r=0.15, p=0.53). However, we noted that oxygen exposure significantly reduced the likelihood of dying if a mouse had apparent seizures after TBI, from 64.8% to 41.2% (X²=12.22, p = 0.007; Fig. 2d). Data were square transformed to pass normality and equal variance.

1.2 Injured mice have a blunted optokinetic response, and oxygen shows partial rescue acutely.

Within the first 7 days after TBI, injured mice have a significantly blunted optokinetic response across all spatial frequencies assessed: 0.12 cpd (p<0.001), 0.24 cpd (p<0.001), 0.32 cpd (p<0.001), 0.39 cpd (p=0.008), 0.46 cpd (p=0.002). Mice given oxygen performed similarly to sham mice (p=0.1) while TBI mice were significantly impaired (p<0.001) at 0.12 cpd. At 0.24 cpd, although both injured groups had blunted OKR responses compared to sham, mice given oxygen performed significantly better than TBI mice (p<0.05). At 0.32, 0.39, and 0.46 cpd both injured groups had significantly blunted OKRs (see fig 3a). Although both injured groups were impaired compared to sham mice at the upper threshold of rodent visual acuity (0.46 cpd), it is worth noting that 13 of the 24 sham mice still produced an OKR while none of the injured mice did, indicating a reduction in not just overall optokinetic function, but also potentially visual acuity for mice not given O₂.

Mice were also tested 3 weeks post injury at which time the benefits of oxygen and the overall severity of injury appear reduced. Effects of injury were now only observed in mice given oxygen at 0.24 cpd (p=0.03) and both TBI (p=0.02) and TBI +O₂ (p=0.005) versus sham mice at 0.39 cpd. Of note, there were fewer responses even in sham mice compared to week 1 (fig. 3b). At 4 weeks, sham mice outperformed TBI mice at 0.24, 0.39, and 0.46 cpd (p=0.03, p=0.003, and p=0.001.

Figure 3. Oxygen modestly rescues acute OKR, but does not prevent sub-acute decline. (A) OKR was measured in the first week after TBI revealing significant impairment at each cpd in injured mice. TBI +O₂ mice performed significantly better than Room Air counterparts at 0.12 and 0.24 cpd (blue boxes). (B) Protective effects were no longer present at 3 weeks post injury or (C) 4 weeks post injury. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.
respectively for room air and \( p=0.02, p=0.006, \) and \( p<0.001 \) respectively for \( \text{O}_2 \)). Animals performed similarly with no significant reduction in OKRs in either injured group at 0.12 or 0.32 cpd (\( p=0.1 \) and \( p=0.3\)).

**Figure 4.** Oxygen prevents acute loss of central retinal cells but not overall loss by 30 days. RGCs were measured in three quadrants of the retina – (A) peripheral, (B) mid-peripheral, and (C) central 7 days post injury. Representative photomicrographs taken at 20x for (D) peripheral RGCs (red) and DAPI (blue) of sham, Room Air, and TBI +O2 mice and (E) central RGCs respectively. From cohorts 2 and 3, 30-day retinas were also stained for Brn3a (red) and analyzed in (F) peripheral, (G) mid-peripheral, and (H) central quadrants. Scale bars represent 100µm. *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \), ****\( p<0.0001 \).
1.3 Oxygen slows acute RGC loss but does not ultimately prevent loss of RGCs.

In retinal tissue collected 7 days after injury, cell counts were analyzed in three regions of the retina in order to assess loss throughout. In the periphery, injury resulted in a significant reduction in Brn3a positive cells ($F_{2,42}=14.89, p<0.001$; Fig. 4a,d) in both TBI ($p=0.001$) and TBI +O$_2$ mice ($p<0.001$) compared to sham. The same was true in the mid-peripheral retina with a main effect of injury ($F_{2,42}=4.71, p=0.01$; Fig. 4b) in both TBI ($p=0.04$) and TBI +O$_2$ mice ($p=0.04$) compared to sham. In the center, however, only TBI mice had significantly fewer RGCs than sham ($p<0.001$) while mice given O$_2$ retained significantly more cells than their injured counterparts ($p=0.01$) and had similar numbers to sham ($p=0.4$; main effect $F_{2,42}=12.84, p<0.001$; fig. 4c,e).

By thirty days, we found similar reduction in Brn3a positive cells in both injured groups, TBI ($p=0.008$) and TBI+O$_2$ ($p<0.001$), compared to sham ($F_{2,42}=12.01, p<0.001$) in the periphery (Fig. 4f). Similar effects were seen in the mid periphery with a main effect of injury ($F_{2,42}=14.58, p<0.001$) in TBI ($p<0.001$) and TBI+O$_2$ ($p<0.001$) versus sham (Fig. 4g). Interestingly, there were no differences between groups in the central retina ($F_{2,42}=0.58, p=0.5$; Fig. 4h).

1.4 Oxygen exposure does not prevent degeneration but may accelerate recovery.

We examined retinal cell projections in the brain for evidence of axonal degeneration using FJ-C and compared tissue from days 7 and 30 in order to assess acute and long-term effects of O$_2$ on TBI. Beginning in the optic tract (OT), we found a main effect of injury ($F_{2,51}=52.66, p<0.001$) and a significant interaction ($F_{2,52}=11.97, p<0.001$) that both injury groups showed significantly higher GFAP immunofluorescence than their sham counterparts ($p<0.001$) and TBI +O$_2$ mice had significantly higher GFAP expression than Room Air mice. By day 30, sham was still significantly lower than TBI ($p<0.001$) and TBI +O$_2$ ($p<0.001$), but there were no differences between injury groups. There was however a significant increase in FJ positive area in TBI mice from 7 to 30 days ($p<0.001$) while TBI + oxygen mice had reduced levels by 30 days ($p<0.001$). Other regions associated with vision including the lateral geniculate nucleus and superior colliculi were also examined and data are presented in the supplementary files associated with this manuscript.

1.5 Astroglial reactivity is significantly reduced in the brain following O$_2$ exposure.

To determine whether oxygen exposure affected astrocytic responses, we stained brain tissue for GFAP and examined retinal projection regions for increased mean fluorescence intensity (MFI) of GFAP staining. In the optic tract, the pattern of increased fluorescence paralleled the axonal degeneration found with FJ-C. At day 7 there was a main effect of injury ($F_{2,33}=97.4, p<0.001$) such that both injury groups showed significantly higher GFAP immunofluorescence than their sham counterparts ($p<0.001$) and TBI +O$_2$ mice had significantly higher GFAP expression than Room Air mice. By day 30 the injury effect ($F_{2,22}=4.27, p=0.03$) was driven only by the difference between Room Air and Sham mice ($p=0.03$) while TBI +O$_2$ mice were not different than their sham counterparts ($p=0.2$; Fig. 6). We also examined microglia morphological changes but found no
effects of oxygen nor any differing results from our previous publications, so data were not included in this manuscript.

1.6 Oxygen exposure promotes IRE-1α pathway activation acutely.

Retinas taken on day seven after TBI showed only significant increases in Inositol Requiring Enzyme 1α (IRE1α) pathway activation in mice given oxygen (p=0.04) compared to sham and room air-exposed mice. There was no detectible increase in the downstream X-box Binding Protein 1 (XBPIu; F_{2,20}=0.75, p=0.5), its splice variant XBPIs (F_{2,20}=0.79 p=0.47), or the ratio of the two within subjects (F_{2,20}=0.5 p=0.6); Fig. 7). By day 30, however, more differences arose in the IRE1α arm of the ER stress response. IRE1α was significantly increased (F_{2,19}=7.3 p=0.004), largely driven by injured Room Air mice (p=0.003) compared to uninjured mice. Total XBPI expression was not altered (F_{2,28}=1.5 p=0.24), but XBPIs showed significant increases in both Room Air (p=0.01) and TBI +O₂ mice (p=0.009) compared to sham animals. In addition, the ratio of total to spliced XBPI
showed significantly higher levels of the spliced form in Room Air (p=0.04) and TBI +O2 mice (p=0.002; Fig 7).

1.7 The PERK pathway is sub-acute elevated after TBI, but supplemental oxygen reduces long-term expression of pro-apoptotic markers.

Seven days post injury the activators associated with the Protein Kinase R-like ER Kinase (PERK) branch of ER stress were similar across groups with no increases in PERK (F(2,21)=0.66 p=0.5), phosphorylated (p)-PERK (F(2,21)=0.22 p=0.8), or the ratio of the two (p=0.12) nor in eukaryotic translation initiation factor (eIF2α; F(2,21)=0.52 p=0.6), p-eIF2α (F(2,21)=0.52 p=0.6), or its ratio (p=0.08; Fig. 8a). Yet, subsequent downstream ER stress factors were elevated in injured mice. Although total activating transcription factor 4 (ATF4) remained stable (F(2.21)=2.87 p=0.08), there was a significant increase in p-ATF4 (F(2,21)=6.24, p=0.007), between sham vs. Room Air (p=0.02) and Room Air vs. TBI +O2 p=0.01. Upon further analysis, this overall increase in phosphorylation in Room Air mice was due to proportional levels of total ATF4 (ATF4 ratio vs. sham p=0.25) while TBI +O2 mouse retinas contained significantly higher phosphorylated levels vs. sham (p=0.005; Fig 8b). In line with this finding, the ER-stress associated apoptotic factor C/EBP homologous protein (CHOP) was only significantly increased in mice given oxygen compared to those not.

Thirty days post injury, PERK pathway activation remained elevated in Room Air mice, but mice given oxygen begin to show adaptive responses (Fig 9). Although total PERK levels are
increased in both injured groups (TBI Room Air p=0.01, TBI +O\textsubscript{2} p=0.01 vs sham), p-PERK was unaltered (F\textsubscript{2,32}=0.14 p=0.87) and the ratio of the two unaffected by oxygen manipulations (F\textsubscript{2,28}=1.56 p=0.23). Yet, the downstream kinase eIF2\textalpha had elevated total protein (F\textsubscript{2,31}=7.9 p=0.002) and phosphorylated protein (F\textsubscript{2,30}=11.21, p<0.001). Post-hoc analyses revealed that this was driven by increases in TBI +O\textsubscript{2} mice (p=0.005 and p<0.001 respectively). This increase not only differentiated them from sham animals, but also from room air mice (p=0.003 and p=0.001 respectively). At this time point, the downstream transcription factor ATF4 was no longer elevated in either injured cohort (total ATF4 F\textsubscript{2,17}=1.46 p=0.26; p-ATF4 F\textsubscript{2,17}=3.2 p=0.06), but CHOP remained elevated (F\textsubscript{2,8}=9.4 p=0.008) in Room Air mice compared to sham animals (p=0.01) and TBI +O\textsubscript{2} mice (p=0.01). Post-hoc probing of retinal tissue for the PERK arm’s feedback machinery, growth arrest and DNA damage-inducible protein 34 (GADD34), did not reveal any significant findings (F\textsubscript{2,26}=1.8 p=0.18, Sup. Fig. 6).

We also asked whether oxygen might have an effect on the redox-sensitive protein folding machinery of the ER. ERO1\textalpha levels were unaltered at day 7 (p=0.52; Fig. 10a, c), but oxygen appears to have a delayed effect as far out from injury as 30 day (F\textsubscript{2,18}=4.19, p=0.03); ERO1\textalpha levels are reduced compared to sham mice (p=0.03; Fig. 10b, d).

Figure 7. IRE1\textalpha Pathway activation after oxygen exposure. (A) Relative densities of total IRE1\textalpha, XBP1 unspliced, and XBP1 spliced show acute activation only in mice given O\textsubscript{2} in 7-day tissue. (B) A representative blot of IRE1\textalpha shows darker bands at the predicted 100 kDa molecular weight in mice given O\textsubscript{2}. (C) Shows a total protein blot image, which was used for normalization. (D) Representative blots of XBP1s at ~55kDa and XBP1u at ~38 kDa with their associated (E) total protein stain are given. (F) Shows similar data in 30-day retinal tissue now with significant elevation of the IRE1\textalpha -ERAD pathway in both injured groups. Where not only total expression of XBP1s was found, but the (G) ratio of spliced to unspliced was significant. (H-I) Representative blots for IRE1\textalpha and total protein are given along with (J,L) XBP1s, and (K,M) XBP1u. C=control lane for intermembrane control brain homogenate, H = water negative control lane, X= lane not measured, kDa = Killa Dalton. *p<0.05, **p<0.01
We previously found that briefly exposing adolescent mice to oxygen after TBI reduced mortality by 40% compared to adult mice. We now show that oxygen partially rescues optokinetic dysfunction acutely, and these early beneficial effects seem to delay the harmful effects of TBI as time goes on. Moreover, astrocytes showed decreased GFAP expression at both 7- and 30-days in mice given oxygen, with reduction in some regions comparable to uninjured mice. We show a potential mechanism through which oxygen exerts these protective effects via activation of adaptive ER stress responses (e.g., XBP1s) and later suppression of apoptotic factors (i.e., CHOP).

### 3.1 Supplemental oxygen reduces mortality

Apnea or agonal breathing is associated with closed-head injury. Interestingly, deaths from apnea are rarely reported in mild-moderate injury paradigms, and only a handful of studies mention use of oxygen to counteract this injury phenotype. Despite the occasional acknowledgement of brief oxygen exposure to help mice survive, little literature has explored the effects of supplemental oxygen to improve survival. Importantly, none of these studies explored how brief oxygen exposure alone might alter injury outcomes. In our previous studies, we observed that mice with prolonged apnea were less likely to recover from the initial impact injury. We found that increasing inhaled oxygen content...
In the current study, we examined other potential consequences of keeping these mice alive who might have otherwise not survived injury. Despite the noted survival increase, there were no correlations between agonal (i.e., labored/shallow) breathing and the time spent in the oxygen chamber, nor did O₂ reduce righting times, suggesting that supplemental oxygen, while improving survivability, did not change recovery of breathing patterns after TBI.

It is important to note that we did not control the exact duration or dosage of oxygen in this study and a few studies show that varying oxygen dosage can alter outcomes. For example, it was shown that in neonates there was a decreased risk of mortality when given only 21% oxygen versus giving 100% oxygen during neonatal resuscitation. Furthermore, a study in rats observed a higher saturation of oxygen (91-95%) seems to be safer than targeting a lower saturation of oxygen (85-89%), with respect to blood cell oxidative stress measures. Future studies should explore varying oxygen exposure taking these factors into consideration. Importantly, these studies should note that too lengthy an exposure to 100% oxygen is associated with oxygen toxicity, particularly in the retina, while a smaller dose of oxygen (i.e., 50-70%) is associated with more beneficial effects without further damage to visual acuity.

### 3.2 Optokinetic Response Outcomes

In addition to the survival benefit, we found behavioral evidence that brief oxygen exposure improved visual performance. We examined the involuntary visually evoked optokinetic nystagmus...
response which is blunted in our model, likely due to optic nerve degeneration and retinal ganglion cell loss observed post-injury.\textsuperscript{15} Optokinetic nystagmus presents as an easily distinguishable movement of the head in line with a rotating drum holding a visual grating. Injured mice have a blunted OKR at all time points examined and for most spatial frequencies, which were additionally used to assess visual acuity. Mice given supplemental oxygen, though, perform similarly to sham mice acutely (assessed during the first week after injury). This acute protection prevented the significant decline in responses indicating that oxygen may have slowed the loss of retina-to-brain connections allowing the OKR to remain functional. Despite this, there was no improvement/retention of visual acuity (shown by zero responses recorded) in both injured groups compared to shams at 0.46 cpd. This distinction becomes relevant when dissecting injury outcomes because acuity can also be affected by loss of photoreceptors.\textsuperscript{41, 42} We did not assess changes in photoreceptors in this study, but future studies should consider whether loss of RGCs is associated with photoreceptor dysfunction in addition to overt RGC loss.

By 30 days after injury, both injured groups have blunted OKRs and reduced visual acuity, and there is no longer a significant difference between mice given supplemental oxygen vs room air only. These results could potentially be explained by RGC cell counts—Brn3a positive cells in the central retina are more numerous in O\textsubscript{2} exposed mice than in room air mice at day 7. By 30 DPI, this pattern has changed, with the three groups being indistinguishable in central retinal cell counts. This finding of similar RGC counts in the central retina at 30 DPI could be due to natural decline in RGCs as mice age,\textsuperscript{43} the prevalence of blood vessels in this region that can limit the number of countable cells, or the fact that Brn3a does not label all RGCs. Future studies could use additional RGC markers to determine whether some cell types are impervious to this decline (e.g. melanopsin expressing RGCs\textsuperscript{44}).

### 3.3 Histology

FJ-C histology suggests that optic nerve axonal degeneration is higher 7 days after injury in mice given oxygen. But how could there be increased axonal degeneration with more surviving RGCs? Previous micro-CT data\textsuperscript{28} coupled with a lack of positive amyloid precursor staining in the brains of our

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**Figure 10.** ERO1L\textalpha reduction 30 days post injury after brief oxygen exposure. (A) Analysis of ERO1L\textalpha marker at 7 days post injury. There are no significant differences between sham, Room Air mice, and mice given oxygen. (B) Mice given oxygen have a significant decrease in oxidoreductase marker ERO1L\textalpha compared to sham mice. (C) Representative blot of ERO1L\textalpha at 7 days post injury. (D) Representative blot of ERO1L\textalpha at 30 days post injury. C=control lane for intermembrane control brain homogenate, X= lane not measured, kDa = Killa Dalton. *p<0.05
mice (data not published) suggests that the location of axon injury in our model is likely inside, or near, the intracanalicular portion of the optic canal. This means that the damage to the axons is occurring between the RGC cell bodies in the eye and their projection targets in the brain. Thus, these data are consistent with recent literature distinguishing the distal Wallerian response to axon injury from proximal cell signaling.\(^{45}\) Wallerian Degeneration (WD) is a mechanism of distal axon degeneration that occurs when a damaged segment of axon is severed and cleared after injury, but it describes little as to why this might cause a cell to survive or become apoptotic. Some cells can survive injury if only a branch of the axon is damaged;\(^{46}\) these cells release fewer death-associated factors after injury, potentially saving cells despite loss of their distal portion. WD also involves several redox-associated mechanisms which might be sensitive to alterations in oxygen delivery, thus leading to the worse distal axon degeneration that we see acutely in room air mice.

Moreover, due to the slow nature of WD in the central nervous system, there may be less potential for axon regeneration without intervention, as compared to the peripheral nervous system. WD mechanisms are tightly linked with the redox state of the axon/injured cell\(^ {37}\) and a mutant mouse with slowed WD is purported to have increased neuroprotection from axon injury due to increased NAD+ activation and thus to decreased oxidative stress.\(^ {38,49}\)

Hypoxia, cerebral edema, and increased intracranial/intraocular pressure all induce oxidative stress.\(^ {48}\) Although our model of TBI is not a hypoxia model, and we have not measured intraocular pressure, the duration of apnea, compression of the orbital bone over the optic nerve, and likely decreased oxygen supply to the brain for a brief period of time could lead to toxic oxidative reactions. Therefore, it is reasonable to ask whether this brief oxygen intervention alters some of the WD mechanisms of axon degeneration to speed up the degeneration of those cells more likely to die (i.e., leading to a fast peak degeneration at day 7) but promoting survival in those that were “savable” (i.e., leading to decreased degeneration by day 30). Future research will need to determine the role of oxidative stress in this model and whether brief oxygen exposure is sufficient to alter this state in the axons.

Another explanation for our results could be that the time points examined do not represent peaks and troughs of degeneration in each group. It is possible that degeneration in the room air mice is progressing at a slower rate, peaking some time before, or around, day 30 where we see higher amounts of FJ-B staining compared to injured O\(_2\) mice. Conversely, day 7 may represent the peak of degeneration for O\(_2\) mice, which could have been accelerated and then diminished by day 30. It is also possible that cell death was not truly affected, and the reduction in FJ-B in oxygen mice is simply the result of improved glial-mediated clearance of axonal debris or further slowing of WD processes. Indeed, we and others have discussed the potentially dysfunctional role of glia after TBI, and we have shown that reactive glia fail to clear axonal debris chronically throughout the visual system.\(^ {50}\) Future research will need to examine tissue at later time points and consider interactions with other cell types to determine whether this reduction given oxygen is associated with more efficient phagocytosis in glial cells. Future research will also need to trace which axons may be resilient to this injury and whether they do eventually die off due to aberrant secondary injury cascades.

Though our intervention is not a hyperbaric chamber, hyperbaric oxygen therapy [HBOT] reduces neuron loss and suppresses microglial inflammatory responses but aggravates astrocyte activation in an animal model of hypoxia.\(^ {51}\) Conversely, in pain models using HBOT, there is reduction of astroglial activation.\(^ {52,53}\) This disease-specific/region-specific response of astrocytes to changing redox environments suggests that a difference in injury or oxygen concentration might lead to unique cellular responses. In particular, we were interested in astrocytes because they are attuned to decreases in oxygen content as small as a few mm of Hg\(^ {54}\) and have been shown to increase activity under oxygen deprivation.\(^ {17}\) In this study, astrocyte reactivity was significantly decreased in mice given oxygen compared to sham animals in nearly all optic-associated brain regions examined at 7 days post injury. But why would this reduced activation state be sustained up to 7 days and subsequently return by 30
days? Perhaps oxygen served less as a direct intervention and more as preconditioning. Indeed, astrocytes pre-exposed to hyperoxia express more glutamate transporters, poising them to more readily clear excess glutamate from synapses, a common event after TBI. Much more research into the mechanistic role of oxygen exposure in the eye and brain are needed to answer these questions, though.

### 3.4 ER Stress

Because of the relationship between hypoxia and oxidative stress, we examined a cellular stress-response mechanism that is sensitive to changes in the oxidative environment and that is elevated after TBI, i.e., ER stress results in a dynamic range of transcriptional and translational signals in response to any disturbance of the protein folding capabilities of the ER. This response is activated when any of three associated receptors – inositol-requiring enzyme 1α (IRE1α), PRK-like Endoplasmic Reticulum Kinase (PERK), or activating transcription factor 6 (ATF6) – senses an abundance of misfolded proteins in the ER lumen. Each of these receptors can activate a signaling cascade to make acute corrections, prolong adaptive change, or induce apoptosis when the burden is too great. Targeting ER stress responses after TBI is predominantly associated with reduced neuron loss/apoptosis, most often in connection with the suppression of IRE1α and PERK branches. Adaptive ER stress responses include the shunting of protein translation by phosphorylation of eIF2α, translation of ATF4, and activation of mRNA splicing XBP1s. Each of these proteins reduces the burden on the ER by preventing new protein translation, upregulation of antioxidant and biogenic protein translation, or destruction of premature mRNA, but their roles in chronic activation are unknown. ER stress-mediated apoptosis, on the other hand, typically subsides in the acute phase as cells that are too damaged are destroyed.

Therefore, much of what we know about ER stress in living organisms focuses on our understanding of acute adaptive responses and apoptosis, leaving a gap in understanding the effects of long-term activation. Our results suggest that ER stress is likely involved in RGC cell fate mechanisms at least 30 days post injury. Injured mice showed increased PERK pathway activation via upregulated ATF4 translation (i.e., total ATF4) but not phosphorylation. This distinction is important because increases in ATF4 translation can be both adaptive (e.g., induction of antioxidant defense) or apoptotic (i.e., activation of CHOP) and because phosphorylation of ATF4 is associated with its degradation and the termination of ER stress activation. Thus, at day 7, ER stress continues in injured mice but may be declining in mice given oxygen. We attempted to further test this hypothesis by measuring PERK pathway feedback using GADD153, which dephosphorylates eIF2α after ER stress subsides. These data were not significant (sup. Fig. 6), but examination of other time points may prove informative.

Although the pro-apoptotic CHOP is still higher in O₂ mice than in controls at 7 days, an adaptive (rather than apoptotic) effect of oxygen is supported by the normalization of CHOP expression at 30-days after injury, as compared to room air mice. Further support for an adaptive effect of oxygen treatment comes from the early upregulation of the IRE1α pathway, which is associated with attempts to decrease/protect against prolonged ER stress. With room air only, this pathway’s response may be delayed as elevated CHOP expression is not seen until 30 days. It will be important for future studies to determine more about this flux in ER stress pathway responses across time since retinal cells appear to remain stressed even though apoptosis mostly subsides between 7-30 days based on Brn3a cell counts.

A related cellular stress pathway relevant to these studies is oxidative stress. In studies assessing effects of brief, acute oxygen exposure, toxicity is correlated with both the concentration of oxygen delivered and the strain imposed on the pulmonary system (e.g., in patients with chronic obstructive pulmonary disease) during exposure. A more taxed pulmonary system, when briefly exposed to oxygen, produces fewer reactive oxygen species than when no oxygen is supplied, significantly reducing free radicals in the lungs. We may thus be able to ask whether brief oxygen exposure is reducing the increased expression of reactive oxygen species typically associated with TBI (e.g., 48-71).
If this is the case, reduced ROS could explain the shift to an adaptive ER over an apoptotic one, due to the overlap of these two systems. Although not a direct measure of free radicals or antioxidant response, we examined protein expression for the highly redox-regulated protein folding machinery of the ER, ERO1Lα. Oxygen significantly reduces ERO1Lα even compared to control mice. In cases of antioxidant interventions, ERO1Lα reduction is related to reduced free-radical formation and a reduction in ER burden. Although little research has been done in disease models to explain the interactions between ER stress and oxidative stress, the two pathways overlap heavily with both upstream and downstream effectors (e.g., integrated stress responses to hypoxia, mitochondrial associations, PERK, and the JNK pathway) that could reasonably control either mechanism (e.g. ).

Given the acute and chronic effects of this minor reduction of oxidative burden on the CNS in our mice given oxygen, future studies will need to consider the role of ROS and begin unraveling the mechanisms of ER stress and oxidative stress in the context of TBI/axon injury.

4 Conclusion

In conclusion, supplemental oxygen might be advantageous after TBI in more ways than simply improving the post-TBI apneic period and enabling increased survival. Several mouse models of TBI report the use of need for supplemental oxygen after TBIs of various severities to promote survival due to this prolonged apnea. However, the potential for other effects of oxygen alone has not yet been thoroughly examined. The current data supports a need to better understand the effects of such oxygen used for post-injury resuscitation. This study also suggests several potential mechanisms through which oxygen may be affecting recovery. These include changes in oxidative and/or endoplasmic reticulum stress responses. Understanding the relationship between oxygen and retinal ER stress responses could potentially make it a target for interventions to improve axonal injury after TBI.

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