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Increased Carbon Dioxide Respiration Prevents the Effects of Acceleration/Deceleration Elicited Mild Traumatic Brain Injury

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Abstract—Acceleration/deceleration forces are a common component of various causes of mild traumatic brain injury (mTBI) and result in strain and shear forces on brain tissue. A small quantifiable volume dubbed the compensatory reserve volume (CRV) permits energy transmission to brain tissue during acceleration/deceleration events. The CRV is principally regulated by cerebral blood flow (CBF) and CBF is primarily determined by the concentration of inspired carbon dioxide (CO_2). We hypothesized that experimental hypercapnia (i.e. increased inspired concentration of CO_2) may act to prevent and mitigate the actions of acceleration/deceleration-induced TBI. To determine these effects C57Bl/6 mice underwent experimental hypercapnia whereby they were exposed to medical-grade atmospheric air or 5% CO_2 immediately prior to an acceleration/deceleration-induced mTBI paradigm. mTBI results in significant increases in righting reflex time (RRT), reductions in core body temperature, and reductions in general locomotor activity-three hours post injury (hpi). Experimental hypercapnia immediately preceding mTBI was found to prevent mTBI-induced increases in RRT and reductions in core body temperature and general locomotor activity. Ribonucleic acid (RNA) sequencing conducted four hpi revealed that CO_2 exposure prevented mTBI-induced transcriptional alterations of several targets related to oxidative stress, immune, and inflammatory signaling. Quantitative real-time PCR analysis confirmed the prevention of mTBI-induced increases in mitogen-activated protein kinase kinase kinase 6 and metallothionein-2. These initial proof of concept studies reveal that increases in inspired CO_2 mitigate the detrimental contributions of acceleration/deceleration events in mTBI and may feasibly be translated in the future to humans using a medical device seeking to prevent mTBI among high-risk groups.

Key words: Traumatic brain injury, Carbon dioxide, Hypercapnia, Compensatory reserve volume, Acceleration/deceleration.

INTRODUCTION

Traumatic brain injury (TBI) is a serious public health concern contributing to over 60 thousand deaths in the United States alone each year (CDC, 2018–2019). Mild TBI (mTBI) and/or concussion account for the vast majority of TBIs incurred annually (Sussman et al., 2018). It is estimated that nearly 25 % of TBI patients have accompanying symptoms persisting for greater than 3 months post-injury (Zemek et al., 2016; Ledreux et al., 2020). These symptoms include cognitive and psychiatric complications such as impaired memory (Chadwick et al., 2021), major depressive disorder (MDD) (Bombardier et al., 2010), anxiety (Bradbury et al., 2021), altered sociability (Anderson et al., 2012), and also can include sleep disturbances (Wolfe et al., 2018), fatigue (Andelic et al., 2021), and persistent, intractable headache (Ledreux et al., 2020; Phipps et al., 2020). Combined, these effects can significantly alter day-to-day functionality and impair the quality of life for those diagnosed with TBI. Currently, there exists a lack of any FDA-approved therapeutics for acute TBI, and therapeutics targeting the aforementioned chronic complications associated with head injury notoriously lack sufficient efficacy (Doppenberg et al., 2004; Ashman et al., 2009; Fann

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Abbreviations: BT, core body temperature; CBF, cerebral blood flow; CO_2, carbon dioxide; CRV, compensatory reserve volume; DEG, differentially expressed gene; GESC, Genomics, epigenomics, and sequencing core; ICP, intracranial pressure; iGEAK, interactive gene expression analysis kit; IJVC, internal jugular vein compression; mTBI, mild Traumatic Brain Injury; RHI, repetitive head impact; RRT, righting reflex time; TBI, Traumatic Brain Injury.

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et al., 2017; Kreitzer et al., 2019). Given the large-scale prevalence of mTBI, our current preventative measures to reduce the rates of head injury are clearly lacking, highlighting a need for an effective method/device designed to prevent brain injury.

To date, helmets (of various designs/technologies) have been the primary external manner of energy/force dissipation for the prevention of TBI among contact sports athletes and other high-risk groups. However, helmets do not convey the required levels of protection to fully prevent mTBI (Sone et al., 2017), and may even exacerbate blast wave exposures through the concentration and propagation of blast overpressures to biologic tissues during specific environmental exposures encountered during military service (Daneshvar et al., 2011; Ganpule et al., 2012; Skotak et al., 2020). The environmental exposures resulting in mTBI are extremely heterogeneous in nature and many causes of mTBI impart acceleration/deceleration inertial forces (linear and rotational) that result in significant strain and shear forces throughout brain tissue (Zhang et al., 2006; Yoganandan et al., 2008; Meaney and Smith, 2011). The transfer of mechanical energy from the environment to brain tissue by these forces generates significant shear-induced tissue damage, an effect greatly increasing the likelihood of unconsciousness (Ommaya and Gennarelli, 1974; Meaney and Smith, 2011). Mitigating the transmission of these forces to brain tissue has been shown to minimize the effects of repetitive head impact (RHI) (Smith et al., 2011; Turner et al., 2012; Myer et al., 2016a, 2016b; Yuan et al., 2017; Yuan et al., 2018; Myer et al., 2019). The recently developed Q-Collar™ utilizes this principle and has received FDA commercial authorization for the prevention of the effects of RHI. The Q-Collar functions by using internal jugular vein compression (IJVC) to result in mild backflow into the cerebral venous capacitance vessels, thereby exhausting the compensatory reserve volume (CRV) of the cranial space and resulting in mild increases in intracranial pressure (ICP) (Smith et al., 2011; Turner et al., 2012; Myer et al., 2016a, 2016b; Yuan et al., 2017; Yuan et al., 2018; Myer et al., 2019).

Although IJVC is one particular mechanism by which to minimize the CRV, the most powerful biologic determinant of cerebral blood flow (CBF), cerebral blood volume, and therefore the CRV is the concentration of inspired carbon dioxide (CO_2) (Asgari et al., 2011). Relatively small increases in inspired environmental CO_2 above the 0.04 % concentration in our atmosphere result in increased bloodstream levels of CO_2 (i.e. hypercapnia ($PaCO_2 \geq 44$ mmHg), increased respiratory end-tidal CO_2, and increased CBF (Kety and Schmidt, 1948; Grubb et al., 1974; Rostrup et al., 1994; Asgari et al., 2011; Sato et al., 2012; Mader et al., 2018; Ogoh, 2019). Further, increases in CBF elicited by experimental hypercapnia act to increase cerebral stiffness (Kreft et al., 2020). These responses, known collectively as "CO_2 reactivity", stem from the well-established ability of the cerebral arterial vasculature to dilate or contract in response to increases or decreases in arterial $PaCO_2$ (Kety and Schmidt, 1948; Raichle and Plum, 1972; Brian, 1998; Godoy et al., 2017). The ability for CO_2 to modulate CBF is well understood, with reports demonstrating that every 1 mmHg increase in $PaCO_2$ results in a 1–2 mL/100 g/min increase in CBF (Grubb et al., 1974). We, therefore, hypothesized that the inherent, biologically driven effects of CO_2 reactivity would prevent the effects of acceleration/deceleration-induced mTBI by minimizing the transfer of energy from external forces to the brain tissue.

Studies contained herein are the first proof of concept studies, aimed at delineating whether harnessing the innate biologic effects of CO_2 reactivity are effective in preventing the detrimental effects of acceleration/deceleration-induced mTBI. Importantly, our studies provide a biologically relevant, novel, and potentially targetable method of mitigating the effects of acceleration/deceleration events that provides a high level of feasibility for translation for the potential future use of a medical device for the prevention of mTBI among high- and at-risk groups.

EXPERIMENTAL PROCEDURES

Animals

Male and female C57BL/6 mice from Jackson Laboratories (stock no: 000664) were group-housed ($\leq$4/cage) with food and water provided *ad libitum*, with a 14:10 h light–dark cycle. Mice were transferred to the animal facility at the University of Cincinnati at 8 weeks of age and acclimated to the animal facility for at least one week. All mice were between 9 and 16 weeks of age at the time of use. Each experiment was conducted after at least a 30-minute acclimation period to the specific testing facility. All procedures were conducted in accordance with the University of Cincinnati Animal Care and Use Program (ACUP), and the standards set by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati, protocol number 20–11-05–01.

Variable gas exposure

Large increases in respired levels of CO_2, such as those utilized for preclinical and clinical hypercapnia studies may elicit detrimental physiological effects that include dyspnea, headache, confusion, and lethargy (Patel et al., 2022). These physiologic effects occur at CO_2 levels many times greater than that required for changes in CBF and manipulation of CRV (Ocamoto et al., 2021). Studies herein, in serving as initial proof of concept studies and in maintaining current field standards, utilized a high level of CO_2 exposure (i.e. 5 % CO_2) that induce readily observable hypercapnic responses in rodents (Bissonnette and Knopp, 2004; Mestre et al., 2020). Exposure of animals to increased environmental CO_2 utilized a constant perfusion exposure chamber (28 cm $\times$ 28 cm) (Fig. 1(**B**)) designed in collaboration with experts in the field of rodent CO_2 exposure (Vollmer et al., 2016; McMurray et al., 2020). This exposure apparatus allows for gas exposure through a diffuser so as not to disturb the animal with any perceivable gas flow. The cham-

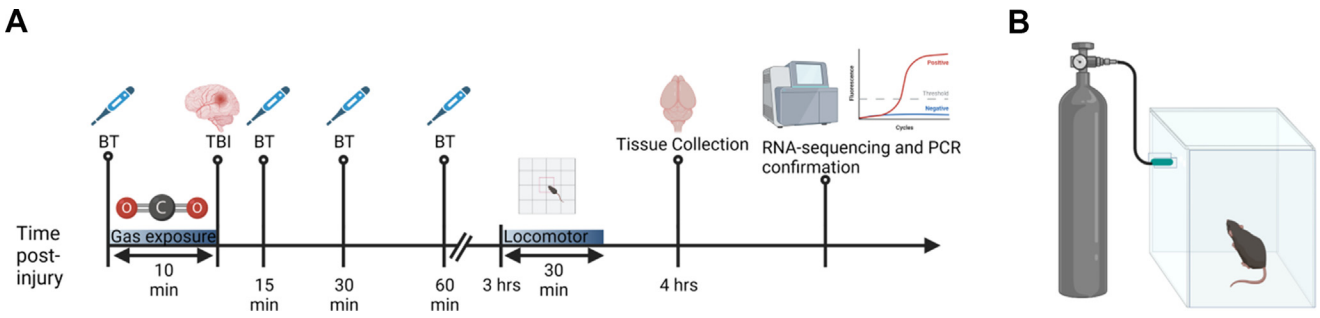


Fig. 1. Experiment timeline and gas exposure chamber. (**A**) Outline of experiment timeline. Baseline core body temperature (BT) is taken immediately prior to gas exposure and subsequent TBI or sham injury. BT readings are also taken at 15, 30, and 60 minutes post-injury. 3 hours post-injury open-field locomotor activity is assessed. 4 hours post-injury tissue samples are collected for RNA sequencing and/or qRT-PCR analysis. (**B**) Gas exposure apparatus consisting of compressed medical-grade atmospheric air or 5% CO_2 mix diffused into a clear acrylic chamber.

ber is clear to allow observation of mice during exposure periods. Briefly, mice were placed into the chamber with constant perfusion of either medical-grade atmospheric air (21 % O_2, 79 % N_2, Airgas #USP200) or a 5 % CO_2 mix (5 % CO_2, 20 % O_2, 75 % N_2, Airgas #X03NI75C2000251) for a period of 10 min. During gas exposure animals were continuously monitored using ANY-Maze video tracking software (Stoelting Co., Wood Dale, IL), and time spent freezing (complete lack of movement for at least 1 s), number of freezing events, and locomotor activity were assessed and analyzed.

Low-Intensity blast induced model for TBI allowing cranial Acceleration/Deceleration

Immediately following gas exposure and locomotor analysis mice were anesthetized using isoflurane (Covetrus, Portland, ME) in a chamber containing the same gas composition as their respective exposure period noted above (medical-grade atmospheric air or 5 % CO_2). Mice were anesthetized and immediately subjected to mTBI or sham procedures as previously described (Logsdon et al., 2020). Mice were placed into a polyvinylchloride (PVC) shielding apparatus perpendicular to the blast wave to shield internal thoracic organs from injury while keeping the head free to move. The device consisted of a 2-piece machined steel shock tube apparatus driven using compressed helium gas (Airgas, #HE 300). The driver and driven section of the apparatus was separated by a clear 76.2 μm Mylar® membrane (ePlastics, San Diego, CA). Shock waves generated were scaled in intensity (≈1160 kPa) and duration (≈1 ms total duration) for mice (Bowen et al., 1968; Lucke-Wold et al., 2017b; Logsdon et al., 2020). Sham animals were exposed to the variable gas exposure paradigm, anesthesia, shielding tube, and noise without exposure to the blast wave. Following mTBI or sham treatments, mice were immediately removed and the time to regain righting reflex (RRT) was measured prior to animals being returned to their respective home cages. Mice fall into one of four treatment groups: medical-grade atmospheric air-sham (Air-Sham), medical-grade atmospheric air-TBI (Air-TBI), 5 % CO_2-sham (CO_2-Sham), and 5 % CO_2-TBI (CO_2-TBI).

It should be noted that the ability to elicit increases in ICP has been observed for halogenated inhalation anesthetics (Marx et al., 1962; McDowall et al., 1968). A primary aspect of our hypothesis is that CO_2 elicits increases in CBF, and by association ICP, thereby minimizing the transfer of energy to the brain and protecting against acceleration/deceleration injury. Previous studies indicate ICP changes elicited by isoflurane however are negligible (Belopavlovic and Buchthal, 1986; Goren et al., 2001).

Locomotor activity analysis

To delineate acute functional effects of mTBI, three hours post-injury or -sham treatments, locomotor activity was assessed using clear acrylic chambers (28 cm × 28 cm) in combination with ANY-Maze video tracking software. Animals were monitored for 30 min and total locomotor activity was quantified.

Core body temperature analysis

Baseline core body temperature readings were collected using a murine rectal thermometer probe model SD-947 (Reed Instruments, Wilmington, NC) immediately prior to gas exposure. Subsequent measurements were collected 15-, 30-, 60-, and 120-minutes following injury. Changes in core body temperature as compared to baseline were calculated and analyzed.

Tissue collection

Four hours following injury, mice from all four experimental groups described above, were sacrificed by rapid decapitation, and bilateral hippocampal samples dissected. Samples were immediately submerged and flash-frozen in liquid nitrogen and stored at −80 °C until subsequent Ribonucleic acid (RNA) extraction.

Ribonucleic acid sequencing

Ribonucleic acid isolation and directional RNA sequencing (RNA-seq) were performed by the Genomics, Epigenomics, and Sequencing Core (GESC) at the University of Cincinnati. RNA was isolated

(N = 3/group) utilizing the *mir*Vana™ miRNA Isolation Kit, with Phenol (ThermoFisher Scientific). The RNA quality was determined by Bioanalyzer (Agilent, Santa Clara, CA). To isolate the polyA RNA, NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Ipswich, MA) was used with a total of 1 μg of high-quality total RNA as input. The NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs) was used for library preparation, which is a dUTP-based stranded library. The library was indexed and amplified under PCR cycle number of 8. After library Bioanalyzer QC analysis and quantification, individually indexed and compatible libraries were proportionally pooled and sequenced using Nextseq 550 sequencer (Illumina, San Diego, CA). Under the sequencing setting of single read 1 × 85 bp, about 25 million pass filter reads per sample were generated. To identify differentially expressed genes, bioinformatic analysis via Illumina BaseSpace was performed by GESC. Once FASTQ files were generated, RNA-seq Alignment App version 2.01 (Illumina) which uses STAR aligner for longer reads mapping was used to create BAM files and data QC report. The BAM files were then introduced to RNA-Seq Differential Expression App version 1.01 (Illumina).

The differential expression data of compiled reference genes merged gene counts, and DESeq2 data were reviewed and downloaded from the online Illumina BaseSpace platform for supplemental analysis. Supplemental analysis was conducted utilizing a R/Shiny-based Interactive Gene Expression Analysis Kit (iGEAK, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) using gene annotations, sample-group information, and raw count matrices from BaseSpace (Choi et al., 2019). Lists of differentially expressed genes (DEGs) were collected from two-group comparisons using a minimum fold change of 1.5 and a p-value of 0.05. A four-way Venn diagram was created using the DEG lists from the following comparisons: Air-Sham vs Air-TBI, Air-Sham vs CO_2-TBI, Air-Sham vs CO_2-Sham, and Air-TBI vs CO_2-TBI within the iGEAK platform. An Over-Representation Analysis based on REACTOME was produced using select DEG lists within the iGEAK platform.

Gene ontology enrichment analysis

Gene ontology enrichment analysis was performed using select DEG lists to determine over/underrepresentation of biological processes using the *Mus musculus* gene ontology set (Ashburner et al., 2000; Mi et al., 2018). Biological processes were identified using a Fisher's Exact test and a false discovery rate of 0.05.

STRING analysis and K-Means clustering

All DEGs from the Air-TBI vs CO_2-TBI comparison were selected and protein–protein interactions were identified using the STRING database of known and predicted protein–protein interactions, representing direct and indirect (physical and functional) associations stemming from computational prediction, knowledge transfer between species, and interactions aggregated from other primary datasets (Szklarczyk et al., 2019).

DEGs were further grouped utilizing K-Means clustering into three groups. Groups were established empirically by categorizing observations of major classes, functions, and families of connected proteins generated within the STRING analysis. The number of groups was optimized to most completely represent the functional heterogeneity of the underlying gene sets without unnecessarily separating gene networks. Disconnected nodes were removed as part of the preprocessing of the data.

TaqMan quantitative real time PCR™ (qRT-PCR) validation of RNA-Seq

In addition to utilizing RNA samples used for RNA sequencing (N = 3/group), bilateral hippocampal samples were collected from separate and distinct experimental cohorts to validate and confirm RNA sequencing results via qRT-PCR. Total RNA was extracted from additional flash-frozen bilateral hippocampal samples (N = 3–4) using TRI Reagent (Sigma-Aldrich, T9424) according to the manufacturer's instructions. Total RNA concentration for each sample was quantified using a NanoDrop™ One (Thermo Scientific, Waltham, MA). Samples of cDNA were prepared by reverse transcription using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA). Each sample reaction included MultiScribe TM Reverse Transcriptase and random primers, and run per manufacturer's directions at the following thermal cycler conditions: step 1 at 25° C for 10 min, step 2 at 37° C for 120 min, step 3 at 85° C for 5 s, and step 4 at 4° C for 10 min.

For PCR amplification, TaqMan® Universal PCR Master Mix and the following probes were obtained from Applied Biosystems (Foster City, CA): *18 s* (Hs99999901_s1) as an endogenous control gene, *mt2* (Mm00809556_s1), and *map3k6* (Mm00522235_m1) The reaction mixture was prepared according to the manufacturer's instructions, with the following thermal cycling conditions: initial holding at 50° C for 2 min, followed by a first denaturing step at 95° C for 10 min, then 40 cycles at 95° C for 15 s, and at 60° C for 1 min. Data from real-time PCR measurements were calculated using the $\Delta\Delta C_t$ method.

Statistical analyses

All statistical analyses were conducted in GraphPad Prism 8 (GraphPad Software, La Jolla, CA). All data are depicted as a box-and-whisker plot, with the box representing the median, 25th, and 75th percentiles, and whiskers representing the 5th and 95th percentile. Normality was assessed using the Shapiro-Wilk test. Equal variance was assessed using an F-test (two samples) or Bartlett's test (more than two samples).

Data from monitoring CO_2 exposure were analyzed using an unpaired t-test, Unpaired t test with Welch's correction (if unequal variance), or Mann Whitney test (non-parametric) dependent on normality and variance.

Two-way ANOVAs (fixed effect) using the factors of Injury (Sham/TBI) × Gas (Air/CO_2) followed by post hoc Uncorrected Fisher's LSD or Tukey's analyses were conducted where applicable. Two-Way repeated measures ANOVA (Group × Time) followed by post hoc Tukey's analysis was used to analyze body temperature alterations. For all statistical analyses, $P \leq 0.05$ was considered statistically significant. All results are presented as Mean ± SEM. Effect size is reported as r for two sample test and % of total variation for ANOVA analyses. Confidence intervals for the difference between means are presented as 95 % unless otherwise specified.

RESULTS

CO_2 exposure elicits increased freezing behavior

Mice exposed to 5 % CO_2 displayed an increased time spent freezing during a 10-minute exposure period compared to their medical-grade atmospheric air-exposed counterparts (Fig. 2(**A**); Unpaired t test with Welch's correction: $t_{(21.78)} = 4.109$, $P \leq 0.001$, CI_{diff}[37.90,115.2], r = 0.661, N = 16–18) as well as an increase in number of freezing episodes (Fig. 2(**B**); Mann Whitney test: U = 38.50, $P \leq 0.0001$, 95.37 % CI of difference between medians[12.00, 39.00], N = 16–18). Unsurprisingly, 5 % CO_2 exposed animals also display a decrease in locomotor activity at this time point (Fig. 2(**C**); Unpaired t test: $t_{(32)} = 3.872$, $P \leq 0.001$, CI_{diff}[-6.955,-2.159], r = 0.565, N = 16–18). These responses serve as quantitative confirmation that mice are being exposed to and physiologically responding to artificially high levels of CO_2 within their respective environment.

CO_2 exposure mitigates acute effects elicited by mTBI

Increases in righting reflex time (RRT) are observed across nearly all preclinical models for TBI and act as a correlate of loss of consciousness, a common facet of human TBI pathology (Alder et al., 2011; Dewitt et al., 2013; Namjoshi et al., 2014). RRT is often linked to injury severity and serves as a confirmation of injury when comparing TBI and sham animals (Siebold et al., 2018). Air-TBI animals experience significant increases in RRT as compared to their Air-Sham counterparts. Mice exposed to 5 % CO_2 prior to injury (CO_2-TBI) display a significant reduction in RRT as compared to mice exposed to medical-grade atmospheric air prior to injury (Air-TBI) indicating significant protection from injury-elicited unconsciousness (Fig. 3(**A**); Air-Sham = 49.875 ± 4.608, Air-TBI = 232.250 ± 44.279, CO_2-Sham = 46.250 ± 5.762, CO_2-TBI = 96.333 ± 15.308; Two-way ANOVA (Injury × Gas): gas, $F_{(1,29)} = 9.876$, $P = 0.0038$, 13.54 % of total variation; Injury, $F_{(1,29)} = 26.37$, $P \leq 0.0001$, 36.15 % of total variation; Tukey's multiple comparison: Air-Sham vs Air-TBI, $q_{(29)} = 8.009$, $P \leq 0.0001$, CI_{diff}[-280.5,-98.27];Air-TBI vs CO_2-TBI, $q_{(29)} = 6.219$, $P \leq 0.0001$, CI_{diff}[54.38,231.50]; Air-Sham vs CO_2-TBI, $q_{(29)} = 2.022$, CI_{diff}[-135.00,42.08], $P = 0.4918$; N = 8–9). Additionally, there is no difference in RRT between either sham group (Air-Sham or CO_2-Sham).

mTBI also elicits acute deficits in locomotor activity 3-hours following injury. Air-TBI animals display a significant decrease in distance traveled during a 30-minute open field locomotor task as compared to Air-Sham mice. CO_2-TBI mice display a significant increase in distance traveled compared to their Air-TBI counterparts, effectively normalizing their locomotor activity to a level no different from that of sham animals (Fig. 3(**B**); Air-Sham = 27.535 ± 2.547, Air-TBI = 11.714 ± 2.440, CO_2-Sham = 31.353 ± 2.998, CO_2-TBI = 27.217 ± 4.890; Two-way ANOVA (Injury × Gas): gas, $F_{(1,28)} = 7.237$, $P = 0.0114$, 15.95 % of total variation; Injury, $F_{(1,28)} = 7.818$, $P = 0.0092$, 17.01 % of total variation; Tukey's multiple comparison: Air-Sham vs Air-TBI, $q_{(28)} = 4.300$, $P \leq 0.05$, CI_{diff}[1.613,30.03]; Air-TBI vs CO_2-TBI, $q_{(28)} = 4.487$, $P \leq 0.05$, CI_{diff}[-28.84,-2.163]; Air-Sham vs CO_2-TBI, $q_{(28)} = 0.08876$, $P > 0.9999$, CI_{diff}[-13.52,14.15]; N = 7–9).

mTBI elicited acute decreases in core body temperature. Air-TBI mice display significant reductions in core body temperature compared to Air-Sham and CO_2-Sham mice 15 min post-injury, an effect distinctly lacking in CO_2-TBI animals (Fig. 3(**C**); Air-Sham = -0.438 ± 0.231, Air-TBI = -2.306 ± 0.444, CO_2-Sham = -0.0525 ± 0.115, CO_2-TBI = -1.061 ± 0.397; Repeated

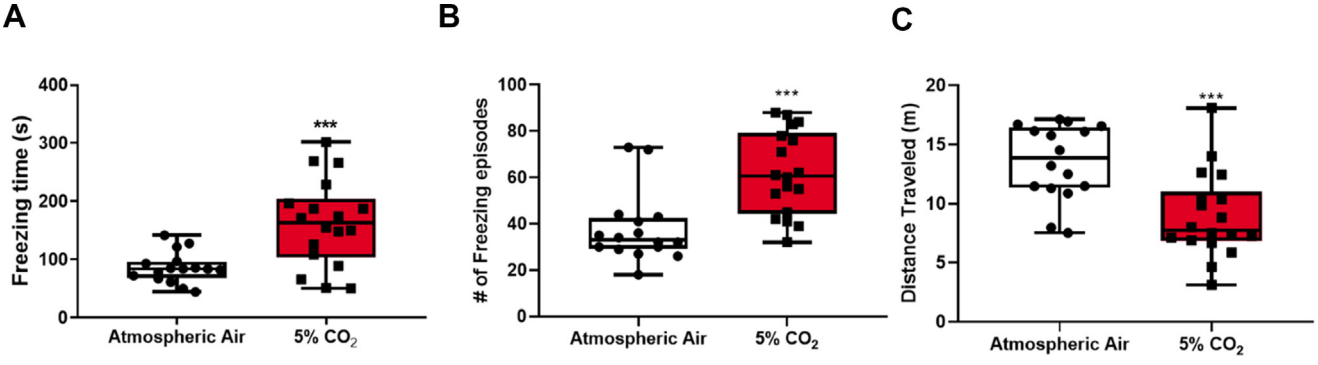


Fig. 2. High concentrations of CO_2 elicit freezing behavior and lethargy. (**A**) Mice exposed to 5 % CO_2 display an increase in freezing time (**B**) the number of freezing episodes. (**C**) Mice exposed to 5 % CO_2 display a deficit in locomotor activity during their exposure period. Atmospheric Air N = 16, 5 % CO_2 N = 18; ***$P \leq 0.001$.

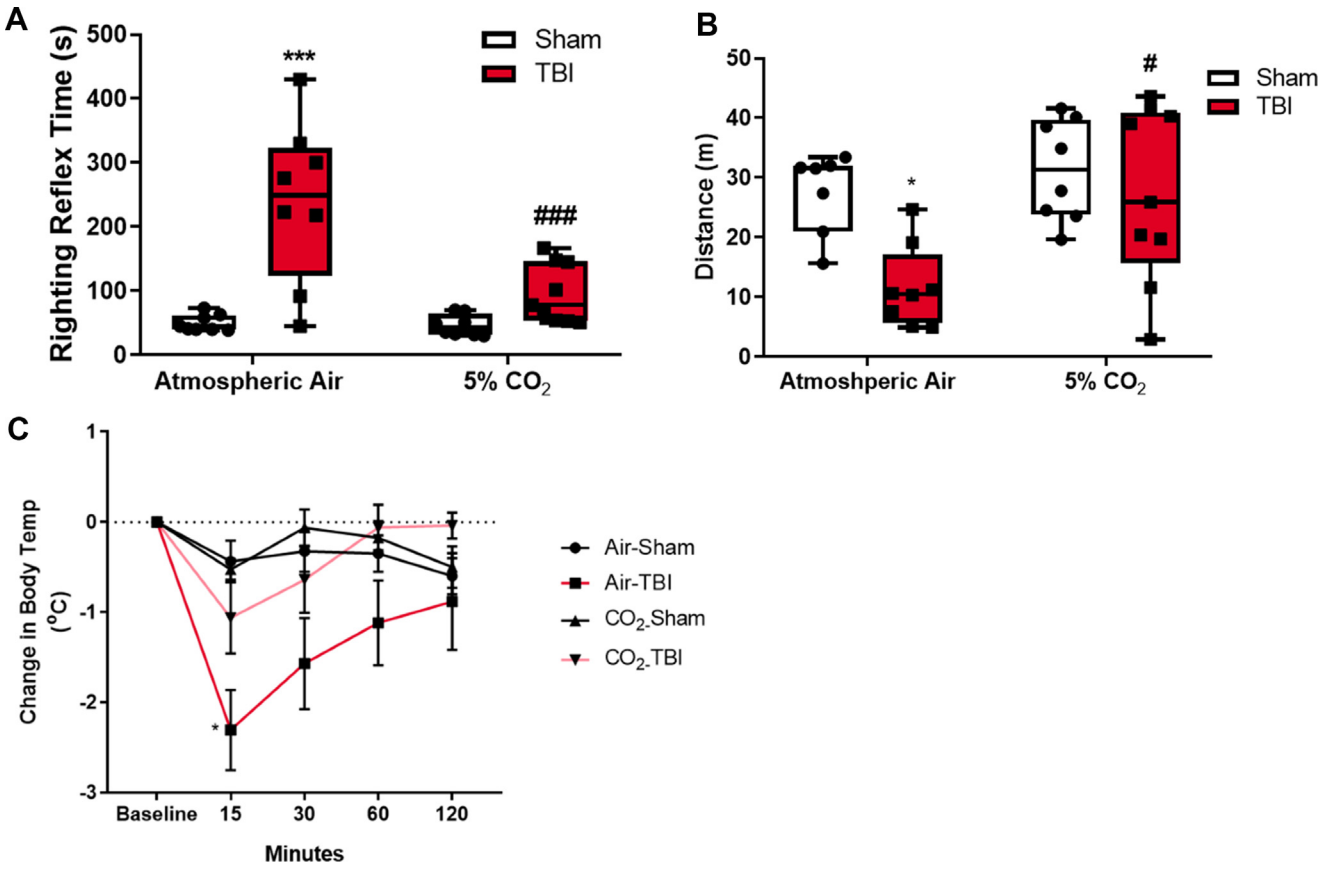


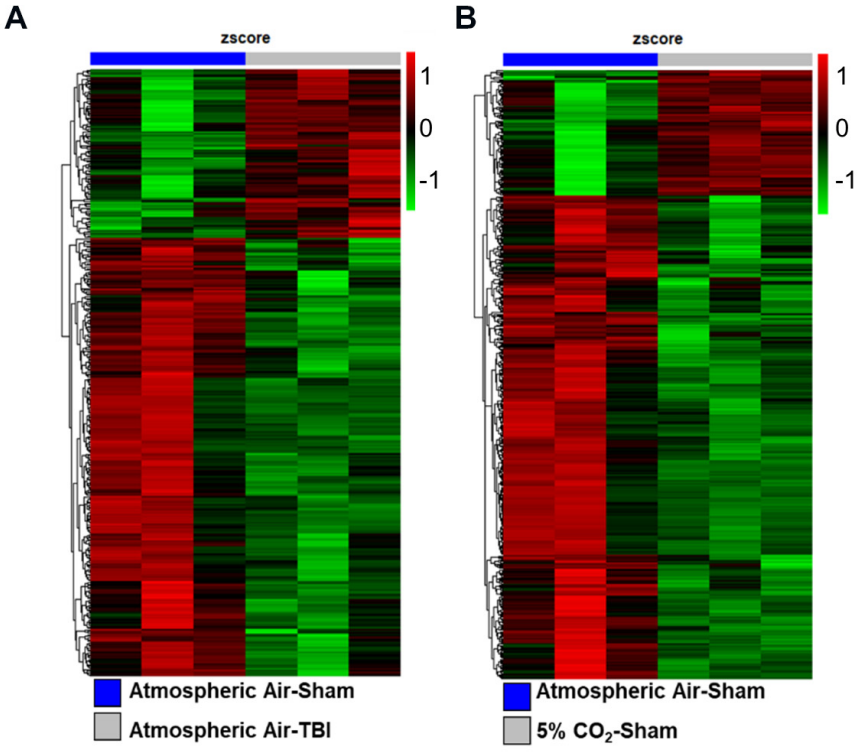
Fig. 3. CO_2 pre-exposure attenuates acute physiologic and behavioral effects elicited by mTBI. (**A**) CO_2 mitigates deficits in RRT, a correlate of unconsciousness, induced by mTBI. (**B**) 5 % CO_2 exposure attenuates reductions in exploratory locomotor activity 3 hours following mTBI. (**C**) mTBI causes transient reductions in core body temperature, an effect that is blocked when mice are exposed to 5 % CO_2 immediately preceding injury paradigm. N = 8–9, Two-way ANOVA (repeated measures for body temperature), followed by post hoc Tukey's multiple comparison tests; *$P \leq 0.05$, ***$P \leq 0.001$ vs Air-Sham; # $P \leq 0.05$, ### $P \leq 0.001$ vs Air-TBI.

measures two-way ANOVA (Group × Time): time, $F_{(2.827,82)} = 16.48$, $P \leq 0.0001$, 12.87 % of total variation; group, $F_{(3,29)} = 3.398$, $P = 0.0309$, 14.34 % of total variation; Tukey's multiple comparison: 15 min. Air-Sham vs Air-TBI, $q_{(10.51)} = 5.278$, $P \leq 0.05$, CI_{diff}[0.3503,3.387]; Air-TBI vs CO_2-TBI, $q_{(14.54)} = 2.954$, $P = 0.2027$, CI_{diff}[-2.970,0.4796]; Air-Sham vs CO_2-TBI, $q_{(12.66)} = 1.920$, $P > 0.5460$, CI_{diff}[-0.7300,1.977]; N = 8–9/group/time point). Together, these data demonstrate that 5 % CO_2 exposure preceding mTBI blocks the acute physiologic and behavioral changes associated with preclinical closed-head neurotrauma.

CO_2 exposure prevents mTBI-Induced hippocampal transcriptional alterations

mTBI results in the rapid activation of cellular and molecular processes within the brain such as neuroinflammation (Kumar and Loane, 2012; Lozano et al., 2015; Thome et al., 2019), glial activation (Karve et al., 2016; Madathil et al., 2018; Xiaoyun Cheng et al., 2019; Mira et al., 2021), endoplasmic reticulum stress (Logsdon et al., 2014; Lucke-Wold et al., 2017a; Hood et al., 2018), and ultimately, neuronal degradation (Omalu et al., 2005; DeKosky and Asken, 2017; Graham and Sharp, 2019). These studies are, to our knowledge, the first to utilize CO_2 pre-exposure to *prevent* the acute effects of acceleration/deceleration injury. Thus, the effects of combined CO_2 exposure and mTBI induction on basal transcription have not been investigated. To elucidate any isolated or synergistic interactions between experimental paradigms, we elected to conduct RNA-sequencing of hippocampal samples. Certain areas of the brain are known to be at higher risk of damage during a TBI than others including the hippocampus. (Jarrard, 1993; Fortin et al., 2002; McAllister, 2011; Girgis et al., 2016; Ratliff et al., 2020). Air-TBI mice display a distinct transcriptional profile as compared to their Air-Sham counterparts noted by a total of 370 DEGs (Fig. 4(**A**)).

An over-representation analysis reveals the most enriched pathways to be involved in extracellular matrix organization, SLC-mediated membrane transport, and platelet-deprived growth factor (PDGF) signaling (Fig. 4 (**C**)). As expected, CO_2 exposure itself alters the gene expression level of many hippocampal transcripts as noted in the comparison between Air-Sham and CO_2-Sham animals (524 DEGs, Fig. 4(**B**)). An over-representation analysis reveals the most enriched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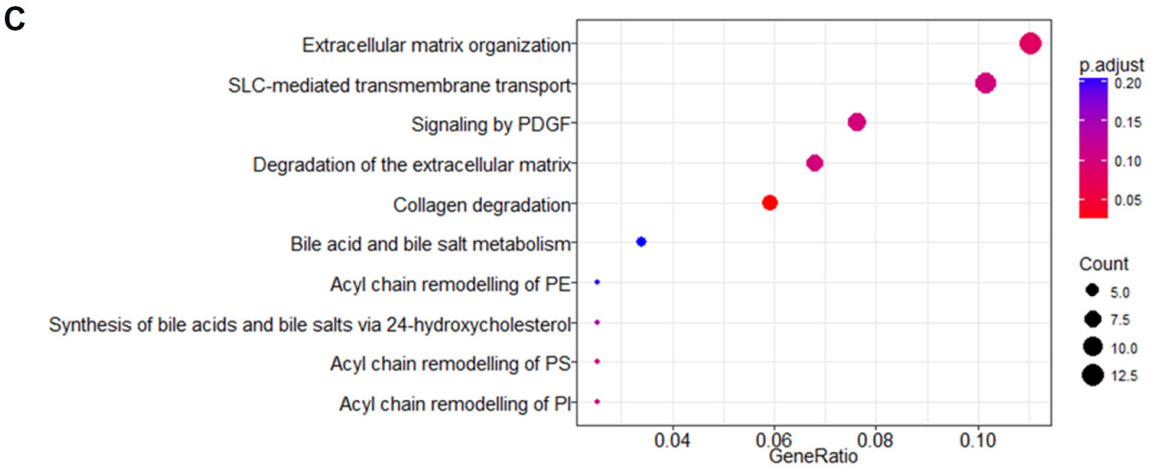
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SLC-mediated transmembrane transport
Signaling by PDGF
Degradation of the extracellular matrix
Collagen degradation
Bile acid and bile salt metabolism
Acyl chain remodelling of PE
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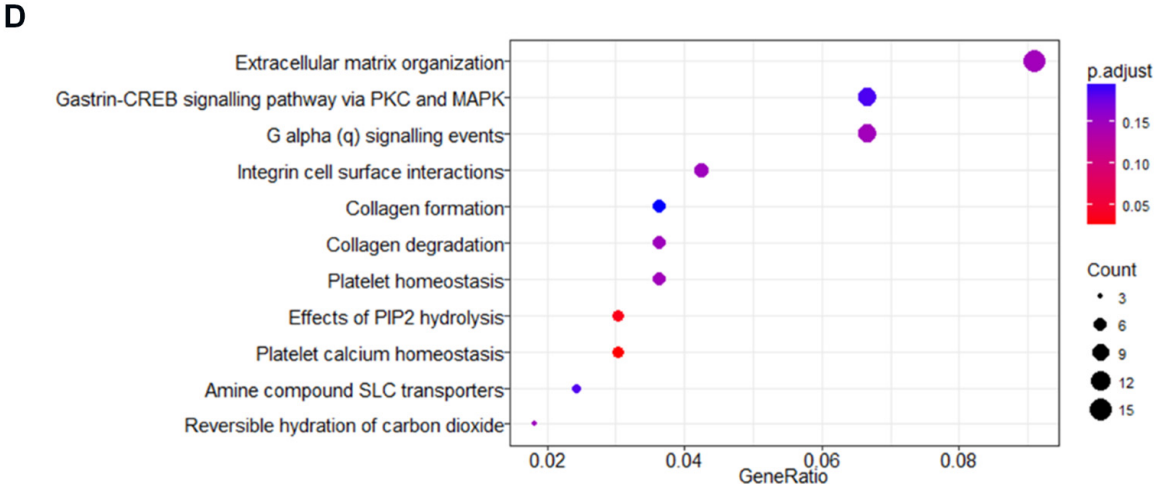
D
Extracellular matrix organization
Gastrin-CREB signalling pathway via PKC and MAPK
G alpha (q) signalling events
Integrin cell surface interactions
Collagen formation
Collagen degradation
Platelet homeostasis
Effects of PIP2 hydrolysis
Platelet calcium homeostasis
Amine compound SLC transporters
Reversible hydration of carbon dioxide
0.02
0.04
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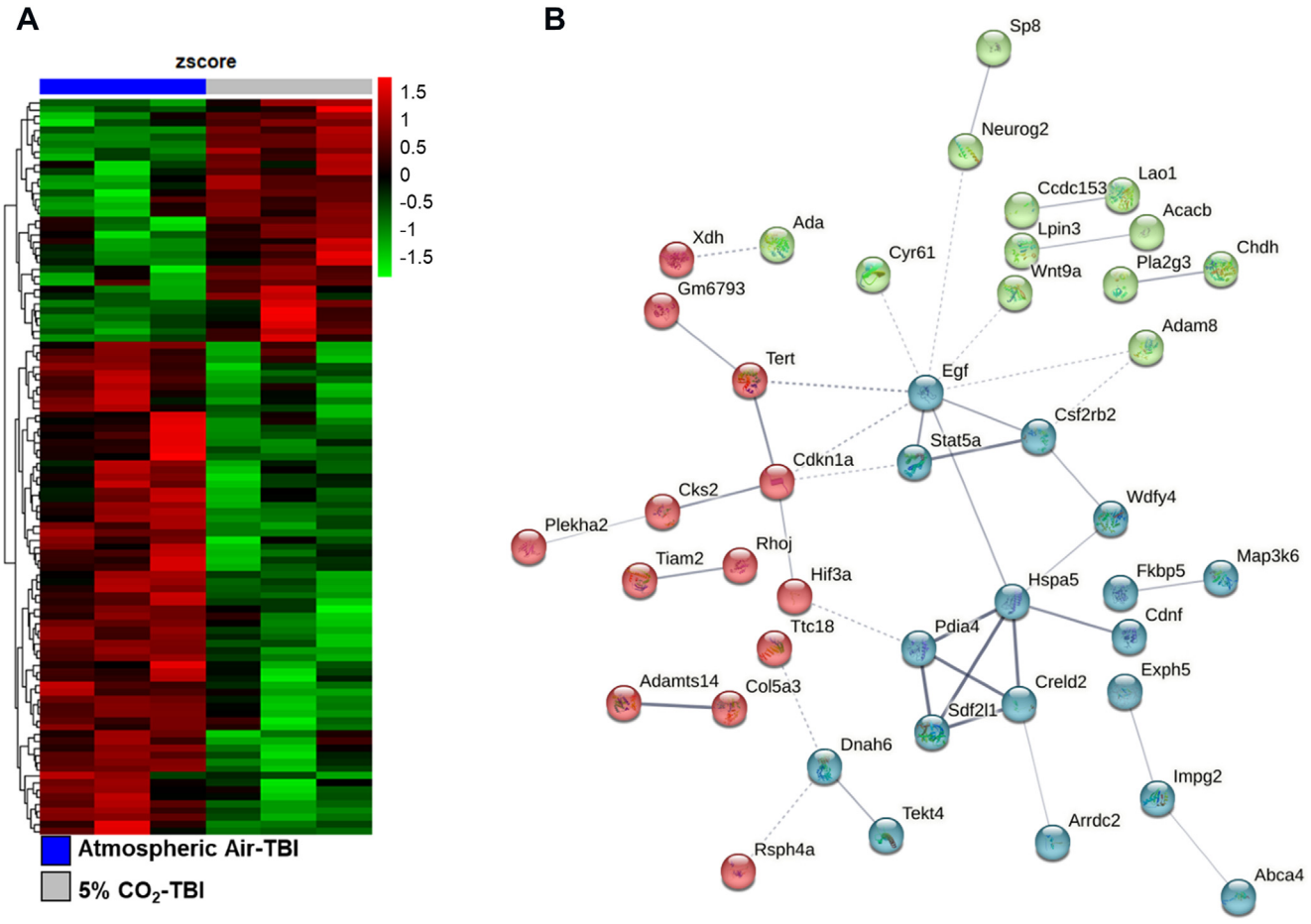

Fig. 5. CO_2 exposed mTBI mice display a distinct transcriptional profile. (**A**) Heat map of DEGs between Air-TBI vs CO_2-TBI. (**B**) String analysis from Air-TBI vs CO_2-TBI DEG list. Clustering analysis reveals gene clusters relating to cell cycle and response to hypoxia (red), immune signaling (blue), and cell proliferation, neurogenesis, and cell adhesion (green). These processes are common altered by TBI and may give insight into the differing outcomes between air and CO_2 exposed TBI animals. DEGs were identified using a minimum fold change of 1.5 and $P \leq 0.05$. N = 3/group.

pathways to be extracellular matrix organization, gastrin-CREB signaling pathway via PKC and MAPK, and G alpha q signaling events (Fig. 4(**C**)). CO_2-TBI mice also show differential gene expression as compared to their Air-TBI counterparts with 108 DEGs, highlighting the ability for CO_2 pre-exposure to prevent, or at the least alter mTBI induced transcriptional alterations (Fig. 5(**A**)).

String analysis was performed on the 108 DEGs between CO_2-TBI and Air-TBI groups and further grouped utilizing K-means clustering for three total groups (Fig. 5(**B**)). The red cluster contains genes related to the cell cycle and involved in response to hypoxia. The blue cluster contains genes involved in immune signaling. Lastly, the green cluster contains genes involved in cell proliferation, neurogenesis, and cell adhesion.

We subsequently generated a Venn diagram using the DEG list from the following comparisons: Air-Sham vs Air-TBI, Air-Sham vs CO_2-TBI, Air-Sham vs CO_2-Sham, and Air-TBI vs CO_2-TBI (Fig. 6(**A**)). The overlap between the Air-Sham vs Air-TBI DEG list and Air-TBI vs CO_2-TBI DEG list reveals a subset of 19 genes that are altered by mTBI and whose alterations are prevented by pre-exposure to 5 % CO_2 immediately preceding injury (Fig. 6(**B**)). Importantly, the expression of those 19 genes is not differentially expressed between Air-Sham vs CO_2-TBI, further indicating that alterations in their expression is prevented by experimental hypercapnia immediately preceding mTBI. The normal function of these genes includes a variety of processes such as immune regulation, DNA damage response, blood brain barrier formation, activation of microglia, and apoptosis. These pathways/processes are all known to be altered by mTBI and are also believed to be common therapeutic targets (Chodobski et al., 2011; McAllister, 2011; Karve et al., 2016; Madathil et al., 2018; Ng and Lee, 2019; Thapa et al., 2021).

Fig. 4. Heat map of DEGs between (**A**) Air-Sham vs Air-TBI and (**B**) Air-Sham vs CO_2-Sham. (**C**) Over-Representation Analysis based on REACTOME for DEGs between Air-Sham vs Air-TBI and (**D**) Air-Sham vs CO_2-Sham. DEGs were identified using a minimum fold change of 1.5 and $P \leq 0.05$. N = 3/group.

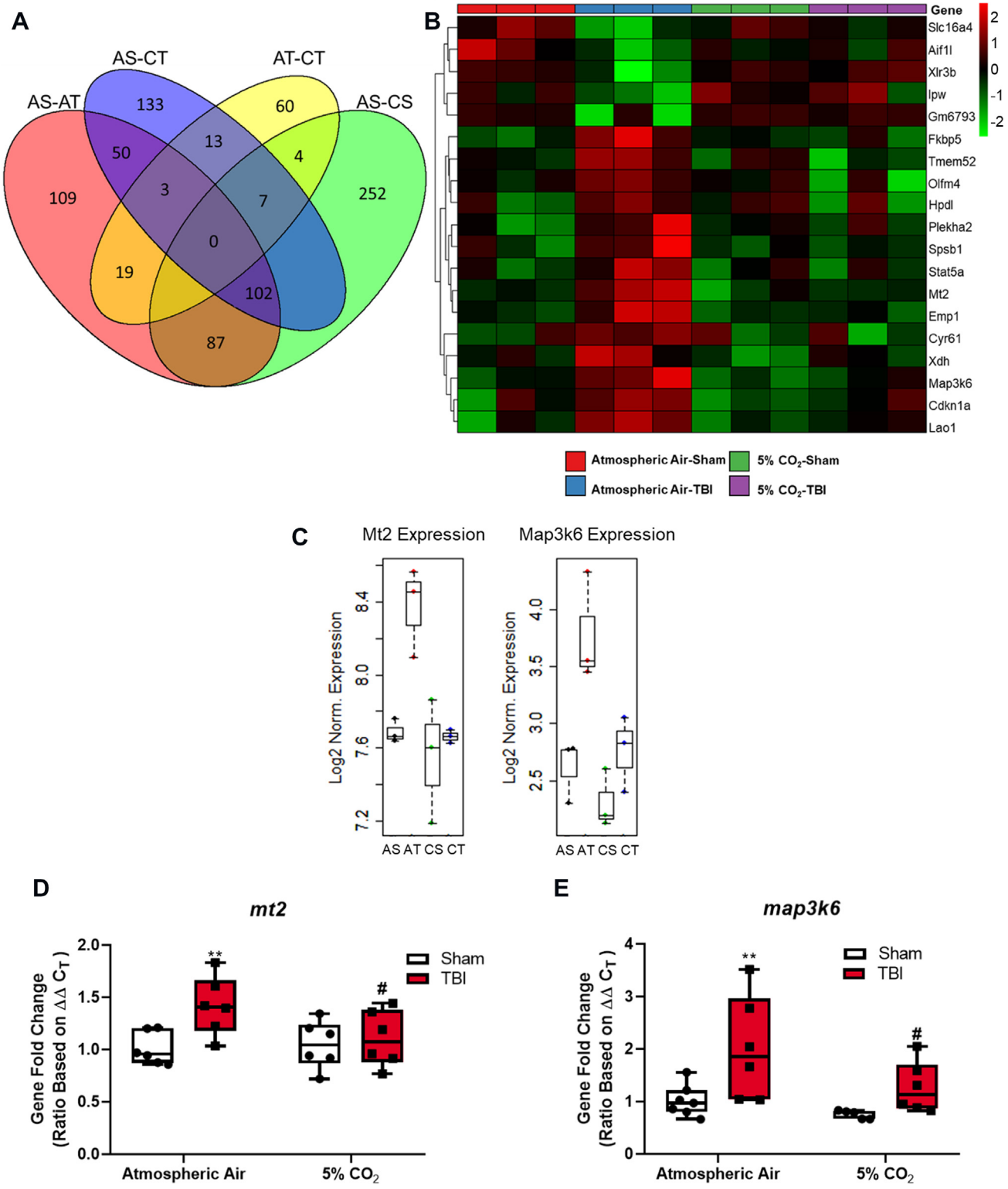

Fig. 6. CO_2 pre-exposure prevents TBI-induced transcriptional alterations. (**A**) Venn diagram showing overlap in DEGs between the Air-Sham vs Air-TBI (AS-AT), Air-Sham vs CO_2-TBI (AS-CT), Air-Sham vs CO_2-Sham (AS-CS), and Air-TBI vs CO_2-TBI (AT-CT) comparisons. (**B**) Heat map of the 19 DEGs overlapping between Air-Sham vs Air-TBI and AT-CT comparisons. These genes are altered by mTBI and that alteration is blocked by pre-exposure to 5 % CO_2 prior to injury. DEGs were identified using a minimum fold change of 1.5 and $P \leq 0.05$. (**C**) RNA sequencing analysis shows alterations in *mt2* and *map3k6* gene expression induced by mTBI. (**D,E**) qRT-PCR confirmed an increase in gene expression induced by mTBI which is also prevented when animals are exposed to 5 % CO_2 prior to injury. N = 3/group for RNA-seq and 6/group for qRT-PCR. Two-way ANOVA, followed by post hoc Uncorrected Fisher's LSD; $^{**}P \leq 0.01$ vs AS; # $P \leq 0.05$ vs AT.

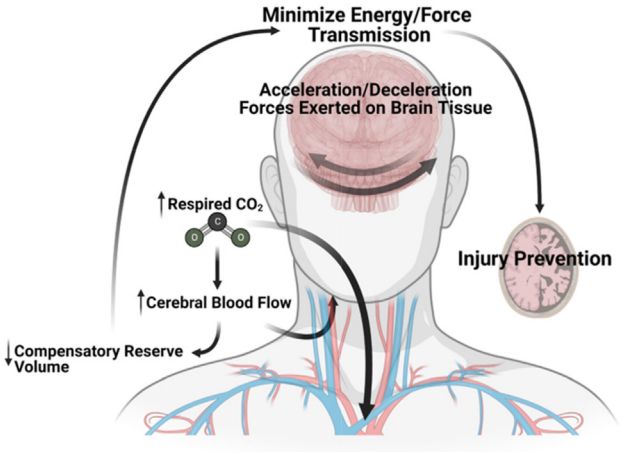

Fig. 7. Hypothesized mechanism of the ability for increases in respired CO_2 to prevent the transfer of energy to the brain during acceleration/deceleration events leading to TBI. Increases in respired CO_2 induce increases in cerebral blood flow (CBF) and cerebral blood volume. These effects reduce the compensatory reserve volume (CRV) thereby minimizing force transmission. An effect that, we hypothesize, reduces energy transmission and injury during acceleration/deceleration events.

qRT-PCR confirmation of select DEGs

In order to validate gene expression, two genes were selected from the list of 19 found in the overlap between the Air-Sham vs Air-TBI DEG list and the Air-TBI vs CO_2-TBI DEG list known to be involved in oxidative stress and inflammation, *metallothionein 2* (*mt2*) and *mitogen-activated protein kinase kinase kinase 6* (*map3k6*), respectively, for PCR confirmation. Both MT2- and MAPK-elicited inflammation have well established roles in oxidative stress and gliosis following TBI across various rodent models (Kim et al., 2004; Stankovic et al., 2007; Bachstetter et al., 2013; Bodnar et al., 2018; Lyons et al., 2018; Tang et al., 2022), leading us to select these two candidates for confirmation studies of our RNA sequencing results. A box-plot analysis from iGEAK reveals an increase in expression of both *mt2* and *map3k6* in Air-TBI mice compared to the other three treatment groups (Fig. 6(**C**)). qRT-PCR showed increased *mt2* mRNA expression in Air-TBI mice compared to Air-Sham, CO_2-Sham, and CO_2-TBI animals (Fig. 6(**D**); Air-Sham = 1.010 ± 0.064, Air-TBI = 1.419 ± 0.114, CO_2-Sham = 1.046 ± 0.093, CO_2-TBI = 1.106 ± 0.109; Two-way ANOVA (Injury × Gas): gas, $F_{(1,20)} = 2.026$, $P = 0.1700$, 6.514 % of total variation; injury, $F_{(1,20)} = 5.846$, $P = 0.0253$, 18.79 % of total variation; Uncorrected Fisher's LSD test: Air-Sham vs Air-TBI, $t_{(20)} = 2.981$, $P \leq 0.01$, CI_{diff}[-0.6951,-0.1228]; Air-TBI vs CO_2-Sham, $t_{(20)} = 2.716$, $P \leq 0.01$, CI_{diff}[0.08645,0.6587]; Air-TBI vs CO_2-TBI, $t_{(20)} = 2.278$, $P \leq 0.05$, CI_{diff}[0.02634,0.5986]; Air-Sham vs CO_2-TBI, $t_{(20)} = 0.7032$, $P = 0.4406$, CI_{diff}[-0.3826,0.1897]; N = 6). Importantly there is no difference between the Air-Sham, CO_2-Sham, or CO_2-TBI groups meaning that CO_2 alone does not alter the expression of *mt2* and that CO_2 pre-exposure prevents the ability of mTBI to alter *mt2* expression. Similar results were seen in *map3k6* mRNA expression profiles in Air-TBI mice compared to Air-Sham, CO_2-Sham, and CO_2-TBI (Fig. 6(**E**); Air-Sham = 1.026 ± 0.079, Air-TBI = 2.012 ± 0.403, CO_2-Sham = 0.966 ± 0.116, CO_2-TBI = 1.267 ± 0.197; Two-way ANOVA (Injury × Gas): gas, $F_{(1,20)} = 4.533$, $P = 0.0459$, 12.78 % of total variation; injury, $F_{(1,20)} = 10.27$, $P = 0.0045$, 28.94 % of total variation; Uncorrected Fisher's LSD test: Air-Sham vs Air-TBI, $t_{(20)} = 3.127$, $P \leq 0.01$, CI_{diff}[-1.665,-0.3326]; Air-TBI vs CO_2-Sham, $t_{(20)} = 3.621$, $P \leq 0.01$, CI_{diff}[0.5338,1.984]; Air-TBI vs CO_2-TBI, $t_{(20)} = 2.248$, $P \leq 0.05$, CI_{diff}[-0.5370,1.437]; Air-Sham vs CO_2-TBI, $t_{(20)} = 0.7944$, $P = 0.4363$, CI_{diff}[-0.9202,0.4126]; N = 6). Again, there is no difference between the Air-Sham, CO_2-Sham, or CO_2-TBI groups. The qRT-PCR results for these select gene transcripts fall in line with the DEG data obtained from RNA sequencing analysis providing ample confidence in the RNA sequencing results.

DISCUSSION

Effective preventative measurements to reduce the rates of TBI among high-risk groups have long been unavailable and/or insufficient. In spite of the recent innovations in helmet technology, mTBI rates remain high among at-risk groups. Thus, there is a clear need for preventative technology that exceeds the capabilities of our current prevention measures. Recent research has highlighted the ability for alterations in CBF to minimize the transmission of energy during mTBI events therefore protecting the brain from injury. The ability for increases in CO_2 respiration to dilate cerebral vasculature and increase CBF has been well documented (Kety and Schmidt, 1948; Raichle and Plum, 1972; Brian, 1998; Godoy et al., 2017). Therefore, we hypothesized that experimentally induced hypercapnia may act to minimize damage to the brain resulting from acceleration/deceleration forces.

Herein, we provide evidence that experimentally-induced hypercapnia to prevent several of the acute physiologic manifestations of mTBI. CO_2 exposure immediately prior to injury mitigates injury elicited loss of consciousness as evidenced by a reduction in RRT. mTBI was found to elicit deficits in locomotor activity 3 h following injury, an effect blocked by experimental hypercapnia induced immediately prior to mTBI. Lastly, mTBI elicited transient reductions in core body temperature. mTBI-induced core body temperature reductions were attenuated in mice exposed to 5 % CO_2 immediately preceding injury. Combined, these effects illustrate a powerful, innate ability of CO_2 exposure to prevent some of the common acute effects of preclinical mTBI.

Consistent with other studies, we report that mTBI induces a myriad of transcriptional alterations within the hippocampus. Importantly, we observed altered expression of map3k6, FKBP Prolyl Isomerase 5 (fkbp5), and the Oncostatin M receptor (osmr) (Boone et al., 2019) within our dataset. Additionally, we demonstrate that exposure to 5 % CO_2 alone induces a variety

of transcriptional alterations while simultaneously normalizing differential expression of DEGs including map3k6 and fkbp5. Previous studies have established the ability for hypercapnia to induce a variety of transcriptional alterations (Casalino-Matsuda et al., 2018; Shigemura et al., 2020). Similar to these reports, differential gene expression induced by 5 % CO_2 in our model (Air-Sham vs CO_2-Sham comparison) results in alterations in biological processes such as cell adhesion, fatty acid biosynthesis, ion transport, and cell differentiation (Casalino-Matsuda et al., 2018). Additionally, we observe alterations in immune response and inflammation including alterations in the TNF signaling pathway (e.g. Tnfaip6, Tnfrsf25, C1qtnf1), a known pathway altered by hypercapnia in mammalian subjects (Shigemura et al., 2020). Previous studies on the effects of hypercapnia on gene expression levels have chiefly focused on lung tissue. Our RNA sequencing analysis presented here builds upon these studies while focusing specifically on brain tissue; thus establishing the first, to our knowledge, RNA analysis delineating the effects of hypercapnia on hippocampal gene expression. We believe these studies afford valuable insight into the effects that acute exposure to higher percentages of CO_2 have on physiologic processes and the mitigation of mTBI pathology.

The role of DEGs induced by mTBI and normalized by CO_2 exposure are relevant in the process of immune regulation, DNA damage response, blood brain barrier formation, activation of microglia, and apoptosis. These processes serve as common preclinical and clinical targets for TBI intervention (Kumar and Loane, 2012; Lozano et al., 2015; Ng and Lee, 2019; Thapa et al., 2021) and preventing these alterations from occurring is likely crucial to the preventative capabilities of CO_2. The normalization of mTBI-induced gene expression changes afforded by CO_2 pre-exposure underscore the viability of CO_2 exposure as a preventative technique. The STRING analysis on the 108 DEGs in the Air-TBI vs CO_2-TBI comparison reveals three clusters of genes relating to cell cycle and response to hypoxia, immune signaling, and cell proliferation, neurogenesis, and cell adhesion. This analysis may highlight some of the key differences between Air-TBI and CO_2-TBI animals and grants insight into the specific aspects of TBI pathology that CO_2 exposure prevents.

Interestingly, there are 60 DEGs that are only differentially expressed between the Air-TBI vs CO_2-TBI comparison and an additional 13 DEGs that are differentially expressed between both the Air-TBI vs CO_2-TBI and Air-Sham vs CO_2-TBI comparisons. These genes are not altered by CO_2 or mTBI separately, however when mice are exposed to 5 % CO_2 prior to mTBI there is an uncharacterized and idiosyncratic interaction driving unique transcriptional profiles unique to CO_2-exposed mTBI mice. This unique interaction may play a crucial role in the ability for CO_2 exposure to protect against mTBI and a thorough investigation of these genes is certainly warranted.

Another interesting occurrence is the relatively large overlap between the DEGs induced by CO_2 (Air-Sham vs CO_2-Sham) and mTBI (Air-Sham vs Air-TBI). There are 189 total genes differentially expressed in both the (Air-Sham vs CO_2-Sham) and (Air-Sham vs Air-TBI) comparisons. Additionally, mTBI and CO_2 both alter gene expression in the same direction; DEGs upregulated by mTBI are also upregulated by 5 % CO_2 exposure.

Hypoxia and ischemia are common occurrences following TBI (Coles et al., 2004; Veenith et al., 2016). Research endeavors have established that ischemic preconditioning and hypoxic preconditioning both induce a degree of tolerance against subsequent ischemic and/or hypoxic events, thus mitigating damage to brain tissue (Chen and Simon, 1997; Liu et al., 2000; Liu et al., 2009). *In vitro* studies have demonstrated elevated levels of CO_2 to inhibit lipopolysaccharide (LPS) induced expression of the proinflammatory cytokines IL-6 and TNF and attenuate nuclear factor kappa B (NF-κB) activity, a regulator of innate immunity (Cummins et al., 2010; Wang et al., 2010). Thus, we hypothesize that CO_2 may be eliciting a pre-conditioning effect (Yokobori et al., 2013; Turner et al., 2017) yielding an emergent protective effect independent of energy transfer minimization.

Studies presented here serve as initial proof of concept studies and in order to capture an effect of CO_2 inhalation and experimental hypercapnia, if one was indeed present, utilized a concentration much higher than necessary to elicit a robust physiologic, CBF response (Kety and Schmidt, 1948; Grubb et al., 1974; Rostrup et al., 1994; Asgari et al., 2011; Sato et al., 2012; Mader et al., 2018; Ogoh, 2019). 5 % CO_2 has been demonstrated to manipulate CBF and increase ICP (Mestre et al., 2020). Additionally, 5 % CO_2 is commonly available and widely used in preclinical rodent models (Kety and Schmidt, 1948; Vollmer et al., 2016; McMurray et al., 2020; Mestre et al., 2020). Thus, we utilized a concentration of 5 % CO_2 for these initial proof of efficacy studies in order to maintain consistency within the field. Consistent with previous studies in rodents we observe freezing behavior and decreased locomotor activity during gas exposure, (Vollmer et al., 2016; Winter et al., 2017). It is important to note that these effects are not typically observed at concentrations of CO_2 below 5 % (Krohn et al., 2003).

Previous established reports have found that every 1 mmHg increase in $PaCO_2$ results in a 1–2 mL/100 g/min increase in CBF (Grubb et al., 1974). As the mechanism behind the ability for CO_2 to increase CBF is very sensitive, it is likely that only slight elevations in CO_2 above the 0.04 % atmospheric concentration are necessary to fill the CRV, increase CBF, increase ICP, and minimize the transfer of acceleration/deceleration forces that would otherwise result in a TBI. We recognize that 5 % CO_2 is much too high for use as a therapeutic in humans and emphasize that these studies serve as proof of concept animal studies. Thus, during future preclinical studies, we envision drastically lower concentrations of CO_2 being utilized, yet still conveying significant protection without the detrimental physiologic effects of hypercapnia.

The authors acknowledge the apparent complication of implementing increases in inspired CO_2 to prevent TBI in humans. The recent COVID-19 pandemic has

produced an abundance of data on N95 and KN95 masks, including evaluations of the CO_2 concentrations individuals wearing these masks are routinely exposed to. These studies have revealed CO_2 concentrations of around 3 % within KN95 (Rhee et al., 2021) masks and between 3 % and 4 % for N95 masks (Laferty and McKay, 2006; Roberge et al., 2010). Counterintuitively, these effects have been found to not negatively impact athletic performance (Epstein et al., 2021; Shaw et al., 2021). We posit that innovative technologies could be implemented to expose those at high risk of TBI to increased CO_2 concentrations without requiring the impractical use of compressed CO_2 We believe that the results contained within this study may ultimately be applicable to preventing aspects of TBI among high and at-risk populations such as military personnel and contact sport athletes.

We show for the first time that manipulating the most powerful, innate regulator of CBF (i.e. inspired concentration of CO_2) may be a viable strategy for minimizing the aberrant effects of acceleration/deceleration forces resulting from mTBI. This mechanism is conceptually similar to the mechanism behind the Q-Collar, however induces a more robust physiologic response. These studies have demonstrated that exposure to CO_2 immediately prior to mTBI blocks acute physiologic, behavioral, and transcriptional alterations induced by acceleration/deceleration injury. Within the current study, we have focused on the acute actions of both CO_2 exposure and mTBI, an important first step in evaluating the potential for CO_2 exposure to serve as a preventative measure against injury. We acknowledge that extensive characterization of the semi-chronic and chronic phases of neurotrauma are important to bolster the findings of the current studies and thus will be a focus of future endeavors in this area.

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Figs. 1 and 7 were created with BioRender.com.

Gene ontology analysis was created with http://geneontology.org/

String analysis was created with https://string-db.org/

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CONFLICT OF INTEREST

David W. Smith reports a relationship with DaltaChase that includes: board membership, equity or stocks, and travel reimbursement. David Smith has patent Device to reduce SLOSH energy absorption and its damaging effects through the reduction of the flow of one or more outflow vessels of the cranium licensed to DeltaChase LLC.

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APPENDIX A. SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2022.10.016.

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