ABSTRACT

CORPS, KARA NICHOLE. Transcranial A2A Agonism Induces a Neuroprotective State that Resists the Damaging Effects of Mild Traumatic Brain Injury (Under the direction of Dr. John M. Cullen.)

Traumatic brain injuries (TBI) frequently result in acute and chronic neurological consequences that affect quality of life. The failure thus far to develop efficacious therapies necessitates further investigation of pathogenic mechanisms following TBI. The mechanism(s) by which adenosine signaling modulates TBI pathogenesis is currently unknown. Studies suggest that this pathway can be targeted to improve central nervous system (CNS) injury outcomes. We utilized our mouse model of closed-skull, compressive focal mild TBI (mTBI) to explore the effects of transcranially promoting or blocking adenosine receptor signaling on cell death in the neocortex. Age-matched C57Bl/6J mice were randomly assigned to vehicle control or test compound groups, anesthetized, and subjected to mTBI. Compounds were incubated transcranially for 8 hours followed by transcranial incubation of propidium iodide (PI) to label dead cells *in vivo*. Brains were collected for frozen histopathology, and PI+ dead cells were quantified following confocal imaging of coronal sections through the mTBI center. We found that A2A receptor agonists were neuroprotective following mTBI by acting in a receptor subtype-specific manner on brain-resident rather than infiltrating cells. After mTBI, A2A receptor agonists did not decrease reactive oxygen species (ROS), protect the glia limitans, increase neocortical glutathione, or impact glutamate levels after mTBI. Instead, A2A agonists protected the CNS parenchyma, but not the meninges, by signaling through protein kinase C (PKC) δ and controlling cellular pH via modulation

of carbonic anhydrase (CA) IX. A2A agonists induce a neuroprotective state that resists parenchymal damage due to mTBI. We describe amelioration of acute cell death following mTBI by transcranial application of A2A agonists, warranting further evaluation of the clinical potential of such compounds.

Transcranial A2A Agonism Induces a Neuroprotective State that Resists the Damaging Effects of Mild Traumatic Brain Injury

by
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DEDICATION

For my family and for Patrick.

BIOGRAPHY

Dr. Corps is a board-certified veterinary pathologist. She completed her Doctor of Veterinary Medicine (D.V.M.) degree at Michigan State University in 2010. While in veterinary school, Dr. Corps completed a M.S. degree in Comparative Medicine and Integrative Biology, focusing on toxicologic pathology. Following completion of veterinary school, Dr. Corps entered the Comparative Biomedical Scientist Training Program through the National Cancer Institute, a Graduate Partnership Program between the National Institutes of Health (NIH) and North Carolina State University, to complete a residency in anatomic pathology and pursue a Ph.D. Dr. Corps completed her anatomic pathology training and didactic Ph.D. coursework in 2012 and joined the laboratory of Dr. Dorian McGavern in the National Institute of Neurological Disorders and Stroke (NINDS), achieving board-certification through the American College of Veterinary Pathologists (ACVP) in 2014. Dr. Corps intends to pursue comparative and toxicologic pathology positions after completion of her Ph.D.

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LIST OF ABBREVIATIONS BY CHAPTER

CHAPTER 1

Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Blood-brain barrier	BBB
Central Nervous System	CNS
Cerebrospinal fluid	CSF
Damage-associated molecular patterns	DAMPs
Mild traumatic brain injury	mTBI
Reactive oxygen species	ROS
Traumatic brain injury	TBI
Uridine diphosphate	UDP

CHAPTER 2

5'-adenosine monophosphate	AMP
A1 adenosine receptor	A1
A2A adenosine receptor	A2A
A2B adenosine receptor	A2B
A3 adenosine receptor	A3
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Blood-brain barrier	BBB
Cannabinoid	CB
Central nervous system	CNS
Chronic obstructive pulmonary disease	COPD
Controlled cortical impact	CCI
Cyclic adenosine monophosphate	cAMP
Danger-associated molecular pattern	DAMP
Ectonucleoside triphosphate diphosphohydrolase 1	ENTPD1
Glial fibrillary acidic protein	GFAP
Mild traumatic brain injury	mTBI
Mitogen-activated protein kinase	MAPK
Nuclear factor kappa-light-chain-enhancer of activated B cells	NFκB
Phosphatidylinositol-4,5-bisphosphate 3-kinase	PI3K
Protein kinase A	PKA
Protein kinase C	PKC
Spinal cord injury	SCI
Traumatic brain injury	TBI
Two-photon laser scanning microscopy	TPLSM
Uridine diphosphate	UDP

CHAPTERS 3, 4 AND 5

4',6-diamidino-2-phenylindole	DAPI
A1 adenosine receptor	A1R
A2A adenosine receptor	A2AR
A2B adenosine receptor	A2BR
A3 adenosine receptor	A3R
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Analysis of variance	ANOVA
Artificial cerebrospinal fluid	aCSF
Bicarbonate	$HCO3^-$
Bone marrow	BM
C57Bl/6J	B6
Carbonic anhydrase IX	CAIX
Central nervous system	CNS
Chloride	Cl^-
Controlled cortical impact	CCI
Dimethyl sulfoxide	DMSO
Diphtheria toxin	DT
Diphtheria toxin receptor	DTR
Glasgow coma scale	GSC
Glutathione	GSH
Glutathione disulfide	GSSG
Immunohistochemistry	IHC
Knock out	KO
Mild traumatic brain injury	mTBI
Paraformaldehyde	PFA
Phosphate-buffered saline	PBS
Propidium iodide	PI
Protein kinase C δ	PKCδ
Reactive oxygen species	ROS
Sulforhodamine	SR
Traumatic brain injury	TBI
Two-photon laser scanning microscopy	TPLSM
Wild type	WT

CHAPTER 1

Inflammation and Neuroprotection in Traumatic Brain Injury

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IMPORTANCE. Traumatic brain injury (TBI) is a significant public health concern that affects individuals in all demographics. With increasing interest in the medical and public communities, understanding the inflammatory mechanisms that drive the pathologic and consequent cognitive outcomes can inform future research and clinical decisions for patients with TBI.

OBJECTIVES. To review known inflammatory mechanisms in TBI and to highlight clinical trials and neuroprotective therapeutic manipulations of pathologic and inflammatory mechanisms of TBI.

EVIDENCE REVIEW. We searched articles in PubMed published between 1960 and August 1,2014, using the following keywords: *traumatic brain injury, sterile injury, inflammation, astrocytes, microglia, monocytes, macrophages, neutrophils, T cells, reactive oxygen species, alarmins, danger-associated molecular patterns, purinergic receptors, neuroprotection,* and *clinical trials.* Previous clinical trials or therapeutic studies that involved manipulation of the discussed mechanisms were considered for inclusion. The final list of selected studies was assembled based on novelty and direct relevance to the primary focus of this review.

FINDINGS. Traumatic brain injury is a diverse group of sterile injuries induced by primary and secondary mechanisms that give rise to cell death, inflammation, and neurologic dysfunction in patients of all demographics. Pathogenesis is driven by complex, interacting mechanisms that include reactive oxygen species, ion channel

and gap junction signaling, purinergic receptor signaling, excitotoxic neurotransmitter signaling, perturbations in calcium homeostasis, and damage-associated molecular pattern molecules, among others. Central nervous system resident and peripherally derived inflammatory cells respond to TBI and can provide neuroprotection or participate in maladaptive secondary injury reactions. The exact contribution of inflammatory cells to a TBI lesion is dictated by their anatomical positioning as well as the local cues to which they are exposed.

CONCLUSIONS AND RELEVANCE. The mechanisms that drive TBI lesion development as well as those that promote repair are exceedingly complex and often superimposed. Because pathogenic mechanisms can diversify over time or even differ based on the injury type, it is important that neuroprotective therapeutics be developed and administered with these variables in mind. Due to its complexity, TBI has proven particularly challenging to treat; however, a number of promising therapeutic approaches are now under pre-clinical development, and recent clinical trials have even yielded a few successes. Given the worldwide impact of TBI on the human population, it is imperative that research remains active in this area and that we continue to develop therapeutics to improve outcome in afflicted patients.

Traumatic brain injury (TBI) is a diverse group of brain injuries that vary in cause, severity, pathogenesis, and clinical outcome. As public awareness of TBI and its consequences increases, there is a growing need to understand the underlying mechanisms and develop therapeutic interventions. Within the United States alone, nearly 2 million people sustain a TBI annually, contributing to one-third of all injury-related deaths. Individuals from all nations and demographics are affected, including athletes, military troops, and individuals with unintentional injuries[1-3]. Traumatic brain injury is a significant cause of mortality in children and young adults, and the incidence in older individuals has increased with the average life span[4]. Mild TBI (mTBI) is the most frequent type diagnosed, typically resulting in post-TBI survival. Traumatic brain injury is suspected to contribute to a variety of chronic degenerative processes, including chronic traumatic encephalopathy, Alzheimer disease, and Parkinson disease[5]. Traumatic brain injury is initiated by the application of mechanical force to the head, which can occur with or without loss of consciousness. This then triggers a series of cerebral events that depend in part on the nature and location of the injury. A major challenge associated with treating patients with TBI is the diverse pathologic and pathogenic mechanisms that become operational after injuries. For example, TBI often promotes disruption of blood-brain barrier (BBB) integrity and the neurovascular unit, which can result in vascular leakage, edema, hemorrhage, and hypoxia. Other pathologic mechanisms include cell death within the meninges and brain parenchyma, stretching and tearing of axonal fibers, and disruptions at the junctions between white and gray matter, stemming from rotational forces that cause shearing injuries[6]. All these primary

pathologic mechanisms are accompanied by cellular and molecular cascades leading to inflammation and additional cell death. This review focuses on our current understanding of the sterile immune reaction to TBI and some clinical successes in treating patients with TBI. We searched articles in PubMed published between 1960 and August 1, 2014, using the following keywords: *traumatic brain injury, sterile injury, inflammation, astrocytes, microglia, monocytes, macrophages, neutrophils, T cells, reactive oxygen species, alarmins, danger-associated molecular patterns, purinergic receptors, neuroprotection,* and *clinical trials.* Clinical trials or therapeutic studies that involved manipulation of the discussed mechanisms were considered for inclusion. The final reference list was assembled based on novelty and direct relevance to the primary focus of this review.

Sterile Immune Reaction to TBI

Central nervous system (CNS) resident and peripherally derived inflammatory cells respond quickly to brain injuries and can even participate in the repair process[7,8]. These responses are commonly referred to as sterile immune reactions. A previous study[9] found that the inflammatory gene expression profile is comparable between mTBI and severe TBI, suggesting a common response to both forms of injury. The acute cellular reaction to TBI includes astrocytes, microglia, monocytes or macrophages, neutrophils, and T cells, which are initially activated in part by purinergic receptor signaling[10,11]. In the following paragraphs, we describe the inflammatory response to TBI in more detail, focusing specifically on traditional immune cell populations. Sterile immune reactions are at least initially designed to be beneficial but can become detrimental in certain situations.

Danger Signals

Pathogens can trigger innate immune activation via pathogen-associated molecular pattern molecules, which are conserved structures within a class of microbes recognized by Toll-like receptors or pathogen-recognition receptors. These innate signaling pathways allow plants and animals to respond quickly to invading microbes. However, it is now recognized that tissue damage in the absence of microbial infection can trigger inflammasome and innate immune activation through the release of damage-associated molecular pattern molecules (DAMPs),sometimes referred to as danger signals[12]. Alarmins are endogenous DAMPs released by cells undergoing nonapoptotic death or by cells of the immune system[13]. Some examples of alarmins include HMGB1, S-100 proteins, adenosine triphosphate (ATP), uric acid, DNA or RNA, and interleukin 1α, among others. After TBI, alarmins are undoubtedly released[14], and this triggers a sterile immune reaction designed to restore tissue homeostasis. However, the severity and duration of injury can foster maladaptive immune reactions that become injurious. A previous study[15] found that ATP release and detection via purinergic receptors elicit an acutely neuroprotective inflammatory response after mild cortical injury, but sustained immune activation may not always be beneficial. For example, therapeutic blockade of inflammasome activation reduced innate immune activation and severe TBI lesion size[16]. Thus, additional research is required to better understand the rules that govern pathogenic vs nonpathogenic innate immune reactions after DAMP signaling in the injured brain.

Purinergic Receptor Signaling

Purinergic receptors are an evolutionarily ancient family of transmembrane molecules that detect ATP, adenosine diphosphate (ADP), or adenosine[10,11]. The receptors are divided into 2 basic classes based on whether they respond to adenosine (P1 receptors) or ATP or ADP (P2 receptors). Because ATP is a cellular source of energy, it is maintained at a high intracellular concentration during steady-state conditions. After tissue injury, ATP is released from damaged cells, which triggers an immune reaction via purinergic receptor signaling. This reaction can be amplified by pannexin and connexin hemichannels that pump ATP from healthy cells into the extracellular space. Sterile immune reactions generally subside as ATP is converted into adenosine through a 2-step reaction that involves ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5′-nucleotidase (CD73). Astrocytes and microglia each express at least one these ectoenzymes[17,18], allowing them to dampen ATP-mediated neuroinflammation.

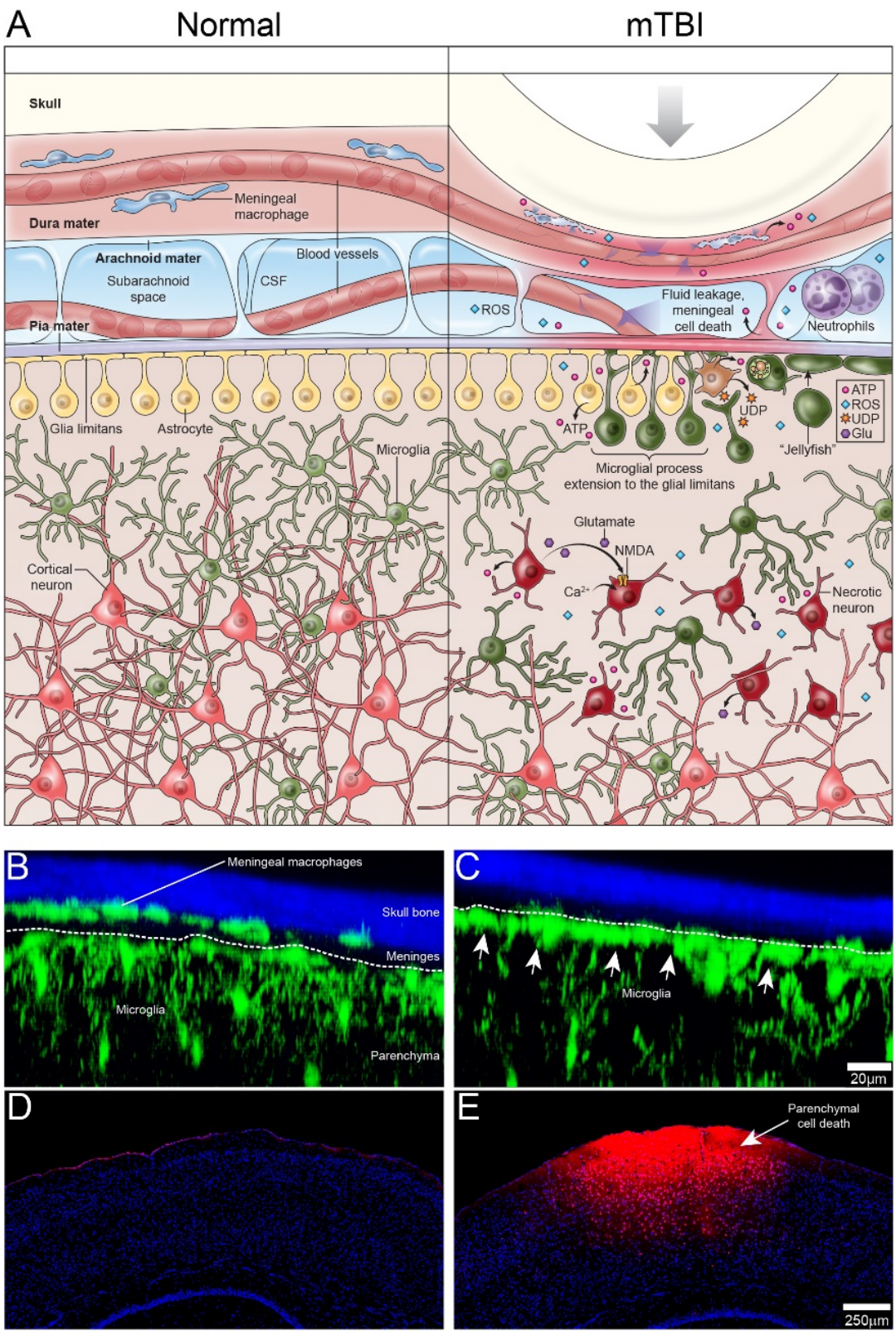
Microglia

Microglia are highly dynamic CNS resident innate immune sentinels that originate from primitive myeloid progenitor cells during development[19,20] . Microglia participate in a variety of homeostatic CNS functions, including synaptic plasticity and learning[21], and are often the first responders to any inflammatory event that occurs within the parenchyma[20]. Microglia mediate neuron removal during development via release of reactive oxygen species (ROS) and can acquire a phagocytic phenotype without an inflammatory response[20]. Microglial expression of genes associated with neuroprotection is upregulated with age[22]. Microglia express

a large number of surface and cytoplasmic receptors, and cumulative signaling through these receptors determines whether microglia remain in a ramified, sentinel state or take on various configurations as a result of activation[23]. In addition, microglia can sense a large repertoire of exogenous and endogenous signals, allowing for dynamic responses to sterile injuries and infectious agents that can be injurious or neuroprotective, depending on the context[22]

During CNS autoimmune disease, activated microglia phagocytose debris and downregulate cellular metabolism in contrast to disease-initiating, peripherally derived macrophages, which appear to play a more destructive role by promoting demyelination[24]. These data suggest that microglia are not inherently neurotoxic during development of a CNS autoimmune disease. After acute focal brain injury in rodents, microglia similarly appear to play a neuroprotective role[15]. Using 2-photon microscopy, we revealed that microglia respond within minutes of brain injury by extending processes to the glial limitans and circumscribing individual astrocytes, resembling a hexagonal honeycomb structure (**Figure 1A-E**, **Figure 2E** and **H**). This reaction was dependent on purinergic receptor signaling ($P2X_4$ and $P2Y_{12}$) and astrocytic ATP-dependent ATP release via connexin hemichannels. In response to cell death (eg, astrocytic cell death in the glial limitans), microglia transformed into phagocytic cells that resembled jellyfish (**Figure 1A-E, Figure 2F** and **I**). Jellyfish microglia were highly mobile and often inserted themselves into the damaged glial limitans in place of dead astrocytes, connecting together to form a phagocytic barrier. This reaction was also dependent on purinergic receptor signaling ($P2X_4$,

Figure 1. Pathogenesis of Traumatic Brain Injury (TBI). A, Comparison of brain anatomy in the meninges and superficial neocortex before and after focal mild TBI (mTBI). The dura mater contains numerous small vessels that are lined by thin, elongated meningeal macrophages. The subarachnoid space contains vessels, fibroblastlike stromal cells, and cerebrospinal fluid (CSF). The glial limitans, composed of astrocytic foot processes, lies beneath the pia mater and forms a barrier between the CSF and underlying parenchyma. Mild focal brain injury mechanically compresses the meningeal space, compromising vascular integrity and inducing rapid necrosis of meningeal macrophages and structural cells. Leakage of fluid from meningeal blood vessels results in edema, and damaged cells within the meninges release reactive oxygen species (ROS) and adenosine triphosphate (ATP), initiating a sterile immune reaction. B and C, Maximum projections (5-μm wide) are shown in the *xz* plane of 2-photon *z*-stacks captured through the thinned skull of CX3CR1$^{GFP/+}$ mice (original magnification ×20). B, A representative image of an uninjured mouse reveals the presence of meningeal macrophages (green) in the dura and ramified microglia (green) in the brain parenchyma beneath the glial limitans (white dotted line). C, Thirty minutes after focal mTBI, meningeal macrophages die and microglia relocate to the injured glial limitans (arrowheads). Skull bone is shown in blue. D and E, Histopathologic analysis of the superficial neocortex by confocal microscopy 8 hours after mTBI (original magnification ×20). D, An uninjured brain is shown for comparison. Dead cells were labeled transcranially with propidium iodide. Cell nuclei are blue. E, A large lesion consisting of numerous dead cells (red) (arrowhead). UDP indicates uridine diphosphate.


A
Normal
mTBI
Skull
Meningeal macrophage
Dura mater
Arachnoid mater
Subarachnoid space
Blood vessels
CSF
Pia mater
ROS
Fluid leakage, meningeal cell death
Neutrophils
Glia limitans
Astrocyte
Microglia
Cortical neuron
ATP
UDP
"Jellyfish"
ATP
ROS
UDP
Glu
Microglial process extension to the glial limitans
Glutamate
NMDA
Ca2+
Necrotic neuron
B
Meningeal macrophages
Skull bone
Meninges
Microglia
Parenchyma
C
Microglia
20μm
D
E
Parenchymal cell death
250μm

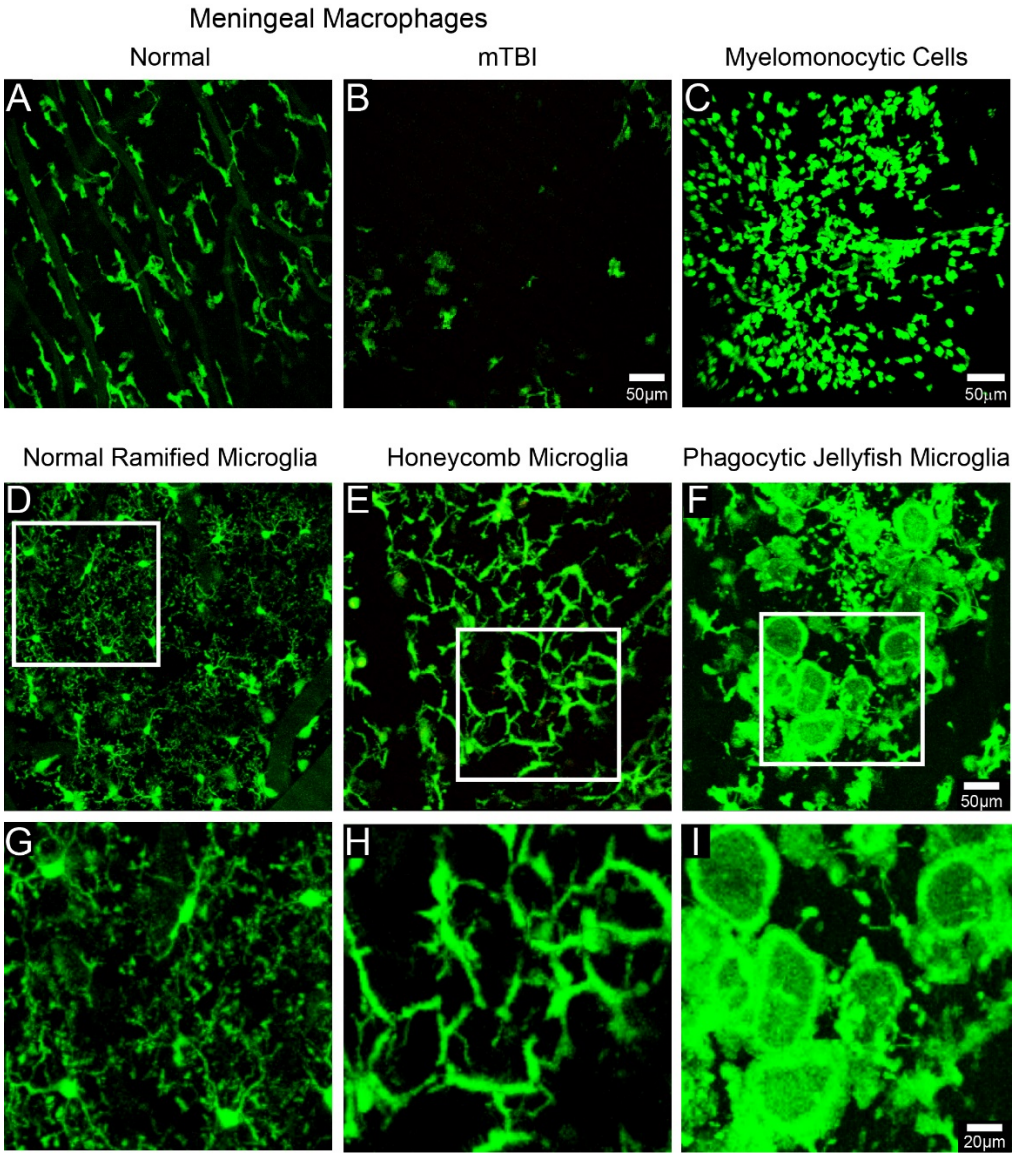


Figure 2. Inflammatory Reaction to Traumatic Brain Injury. A-I, The 25-µm *xy* maximum projections from CX3CR1GFP/+ (A, B, and D-I) or LysM$^{GFP/+}$ (C) mice were captured by 2-photon microscopy through a thinned skull. A, Meningeal macrophages (green) are thin, elongated cells that reside along the dural blood vessels in the uninjured brain. B, After focal mild traumatic brain injury (mTBI), meningeal macrophages undergo necrosis within 30 minutes and disappear from the field of view. C, Myelomonocytic cells (green) invade the damaged meninges within an hour of brain injury. D and G, In the uninjured brain, microglia (green) have small cell bodies and are highly ramified. Focal brain injury induces the rapid transformation of microglia into at least 2 distinct morphologic patterns. E and H, Honeycomb microglia extend processes that circumscribe the borders between individual astrocytes in the glial limitans. F and I, Phagocytic jellyfish microglia are generated in response to cell death and form a film across the damaged glial limitans. High-magnification views in panels G through I are denoted with white boxes in panels D through F (original magnification ×20).

$P2Y_6$, $P2Y_{12}$) and connexin hemichannels. When these microglia responses were inhibited locally through blockade of purinergic receptor signaling or connexin hemichannels, the pathologic mechanisms observed after brain injury were more severe. One of the most notable changes was increased leakage of materials through the glial limitans into the brain parenchyma. These data suggest that microglia not only clean up debris from the injured brain but also help maintain glial limitans barrier integrity by sealing the gaps that result from dead or damaged astrocytes. Moreover, our data are consistent with previous studies[25-28] that link microglia injury responses to ATP release and purinergic receptor signaling. Although it is conceivable that microglia responses become maladaptive over time or after exposure to different combinations of stimuli[29], we propose that the acute role of microglia in the focally injured brain is neuroprotective.

Monocytes and Macrophages

Monocytes are a multipotent population of circulating bone marrow–derived leukocytes capable of differentiating into macrophages or dendritic cells after invasion of an infected or injured tissue[30]. They are also known to participate in diverse functions, such as phagocytosis, cytokine or chemokine release, antigen presentation, immune modulation, and tissue repair. In the naïve brain, there are also populations of specialized macrophages that reside in the meninges, choroid plexus, and perivascular spaces[31]. Their role in TBI pathogenesis is unknown. Another study[15] also found that meningeal macrophages are among the first cells to die after focal cortical injury and may serve as an early source of alarmins and ROS (**Figure 1A-C**, **Figure 2A** and **B**). Monocyte-derived macrophages coming from the

blood do not reach peak numbers in the damaged brain of animals and humans until 24 to 48 hours after injury[32,33]. Monocytes are capable of crossing the blood–cerebrospinal fluid barrier with neutrophils into the injured brain as a result of CCL2 production by choroid plexus epithelium[34]. CCL2 is significantly increased in the cerebrospinal fluid of patients with TBI[33]. Examination of $CCL2^{-/-}$ mice after TBI revealed slight alterations in cytokine expression but no changes in lesion size within the first week of injury[33]. However, when followed for a longer timeframe of 2 to 4 weeks, $CCL2^{-/-}$ mice had improved functional recovery, suggesting a pathogenic role for macrophages during the chronic phase of TBI. Similar results were obtained in $CCR2^{-/-}$ mice after TBI[35]. CCR2 is the receptor for CCL2, and deficiency significantly reduced the number of lesion macrophages and increased hippocampal neuronal densities, spatial learning, and locomotion when measured several weeks after brain injury. Collectively, the data obtained in CCL2 and CCR2 knockout mice suggest that monocyte-derived macrophages play a pathogenic role in the chronic phase after TBI. Additional studies are required to determine whether these cells can participate in brain repair after TBI similar to what has been described in models of spinal cord injury[36]. Whether a macrophage is pathogenic or beneficial after tissue injury likely depends on its state of differentiation.

Neutrophils

Neutrophils are an abundant population of circulating leukocytes that are usually among the first responders to tissue injuries in the periphery and CNS[37]. Neutrophils are often viewed as a proinflammatory cell population but are known to play a vital role in wound healing through their involvement in phagocytosis,

metalloproteinase release, and growth factor production. After tissue injury, neutrophils can help prepare the damaged environment for repair. Neutrophils are rapidly recruited to the CNS after TBI and enter through meningeal blood vessels and the choroid plexus[15,32,38,39]. They can also facilitate the recruitment of monocytes[37]. A previous study[40] focused on sterile injury of the liver found that ATP released from the damaged tissue induced inflammasome activation in a $P2X_7R$-dependent manner. This activation in turn promoted rapid recruitment of neutrophils through release of chemoattractants (CXCL1 and CXCL2) and formyl peptides that guided these cells to the site of injury. After focal TBI, we observed that neutrophils are similarly recruited in a $P2X_7R$-dependent manner and arrive within 1 hour of injury (**Figure 2C**)[15]. Visualization of cellular dynamics and localization by 2-photon microscopy revealed that neutrophils localized primarily to the damaged meninges (instead of the parenchyma), where they swarmed the area and interacted with dead cells. Antagonism of this response by blocking $P2X_7R$ signaling increased the amount of cell death in the meninges, suggesting a protective role for neutrophils in the meningeal space after focal cortical injury.

Neutrophils are not always neuroprotective and have the capacity to break down the BBB by releasing metalloproteinases, proteases, tumor necrosis factor α, and ROS. Inflammatory mediators released after brain injury can facilitate this process by inducing a hyperactivated state that allows neutrophils to breach the BBB and enter the CNS[41]. On arrival, neutrophils have the potential to induce neuronal cell death using the same soluble mediators that break down the BBB[42]. A previous study[43] revealed that neutrophils are the most abundant cell population in circulation

after TBI and cause increased expression of oxidative enzymes indicative of activation. Depletion of neutrophils with anti-Gr-1 antibodies after controlled cortical impact in rodents reduced edema, microglia and macrophage activation, and TBI lesion size, but did not affect vascular leakage at 24 to 48 hours after injury[44]. These data reveal that neutrophils can be pathogenic after open-skull cortical impact. However, the contribution of neutrophils to a CNS lesion may depend on their precise localization and state of activation. Open-skull controlled cortical impact is highly disruptive to meningeal architecture and likely favors neutrophil recruitment to the heavily damaged brain parenchyma. These findings contrast with mild closed-skull cortical injury, which maintains meningeal architecture and fosters a more selective pattern of neutrophil recruitment[15]. To definitively establish the contribution of neutrophils to TBI pathogenesis, these cells should be evaluated in many different models of brain injury. It is conceivable that their contribution will differ based on the nature of the injury.

T Cells

Although T cells play diverse roles in adaptive immune responses and the regulation of inflammation, their role (if any) in TBI pathogenesis is not clear. It has been proposed that autoreactive T cells against CNS antigens, such as myelin basic protein, can be neuroprotective after spinal cord injury[45]. After brain injury, activated T cells are recruited to sites of damage[46], and ROS release may facilitate this recruitment by activating endothelial barriers[47]. To address the role of T cells in TBI, a previous study[48] examined the response to closed-skull head injury in *RAG1* knockout mice that lack mature T and B cells. No difference in any pathologic or

neurologic parameters was observed between wild-type and *RAG1*-deficient mice for up to 1 week. These data suggest that T cells play no role in early TBI pathogenesis. Additional studies are required to determine whether T cells actively participate in chronic TBI lesions (beyond 1 week) and/or the reparative process.

Therapeutic Modulation of TBI Pathogenesis

The pathogenesis of TBI is complex as reflected by the number of clinical trials that have failed to improve outcomes in humans[49,50]. The many reasons for these failures have been discussed in other reviews[49,50]. Rather than focus on the reasons for prior failures, we instead briefly discuss some successes that pertain to mechanisms of pathogenesis and inflammation covered in this review.

The concept of free radical–mediated damage of CNS tissue after injury has existed for several decades[51,52]. Administration of effective antioxidants has the potential to significantly limit the spread of damage and inflammation if given soon after brain injury. In animal models, a number of previous studies[53,54] have yielded promising results with antioxidants that neutralize ROS. For example, intravenous administration of the small-molecule free radical scavenger edaravone at 2 and 12 hours after weight drop–induced TBI resulted in significantly reduced inflammation, edema, BBB breakdown, lesion size, and neurologic deficits[53]. Inhibition of NADPH oxidase complex assembly with apocynin also reduced ROS production, BBB breakdown, and neuronal cell death after weight drop-induced TBI[54]. The only caveat of this study was that the apocynin was injected intraperitoneally 15 minutes before injury. Nevertheless, the favorable outcome implicates NADPH oxidase as a potential source of ROS after brain injury.

Using a new model of mild cortical injury, we found that transcranial administration of the antioxidant glutathione at 15 minutes or 3 hours after injury significantly reduced inflammation, glial limitans breakdown, and parenchymal (but not meningeal) cell death by up to approximately 70%[15]. Pretreatment with glutathione reduced meningeal cell death by approximately 50%. These data indicate that ROS are a primary inducer of cell death and inflammation after focal brain injury and that an antioxidant can have a major effect on lesion expansion if given early. The advantage of passing a neuroprotective compound directly through the skull bone (transcranial delivery) is that a high local drug concentration can be achieved in the CNS with a limited off-target effect on the periphery.

Previous studies[55,56] have supported antioxidants as neuroprotective agents in rats and humans, revealing that administration of *N*-acetylcysteine reduces brain damage and improves recovery after TBI. *N*-acetylcysteine is the cellular precursor to glutathione. A randomized, double-blind, placebo-controlled clinical trial[55] was performed to assess efficacy in members of the military who experienced a mTBI that resulted from blast exposure. Patients who received *N*-acetylcysteine within 24 hours had significantly improved recovery during a 7-day period when compared with a placebo control group. These findings were corroborated in 2 different rodent models of TBI (weight drop and fluid percussion), which revealed that *N*-acetylcysteine reversed the behavioral deficits associated with mTBI and moderate TBI[56]. Further studies are needed to determine whether this promising neuroprotective intervention will be efficacious in patients with diverse types of brain injury.

Many clinical trials have been completed or are under way to assess the role of excitotoxic mechanisms in TBI pathogenesis[49,50]. With the exception of amantadine, all drugs in this class tested to date have not been effective in promoting recovery in patients with TBI. Amantadine is thought to act as an *N*-methyl-D-aspartate receptor antagonist and an indirect dopamine agonist. When patients with TBI were treated during a 4-week period beginning 4 to 16 weeks after injury, amantadine improved recovery relative to the placebo control. The mechanism underlying this positive effect remains unclear. Prevention of *N*-methyl-D-aspartate receptor–mediated excitatory damage seems unlikely given that the drug was administered a month or more after the initial injury[57].

Manipulation of purinergic receptor signaling is another therapeutic approach worth considering. Use of specific purinergic receptor agonists and antagonists should allow therapeutic amelioration of different TBI lesion parameters. A previous study[15] found that microglia responses after mTBI were dependent on $P2X_4$, $P2Y_6$, And $P2Y_{12}$ receptors, whereas $P2X_7R$ signaling was necessary for neutrophil recruitment. It might be possible to promote neuroprotective inflammatory responses through therapeutic agonism of these pathways after brain injury. The challenge, however, with purinergic receptor manipulation is that specific receptors are often expressed on multiple cell populations. A purinergic receptor agonist or antagonist will likely affect multiple cell populations simultaneously. As an example, a previous[58] study found that $P2X_7R$ localized to astrocytic end feet and antagonism of this receptor reduced astrocyte activation, cerebral edema, and neurobehavioral

abnormalities after controlled cortical impact–induced TBI. A similar protective effect was obtained by blocking $P2X_7R$ after spinal cord injury, which was linked to receptor expression on spinal cord neurons[59]. However, $P2X_7R$ is also expressed on inflammatory cells, and a previous study[15] found that antagonism of this pathway increased meningeal cell death after mTBI, likely due to diminished neutrophil recruitment. Thus, purinergic receptor modulation can positively affect one CNS environment and negatively affect another. It will therefore be important in future studies to map out the exact contributions of specific purinergic receptors to different TBI lesion parameters before deciding which (if any) are best to target therapeutically in patients.

Discussion

The pathogenesis of TBI is initially induced by a mechanical injury that sets into motion a complex secondary reaction mediated by ROS, purines, calcium ions, excitatory amino acids, and DAMPs, among others. This pathogenesis in turn triggers a robust sterile immune reaction that consists of CNS resident and peripherally recruited inflammatory cells. The response is designed to be neuroprotective and promote wound healing but can become maladaptive over time, especially if the lesion remains active for weeks. Among the earliest soluble mediators are ROS and purines. Both are released within minutes of brain injury and initiate an inflammatory cascade. Even after mild focal cortical injury, ROS can damage the glial limitans that separate the meninges and parenchyma, which results in lesion expansion within brain tissue. Vascular damage and leakage represent

another early hallmark of TBI pathogenesis that can foster edema, hypoxia, and tissue destruction. After brain injury, the innate immune system quickly mobilizes in response to purines and alarmins, and astrocytes help orchestrate this response by serving as inflammatory amplifiers. Within minutes, resident microglia are among the first to react by fortifying CNS barriers and participating in phagocytic cleanup. Neutrophils and monocytes arrive shortly thereafter and preferentially survey injured meningeal spaces if the CNS architecture remains intact. Focal brain injury elicits an anatomically partitioned immune reaction (at least acutely) with myelomonocytic cells tending to the damaged meninges and microglia responding within the parenchyma. Eventually, myelomonocytic cells can enter the damaged brain, and studies[40-42] have found that their presence there is sometimes neurotoxic. However, sterile immune reactions are not inherently neurotoxic and are usually elicited to prepare a damaged tissue for wound healing. Thus, the entire contribution of immune cell subsets to TBI lesions needs to be considered before targeted therapeutic interventions can be intelligently designed. Another important variable is time. The exact contribution of immune cells to a TBI lesion may in fact shift over time. For example, an initially neuroprotective immune response may become maladaptive as secondary inducers of tissue destruction diversify.

Although TBI has proven difficult to treat, promising interventions lie on the horizon. Given the importance of ROS in TBI pathogenesis and the success with *N*-acetylcysteine in patients with mTBI, clinical pursuit of antioxidant therapy seems warranted. The likely key to success is early treatment with antioxidants so that TBI lesion expansion and subsequent inflammation can be stopped as soon as they are

initiated. Because TBI lesions begin to expand within hours of injury, development of strategies to rapidly preserve brain tissue is paramount. The kinetics of lesion expansion must be similarly considered when attempting to manipulate purinergic and excitatory neurotransmitter pathways, which engage rapidly after injury. Therapeutic targeting of these pathways has the greatest likelihood of working if administered soon after injury. For the chronic phase of TBI pathogenesis, more research is required to understand lesion dynamics. Over time, it may become necessary to dampen maladaptive inflammatory responses and attempt to promote wound healing reactions, which would be challenging to achieve without having a better understanding of chronic lesion dynamics.

Conclusions

Traumatic brain injury encompasses a complex spectrum of injuries that tax the neural-immune interface and can result in permanent neurologic dysfunction. Detailed knowledge of this interface during the acute and chronic phases of TBI will help us design the most efficacious interventions.

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CHAPTER 2

Purinergic Signaling in Traumatic Brain Injury

Purinergic Receptors and Signaling

Extracellular signaling of neurons via purines was proposed initially in 1972 and subsequently demonstrated in a variety of cell types[1,2]. The three types of purinergic receptors were identified and described two decades later[3]. These include the P1, or adenosine, receptors; the P2Y receptors, which are transmembrane G protein-coupled receptors; and the P2X receptors, which are ion-gated transmembrane channels. Currently, there are eight recognized P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) and seven P2X receptor subtypes ($P2X_{1\text{-}7}$). Additionally, a recently characterized "orphan" G protein-coupled receptor, GPR17, binds uracil nucleotides and exhibits signaling behavior very similar to P2 receptors and is therefore currently discussed with purinergic receptors despite this being a misnomer[4]. Adenosine triphosphate (ATP) was the first identified ligand of the P2Y and P2X receptors, though numerous groups have since demonstrated binding of adenosine diphosphate (ADP) and pyrimidines to the P2Y receptors[5]. P2Y and P2X receptors drive immune and inflammatory reactions in response to heterogenous injurious tissue processes. Pannexin and connexin hemichannels propagate these responses by allowing healthy or injured cells to pump ATP into the extracellular space, enhancing signaling on local and infiltrating cells via a danger-associated molecular pattern (DAMP)-like mechanism[6,7]. Purinergic receptors have been implicated in critical development processes[8,9], immune and inflammatory diseases[6], neuropathic pain[10,11], memory[12], anxiety and

fear[12,13], diabetes[14], renal function and pathology[15], bone remodeling and osteoporosis[16], vascular and blood flow dynamics[17], cancer[18], numerous central and peripheral nervous system functions and neurodegenerative processes[19,20], and a variety of other pathophysiological processes too numerous to list here[5]. In normal immune responses, cessation of purinergic receptor-driven signaling is facilitated by the two-step hydrolysis of ATP by ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, CD39) and ecto-5´-nucleotidase (CD73), eventually resulting in adenosine[6]. Like purinergic receptors, CD39 and CD73 are expressed on numerous cells types throughout the body and are involved in many processes involved in both normal homeostasis as well as pathology[21]. The ectonucleotidases are very highly expressed on cells involved in release of ATP for signaling, including astrocytes in the glia limitans and found throughout the CNS[22]. The vast involvement of purinergic receptors, CD39 and CD73 in pathophysiological processes has made them attractive targets for therapeutic development, particularly in the central nervous system[5,23,24]. Collectively, the purinergic system allows very tight regulation of cell signaling, immune responses and inflammatory reactions, with adenosine most commonly producing an effect opposite that of ATP[6].

P1 Adenosine Receptors

Adenosine is produced in the extracellular environment by hydrolytic degradation of ATP through ADP and the immediate precursor 5′-adenosine monophosphate (AMP). Intracellular production of adenosine is accomplished by reversible conversion of AMP by 5′-nucleotidase. Adenosine moves bidirectionally through specific nucleoside transporters, allowing active alteration of extra- or

intracellular concentration. Passive transporters are highly expressed in the CNS, while sodium-dependent active transporters are expressed in brain, lung, T cells, liver, kidney, and intestines and move additional adenosine to areas of high concentration. Adenosine can be metabolized intracellularly via two main pathways, resulting in production of uric acid by xanthine oxidase or production of inosine and hypoxanthine by adenosine deaminase. Adenosine is maintained at concentrations between 10-200 nM in the extracellular environment but can increase to 10-100 μM with tissue damage. Like its precursors, adenosine has highly varied effects in many cells types and is involved in numerous developmental, homeostatic and degenerative processes[25-27].

The four adenosine receptors (A1, A2A, A2B, and A3) are G protein-coupled receptors expressed throughout the body, with relatively high expression levels on cells including bone marrow-derived cells, endothelium, smooth muscle, cardiac muscle, bone, neurons, and all examined types of glia. The A1 and A3 receptors are considered broadly inhibitory in that they are linked to G_i proteins and predominantly decrease cyclic adenosine monophosphate (cAMP). The A2A and A2B receptors are coupled to G_s proteins, considered broadly stimulatory and increase cAMP upon agonist binding. Ligand binding at adenosine receptors results in signaling cascades through numerous downstream targets and mediators, some of which are cAMP independent. These include but are not limited to protein kinase A (PKA), protein kinase C (PKC), Ras, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), calcium, mitogen activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and cell cycle proteins[6,28-31]. The specific

signaling cascades and results thereof are significantly more complex than the relatively small number of receptors would suggest, due in part to formation of a variety of heterodimers and heterotrimers, particularly in the brain. In addition to coupling of different adenosine receptor subtypes, links between the following have been reported: A1 receptors and β1 adrenergic receptors, A1 receptors and P2Y receptors, A2A receptors and cannabinoid (CB)-1 receptors, A2A receptors and glutamate receptors, A2A receptors and dopamine receptors, and heterotrimers of A1/A2A and dopamine receptors[25,30,32]. The diversity of these hybrid receptors and potential combinatorial effects of signaling through multiple adenosine receptor subtypes simultaneously provides a mechanism of exponential complexity for tight control of functions in the CNS and other tissues. Despite this, most agonists and antagonists of adenosine receptors, including those considered relatively selective, exhibit overlap in receptor binding. There is significant interest in development of highly specific and selective adenosine receptor ligands for a variety of clinical applications despite the potential difficulty in elucidating mechanisms of action, an issue further complicated by moderate sequence homology between species and relatively low homology between receptor subtypes, giving rise to variable receptor binding properties[25,27,31].

The A1 receptor binds adenosine with an EC_{50} of 0.2-0.5 μM[6] and is the most conserved among examined species[31]. It is widespread throughout the body but is expressed most highly in the brain, particularly on neurons[27]. In addition to cAMP-dependent functions, this receptor has been demonstrated to activate ion channels and a variety of kinases independently[33]. Perhaps the best-known functions of this

receptor involve inhibition of neurotransmitter release in both the CNS and in cardiac muscle[34], though it has been implicated in numerous other physiological and pathophysiological processes including vasoconstriction and bronchoconstriction[35,36], reduced heart and respiration rates[34,37], bone homeostasis[38], sleep[39], and primary and cardiac dysfunction-induced renal disease[27,40]. The A1 receptor is frequently studied with the A2A receptor due to high expression of both in the brain and potential synergistic and antagonistic effects[41].

The A3 receptor has similar binding affinity for adenosine with an EC_{50} of 0.2-0.5 μM[6,29]. Its distribution appears to be highly species dependent, though it has been identified in most tissues including several cell types in the brain. The A3 receptor appears to play a special role in mast cell degranulation in rodents that is not recapitulated in humans[33]. Despite relatively low expression, numerous authors have reported A3 receptor effects in cardiovascular disease[42], pulmonary disease and injury including chronic obstructive pulmonary disease (COPD)[43], immune-mediated and rheumatologic diseases[44], osteoarthritis and other musculoskeletal diseases[45], cancer[46], ocular disease[47,48], and CNS diseases[29]. Temporal- and dose-dependent factors have complicated study of this receptor due to seemingly opposite effects in the same system. For example, agonizing this receptor appears to have cardioprotective effects under ischemic conditions yet causes deleterious vasoconstriction in some models[42,49,50]. Similarly, both agonists and antagonists have proven beneficial in inflammatory ocular diseases[47]. Like the other adenosine receptors, advances in understanding of the role of A3 in health and disease is expected to increase with development of additional receptor-specific compounds.

The A2B receptor binds endogenous adenosine with relatively low affinity, exhibiting an EC_{50} of 16-64 μM[6,51]. It is most highly expressed in blood vessels, immune cells, aortic smooth muscle, large intestine, urinary bladder, and brain, though expression levels are low overall compared to the other adenosine receptors[27,52]. Important roles for the A2B receptor have been found in lung injuries and diseases, vascular diseases, cancer, renal disease, and diabetes. Generally, signaling via A2B receptors tends to decrease proinflammatory cytokine production and protects against sequela of inflammatory conditions such as fibrosis, showing promise in both acute and chronic renal diseases[52,53]. Groups have demonstrated a multifaceted protective role for the receptor in vascular injury diseases and regulation of platelet aggregation[54,55]. Mixed results have been demonstrated in several *in vitro* models of cancer and *in vivo* models of diabetes[52]. As with other purinergic receptors, conflicting evidence suggesting beneficial effects of both agonizing and antagonizing A2B signaling has been demonstrated in several diseases and is likely dependent on numerous factors including receptor specificity of A2B-binding compounds, *in vitro* vs. *in vivo* models of disease, tissue microenvironment, and disease-specific factors.

The A2A receptor exhibits moderately high binding affinity for adenosine with an EC50 of 0.6-0.9 μM[6,51]. This receptor is highly expressed on leukocytes, platelets, endothelial cells, thymus and spleen immune cells, and cells in the striatum of the brain, but is also found in blood vessels, peripheral nerves, heart, and lung[27,50,56]. In some cases, very low expression of this receptor has been discovered as a result of organ-specific effects of agonists or antagonists, such as the

unexpected effects reported in the kidney[15]. Receptor subtype-specific effects have also been demonstrated in the absence of detectable A2A mRNA in the brain[57]. The A2A receptor has been implicated in diverse functions and diseases, including potent vasodilation of systemic and coronary vessels[58,59], physiological and pathological angiogenesis[60,61], fibrosis and wound healing[60,62], locomotion[58], and platelet function[58]. A2A signaling has been shown to decrease pulmonary inflammation as a sequela of sickle cell disease[63,64]. Several groups have reported that A2A signaling contributes to an immune-suppressed microenvironment in cancer, conferring a survival benefit to some neoplasms[28]. This receptor is involved in both control of immune responses and in inducing immunosuppression[65], and has been shown to decrease inflammation in diabetic nephropathy[66]. Activation of the A2A receptor is under investigation as a mechanism to decrease the occurrence of graft-versus-host disease in patients receiving hematopoietic cell transplants[67]. Numerous groups have independently reported a role for A2A signaling in the integrity of the blood-brain barrier (BBB), a property that is being exploited pharmacologically to provide better CNS penetrance of chemotherapeutic agents[68-70]. The role of the A2A receptor in the CNS is of great interest within the biomedical research community. In the striatum, the A2A receptor controls maintenance of certain types of memory[71]. This receptor is upregulated in the brains of individuals with disorders like schizophrenia and has been implicated in development and maintenance of chemical dependence and addiction[72,73]. Even though caffeine is a nonspecific adenosine receptor antagonist with strongest binding to both the A1 and A2A receptors, it has been demonstrated that the wakefulness and stimulant effects

of caffeine are mediated through the A2A receptor[27,74]. Additionally, there is strong evidence that the A2A receptor is a potentially valuable target for treatments addressing neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease[72,75-80]. Mice deficient in the A2A receptor exhibit cognitive deficits[81]. As with the other adenosine receptors, studies in similar animal models report both beneficial and deleterious effects of the A2A receptor agonists and antagonists, warranting further careful study and elucidation of the mechanisms underlying observed results.

P2 Receptors in Sterile Injuries of the CNS

Sterile injuries in the CNS include ischemic stroke and TBI, which are grouped due lack of invading or infectious agent causing tissue injury. Rapid release of ATP and adenosine has been demonstrated following sterile injury, effectively converting molecules normally used as neurotransmitters in the CNS to DAMPs[57,82]. Purinergic receptors and ectonucleotidases are found on all CNS cells thus far examined at varying levels of expression and facilitate glia-glia and glia-neuron communication[83]. Like the effects in other organs and systems, ATP and adenosine signaling tend to have oppositional effects in the CNS following insult, with ATP mediating inflammation and adenosine modulating and dampening these responses.

CNS injury results in numerous cascades resulting in infiltration of immune cells from the periphery, with facilitation and fine tuning influenced by purinergic signaling. All examined peripheral immune cells express purinergic receptors, with P2Y and P2X receptors regulating chemotaxis in neutrophils and macrophages, and pannexin-released ATP-mediated signaling amplification on T cells via

predominantly P2X receptors[6]. $P2X_7$ activation induces chemokine and cytokine production by microglia in response to neighboring cell injury[84] and results in neutrophil infiltration into the meninges between 1 and 3 hours post-injury in models of mTBI[85,86]. $P2X_7$ inhibition exacerbates acute cell death in the meninges, demonstrating the importance of early peripheral immune responses in controlling the spread of tissue damage[85]. Influx of other peripheral immune cells occurs much later following brain injury and is likely influenced by purinergic signaling-induced production of proinflammatory cytokines and chemokines by CNS resident cells[19,86].

Signaling via purinergic receptors plays numerous important roles in oligodendrocytes, including development and maturation of many precursor cell types[9]. These cells express numerous P2 receptors and all P1 receptors which strongly regulate myelin production. ATP released from large axons has been shown to regulate proliferation and differentiation of mature myelinating cells, indicating a likely role in post-injury recovery in white matter that is under current investigation by several groups[87]. $P2X_7$ receptors negatively regulate precursor proliferation at high extracellular ATP concentrations following traumatic white matter damage, but promote migration and differentiation of existent precursors. Depending on the severity of the injury and other microenvironmental factors, the $P2X_7$ receptor may exacerbate excitotoxic injury to mature oligodendrocytes, which can be quite sensitive to excessive glutamate levels[87,88]. In contrast, ischemic injury results in $P2X_7$-mediated necrotic and apoptotic death of oligodendrocyte precursors[88]. Much of the effect of ATP signaling via $P2X_7$ appears to depend on expression levels on mature or precursor oligodendrocytes in sterile injury and other non-purinergic

factors intrinsic to the injurious process[88]. The P2-like receptor GPR17 is frequently associated with effects following sterile CNS injuries that are seemingly at odds. For example, stimulation of this receptor correlates with proliferation of oligodendrocyte precursors at the periphery of damaged tissue following traumatic injury, yet oligodendrocytes, astrocytes and neurons exhibit an expression level-dependent susceptibility to ATP-induced death following stroke, spinal cord injury (SCI) and TBI[87,89,90].

Multiple types of P2 receptors are involved in initial reactive astrogliosis and long-term astrocyte changes following sterile injury. ADP activation of $P2Y_1$ receptors mediates cytokine and chemokine production. ATP activation of GPR17 and $P2Y_2$ receptors governs activation, increased glial acidic fibrillary protein (GFAP) expression, morphological changes and migration following injury. $P2X_7$ receptors are expressed following traumatic insult, leading to excessive release of glutamate and potentially cell death[19,84,87,89]. Multiple types of P2Y and P2X receptors that are not detectable at the mRNA level under homeostatic conditions are upregulated on astrocytes following either traumatic or ischemic injury[57]. P2 receptors have been implicated in detrimental glial scar formation, in astrocytic activation in neurodegenerative diseases and in reactive astrocytosis needed for BBB integrity restoration and neuronal recovery after injury[84,91]. Additionally, ATP and ADP signaling upregulate growth factor receptors and production of proinflammatory cytokines by astrocytes, in some cases through multiple purinergic receptors[87]. Interestingly, microglia have been demonstrated to convert reactive astrocytes to a neuroprotective phenotype by downregulating astrocytic $P2Y_1$[92]. The role of this

receptor on astrocytes in TBI models is currently controversial, with one report of pharmacological inhibition of $P2Y_1$ resulting in improvement in a preclinical TBI model[23], and others demonstrating reduction of edema, gliosis, and neuronal damage upon $P2Y_1$ agonism in ischemic and traumatic brain injuries[93,94].

ATP and UDP signaling via purinergic receptors mediates important immune and inflammatory functions of microglia, the first cell type in which a purinergic receptor ($P2X_7$) and a proinflammatory cytokine (IL-1β) were linked[84]. Purinergic receptors mediate microglial activation (GPR17 and $P2Y_{12}$)[89,95], degree of process ramification (ectonucleotidases)[96], process retraction and polarization ($P2Y_{12}$)[97-99], migration ($P2Y_{12}$)[99,100], and phagocytosis of debris ($P2Y_6$)[101]. Release of ATP by astrocytes is critical for microglial activation following sterile injury[97]. ATP appears to selectively promote a microglial response over recruitment of peripheral macrophages early following CNS insult[102]. Short-term activation of $P2X_7$ on microglia acts as a danger sensor for adjacent neurons, astrocytes and oligodendrocytes, facilitating local and distant chemokine and cytokine responses. Chronic activation of $P2X_7$ results in formation of a membrane pore complex and leads to cell death on most cells including microglia, though paradoxically can also lead to local microglial proliferation[87]. Increased expression of $P2X_4$ following ischemic injury has been demonstrated, though the consequences of this are unclear[5]. In our model of mTBI, formation of honeycomb microglia is mediated by $P2X_4$ and $P2Y_{12}$ receptors, and blockade of $P2Y_6$, $P2Y_{12}$ and $P2X_4$ receptors prevents formation of highly motile jellyfish microglia. Consistent with these results, pharmacological inhibition of release of nucleotides via connexin hemichannels

prevents morphological microglial changes entirely. Perhaps somewhat unexpectedly, inhibiting early microglial responses is highly detrimental to the neocortex, resulting in increased cell death[85].

Neuronal signaling and neurotransmission relying on ATP, ADP, adenosine and other nucleotides is well-documented, as is expression of purinergic receptors on neurons, where these receptors were first identified[1,5,19]. Purinergic signaling and receptor expression is highly heterogeneous in different brain regions and on different types of neurons. Understanding of the intricate complexities of purinergic dynamics of neurons suffers from lack of complete characterization, but current knowledge indicates highly diverse and important functions. Neurogenesis during development and in niches in the adult brain are influenced by purinergic signaling, and receptor expression has been demonstrated on neuronal precursors in the hippocampus[83]. Roles in neuronal differentiation, homeostatic survival and plasticity have also been described, wherein the effects of ATP resemble those of classical growth factors[83,103,104]. Both P2Y and P2X receptors have been found on presynaptic membranes and regulate the release of other neurotransmitters, including glutamate[105]. Due to these functional complexities and to the high demand for cellular energy in the brain, injury has the potential to result in release of very large quantities of ATP, with extracellular quantities in millimolar ranges reported[83,106]. Upregulation of $P2Y_1$, $P2X_3$ and $P2X_7$ have been reported on neurons in the hippocampus and striatum following traumatic injury and contribute to failure of neurotransmission, susceptibility to excitotoxic mechanisms and neuronal death[57,105,106]. $P2X_4$ receptors on precursor cells are activated following injury and

may contribute to neuroregeneration[5]. $P2Y_{13}$ signaling activates a Nrf2-dependent mechanism that prevents oxidative neuronal death, a pathogenic process demonstrated in sterile CNS injuries[85,107]. Many effects of P2 receptor signaling impacting neurons, such as increased susceptibility to excitotoxicity, initiate in other cell types, leading to complex interactions that may contribute to death of multiple cell types simultaneously[57].

P1 Receptors in Sterile Injuries of the CNS

The role of adenosine receptors and signaling in sterile injuries has been of significant interest due to the potential therapeutic value of agonists and antagonists, particularly targeting the A2A receptor, in neurodegenerative diseases. Unlike signaling through P2 receptors, the broad effects of adenosine signaling do not tend to generalize across different types of sterile injuries. Both *in vitro* and *in vivo* experimental findings have proven controversial due to results demonstrating opposite effects, frequently involving signaling via the same receptor[72,79,108]. This is not necessarily unexpected given that neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells express all four adenosine receptors and therefore the global result of adenosine signaling has the potential to be exceptionally complicated and influenced by a multitude of inputs, both cellular and microenvironmental[27,108]. In addition, adenosine plays many important and tightly-controlled physiological functions in the CNS, including mediation of glutamate release by neurons, glutamate uptake by astrocytes, synaptic plasticity of neurons, early activation and process retraction of microglia, control of proliferation and migration of oligodendrocyte precursors, communication between glia and cerebral

vasculature, fine tuning of communication between neurons and glia, and acute responses to changes in homeostatic state[19,22,27,87,108-110].

In general, increased adenosine signaling due to release and metabolism of ATP, in conjunction with release of pre-existent adenosine, tends to have tissue-protective effects acutely following injury. In contrast, continued and/or chronic adenosine following injury tends to produce deleterious effects[19,27,108]. Such a temporal switch has been demonstrated for all adenosine receptors in different diseases[27,29]. Despite evidence suggesting a connection between acute sterile injuries and chronic neurodegenerative diseases, other findings point to differing pathogenic mechanisms and therefore potentially different therapeutic targets. Caution is warranted in interpretation of studies extrapolating positive results from one entity to another[5,19,111].

In acute ischemic brain injuries, signaling through the A1 and A2A receptors induces a state in which most cells become less susceptible to hypoxic and oxidative injury, and A1 and A3 receptor signaling decrease synaptic communication in neurons to decrease susceptibility to excitotoxicity[27,29,111]. In the same or similar models, activation of the A1 receptor prevents apoptosis of astrocytes, A2A receptor signaling mediates reactive astrocytosis, and the A2B receptor influences astrocytic activation state in a TNFα-dependent matter[87]. At low agonist concentrations, the A3 receptor promotes survival, activation and resistance to oxidative damage in astrocytes and better functional outcome in animal models. These effects are reversed at long-lasting and high agonist concentrations[87]. A3 receptors tend to promote a proinflammatory, activated state in microglia but have a more moderate

effect when in combination with signaling through other adenosine receptors[29]. Both activation and inactivation of A2A receptors have been reported as protective following ischemic injury[111]. A2A receptors are upregulated on microglia following inflammation in ischemic brain injury models and promote movement away from ATP-rich tissue damage. This fits with other reports of anti-inflammatory microglial effects facilitated by A2A signaling, and yet inhibition of this receptor is protective following ischemia in some models. Some groups propose that this is due to removal of pathways that counteract neuroprotective A1 signaling, while others have shown a decrease in the production of proinflammatory mediators like nitric oxide by activated microglia[100,112,113]. Reduced release of glutamate and therefore decreased excitotoxic potential has also been demonstrated upon A2A inhibition, though the same authors report that A2A agonists administered prior to ischemia and immediately following injury are highly protective at very acute time points[111,114].

The A1 and A3 receptors likely mediate similar astrocytic and microglial responses in traumatic spinal and brain injuries as they do in ischemic injury, though these two receptors have been minimally investigated in models of SCI and TBI[87]. A similar dearth of information currently exists regarding the role of A2B in sterile CNS injuries. A single publication reports increases in astrocytic production of interleukin-6 in the striatum upon treatment with an A2B agonist, though this work was not performed in a TBI model[115]. Activation of A1 receptors decreases microglial proliferation following TBI, exerting control on neuroinflammation[116]. Treatment of rat hippocampus neurons with an A1 agonist attenuated cell death after traumatic

injury[117]. The A1 receptor is most commonly associated with post-traumatic epilepsy, and A1-deficient mice tend to develop fatal, intractable seizures after TBI[118].

Targeting the A2A receptor in SCI and TBI has produced paradoxical results in protection after different sterile injuries, though some of this is likely explained by the temporal shift in which agonists transition from protective to deleterious and antagonists become protective at more chronic time points. Particularly important in this context is the relationship between glutamate concentrations in the environment and the effect of treating with A2A ligands, in which A2A agonists are protective with low extracellular glutamate and A2A antagonists are protective when extracellular glutamate is high in the CNS[119]. Numerous groups have reported protection or improvement of both lesion parameters and/or cognitive and behavioral measures with A2A antagonists or genetic inactivation following injury in multiple models, including blast-induced TBI[120] and controlled cortical impact (CCI) TBI in rodents[121-123], though these models tend to induce moderate to severe injury or are assessed at chronic time points[124]. In SCI, early application of A2A agonists and late application of A2A antagonists confer lesion and functional improvement[125,126]. In one model of SCI, the actions of an A2A agonist on oligodendrocytes led to decreased MAPK signaling and decreased severity of demyelination[127]. Dai et al. reported that inactivation of the A2A receptor on peripheral immune cells had a beneficial effect following CCI, likely due to modulation of activation of these cells and subsequent inflammation. Reconstitution of A2A-competent bone marrow-derived cells in global A2A null mice does not recapitulate the lesion found in wild type mice[123]. Li et al. published somewhat contradictory results, showing that

inactivation of the A2A receptor in the spinal cord is protective but activation via A2A agonist on bone marrow-derived cells improved injury outcome[128]. **(See Appendix Table 1)**.

Adenosine receptors represent an area with significant potential for therapeutic development in the TBI field. Unfortunately, most studies reporting effects of agonizing or antagonizing one of the four adenosine receptors do not include *in vivo* investigations of molecular mechanisms. We therefore formulated the following working hypotheses and specific aims to investigate use of adenosine receptor-targeting compounds in our model of mild traumatic brain injury.

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WORKING HYPOTHESES AND SPECIFIC AIMS

We hypothesize that the early pathology of mTBI is mediated by reactive oxygen species (ROS) and danger-associated molecular patterns (DAMPs) signaling, leading to cell death and the development of secondary injury-induced lesions.

SPECIFIC AIM 1

Conduct a comparative pathological assessment of the mTBI lesion and develop a quantitative and qualitative assay to assess the lesion produced by mTBI.

We hypothesize that the lesion in our model of mTBI cell death will increase in size over time, spreading laterally and to deeper neocortical layers.

SPECIFIC AIM 2

Investigate the role of adenosine signaling through adenosine receptors in a model of mTBI.

We hypothesize that agonizing the A2A receptor will decrease neocortical cell death following mTBI.

SPECIFIC AIM 3

Determine the mechanism by which A2A agonists protect the neocortex following mTBI.

We hypothesize that A2A agonists protect the neocortex by decreasing reactive oxygen species following mTBI.

CHAPTER 3

In Vivo Cell Death Assay & Histopathological mTBI Time Course

Introduction to Mild Traumatic Brain Injury

Greater than 2 million individuals sustain a TBI in the United States every year[1]. Clinically, TBI are classified as mild, moderate or severe based on several criteria including Glasgow Coma Scale (GCS), which involves assessment of motor, verbal and vision capabilities. Also incorporated into initial clinical determination of TBI severity are awareness at presentation and loss of consciousness and duration thereof. Further characterization may be accomplished by standard imaging modalities, such as computed tomography or magnetic resonance imaging, to assess for presence and severity of lesions[2-5]. Harmonizing nomenclature for discussions about head injuries and diagnostic criteria for each TBI severity level is an ongoing challenge for the research and medical communities[3,4]. It is generally agreed upon that an injury should be classified as mild when there is a GCS score of 13-15, loss of consciousness for no more than 30 minutes, minimal post-traumatic amnesia, and imaging findings that are categorized as minimal abnormalities or within normal limits. A penetrating head injury or skull fracture typically excludes a TBI from this category[4,5]. Clinical categorization of a TBI does not reflect the diversity of head injuries that may be labeled mTBI, nor does it describe the pathological mechanisms, immune responses and degree of microscopic changes found in an individual injury[4]. The long-term sequela and challenges patients may experience regardless of TBI severity are increasingly well recognized and are linked to highly heterogenous pathology[6]. This complicates experimental study of TBI and

development of efficacious therapeutics, necessitating an understanding of selected experimental models prior to studies intended to reveal potential mechanisms to target pharmacologically[7].

Review of Models of Traumatic Brain Injury

Interest in the cascade of events following TBI has led to development of a variety of *in vitro* and *in vivo* models, as well as recent advances in computer and mathematical modeling of human brain tissue[2,8]. *In vitro* models of TBI include immortalized cell cultures, primary dissociated cultures and organotypic models such as living brain slices. Both single and repetitive injuries have been studied *in vitro.* These models have been used to study molecular mediators such as oxidative stress, mechanisms of cell death such as apoptosis and excitotoxicity, reaction of multiple cell types to simplified injury modalities such as excessive stretch, and treatment response following injury[9-12]. Despite a lack of anatomical architecture and therefore physiological and mechanical relationships of structures within intact brain tissue, *in vitro* results have predicted certain *in vivo* findings with relatively high fidelity, such as axonal injury and oxidative stress[2,11].

There are numerous animal models of TBI that range in severity from mild to severe. A thorough review was recently written on this topic[13], and thus only a brief synopsis of existing animal models will be provided here. Recently, models of both mild and severe TBI have been developed in *Drosophila melanogaster*, including repetitive mTBI. These models have proven highly reproducible and have allowed study of neuronal injury and innate immune responses, but have the obvious limitation of significant anatomical differences from mammalian models[14,15]. There

are several rodent models utilizing speed-controlled and gravity-based weight drops that contact the skull or cortical parenchyma and result in mild to severe injury. The injuries are relatively diffuse in nature and tend to recapitulate many aspects of human TBI pathology, but suffer from a lack of reproducibility[3,13,16]. Another TBI model referred to as fluid percussion requires a craniotomy followed by the stereotactic insertion of fluid nozzle above a brain region of interest. Afterward, a calibrated weight or piston is fired, compressing the loaded fluid through the nozzle, directly onto cortical brain tissue. Fluid percussion produces focal to diffuse injury, and tends to result in higher animal mortality than some other methods. Fluid percussion has been applied to multiple species, including mice, rats, dogs, and pigs[13,17]. Controlled cortical impact (CCI) is one of the most commonly used TBI methods. An air-driven piston is applied to the parenchyma through a craniotomy window. Due to high reproducibility and a relatively low mortality rate, this method has also been applied to several animal species[13,18]. More recently, CCI was used with an intact skull to induce repeated injuries[19]. Blast injuries are also of significant interest in the research community due to the types of injuries commonly sustained by military personnel. Therefore, several animal models have been developed to facilitate study of diffuse blast injuries. For these models, animals are placed within a chamber and exposed to explosive or concussive air blasts. These closed-skull models produce less severe forces than those experienced by humans exposed to blast injuries due to anatomical differences[20,21]. Finally, our lab has recently developed a closed-skull, focal compressive model of mild TBI. A focal lesion is induced by thinning the skull bone to ~30 microns and then applying downward

pressure to promote concavity in the bone. The advantages of this model include its focal nature, high degree of reproducibility, ability to visualize the ensuing injury response by two-photon laser scanning microscopy (TPLSM) through the thinned skull window, and ability to pass a variety of compounds and dyes through the thinned skull window and into the meninges and parenchyma[22]. Our closed-skull, focal compressive mTBI model was used for all experiments described herein.

Development of an *In Vivo* Propidium Iodide Quantitative Cell Death Assay

Our lab previously established that solutions up to 40,000 MW can pass through an intact skull and into the meninges, typically stopping at the intact glia limitans. The astrocytes of the glia limitans are injured and frequently die in our mTBI model, causing loss of glia limitans integrity and allowing compounds to pass into the underlying neocortical parenchyma [22]. As a first step to approach this project, we took advantage of these conditions to develop an *in vivo* histopathological assay following mTBI, allowing quantification of cell death in both the meninges and neocortical parenchyma.

Briefly, mTBI injury in our model was induced as follows. Following induction of surgical anesthesia, the mouse heads were shaved between the ears to just above the eyes. A vertical midline surgical incision was made using scissors and the skin overlying the skull was parted, revealing the underlying skull. A pair of pointed forceps was used to gently remove the periosteum covering the right parietal bone. A stainless-steel bracket with a 2-millimeter central hole was affixed to the center of the right parietal bone using surgical glue. An Ideal micro-drill (Braintree Scientific, Braintree, MA) with a 0.7-millimeter burr (Fine Science Tools, Foster City, CA) was

used to drill a 1 millimeter diameter window into the skull in approximately 45-60 seconds. The skull comprising the window was drilled to a final thickness of approximately 30 micrometers and then compressed onto the underlying parenchyma 10 times using the blunt side of a round-tip, microophthalmic surgical blade (Surgistar, Knoxville, TN).

To assess the lesion induced in the meninges and parenchyma following mTBI, we developed an assay using propidium iodide (PI, ThermoFisher Scientific, Waltham, MA) as an *in vivo* indicator of cell death. PI has a molecular weight of 668.4, is live cell-impermeant and is fixable via aldehyde fixatives including paraformaldehyde and neutral buffered formalin. It binds to both DNA and RNA by intercalating between bases, resulting in fluorescence excitation and emission of 535 nm and 617 nm, respectively. At the desired time point following mTBI, any transcranial treatment was removed from the skull and 200 microliters of 1.5 mM PI were applied to the skull and allowed to incubate for 45 minutes.

After PI incubation, a single wash with artificial cerebrospinal fluid (aCSF; catalog # 59-7316, Harvard Apparatus, Cambridge, MA) was followed by collection of a high-resolution z-stack with a total depth of 250 micrometers via intravital two-photon laser scanning microscopy (TPLSM). These stacks were collected at a wavelength of 920 nanometers with offset and gain optimized for each experiment and then maintained at consistent levels for all animals that study. TPLSM z-stacks were assessed using Imaris (Bitplane, South Windsor, CT) image analysis software to quantify PI-positive dead cells in the meninges. 250-micrometer stacks included the skull at the center of the lesion, the meninges and approximately 50 micrometers

of neocortical layer one. Matlab plugins embedded in Imaris were used to create a surface object representative of the skull and apply a dot object to each individual dead cell. A filter was then applied to exclude any dead cells overlapping with the skull and to include only dead cells within 30 micrometers below the skull (those within the meninges). These dots were then quantified to give the number of dead cells in the meninges at the center of the lesion.

Following TPLSM scanning for meningeal cell death, mice then underwent deep anesthesia via intraperitoneal (IP) injection of 3.8% chloral hydrate and transcardiac perfusion fixation with 4% paraformaldehyde (PFA). The head was removed from the body and the mandible and skin removed. Cleaned heads were placed in 4% PFA overnight for immersion fixation. Heads were then processed in one of two ways. Method 1: heads were dried and the lesion site marked with an industrial paint marker, then placed in 30% sucrose for storage and transport to Histoserv, Inc. (Germantown, MD). The heads were decalcified for 24 hours using a combination of formic acid and sodium hydroxide, then returned to sucrose for 24 hours prior to embedding. A histology technician trimmed the heads for coronal sectioning to include the marked lesion and a small zone of skull on either side. Method 2: heads were dried and the brain carefully removed and placed into a 2 mm stainless steel brain sectioning mold (Zivic Instruments, Pittsburgh, PA). A 4 mm segment of brain containing the concave mTBI lesion was taken and placed in 30% sucrose for transport to Histoserv, Inc. the same day. A histology technician then embedded the brain segment. Slides were made by a combination of step- and serial-sectioning, beginning at the rostral-most portion of the marked lesion site.

Steps were approximately 40 micrometers and serial sections were 8 micrometers thick to provide two examples at each step through the lesion. This resulted in 10-12 slides with two sections each per lesion.

Upon receipt of the unstained slides cut from the mTBI lesion, the slides were coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) within 3 days. Slides were stored at -80 degrees Celsius until coverslipping. The slides were scanned using an Olympus Fluoview FV1200 laser scanning confocal microscopy system, using a UV laser to excite DAPI and a 488 nm laser to excite PI. The widest diameter of the thinnest skull was identified in these slides, and both serial sections representing the center of the lesion were collected for quantification. The number of PI-positive dead cells colocalizing with DAPI-positive nuclei in neocortical layers 2-6 were quantified using Imaris image analysis software. Dots were applied to DAPI-positive nuclei and PI-positive dead cells and two new "channels" produced from these dots. A colocalization algorithm available in Imaris was used to determine the number of dead cells in a scanned section. This method was used to execute a descriptive time course of our mTBI model and for any experiment requiring quantification of cell death.

Source of Cell Death in mTBI Model

Our model of mTBI relies on consistency in executing the rapid drilling and compression of the skull to induce reproducible lesions for experimental assessment. In our experience, accurate reproduction of both drilling and compression are important within a single study and between experiments, but the

contribution of each phase of injury to the final mTBI lesion were unknown. To determine the contribution of each phase of mTBI induction to the total number of dead cells in the neocortex at 8 hours after injury, 7-week-old male C57Bl/6J male mice were randomly assigned to three groups. One group of mice was subjected to mTBI and a second group was thinned by drilling in a manner consistent with mTBI mice but was not subjected to compression. The third group was produced by member of our laboratory with numerous years of experience performing non-injurious skull thinning preparations for intravital imaging in age- and sex-matched mice. All mice were maintained under anesthesia for 8 hours and their skulls were incubated with aCSF. At 8 hours post-mTBI, mice were prepared as described above for the *in vivo* quantitative cell death assay, with particular attention paid to the neocortex (**Fig. 1a-d**).

All mice subjected to non-injurious skull thinning had fewer than 5 total dead cells in their neocortices (mean 1.4/mouse; **Fig. 1a**). In the drill-only group, there were a mean of 128 dead cells/mouse in examined neocortical sections (**Fig. 1b**). All mice in this group had fewer than 230 dead cells total. All mTBI mice (drilling + compression) had greater than 400 dead cells in their neocortices (mean 484/mouse; **Fig. 1c**). Somewhat surprisingly, rapidly drilling the skull resulted in relatively little cell death in the neocortices. Compression accounts for approximately 75% of the cell dead in our model of mTBI (**Fig. 1d**).

Histopathological Time Course of mTBI

To adequately assess progression of the lesion in our mTBI model at various time points and determine if transcranial PI would penetrate through all neocortical

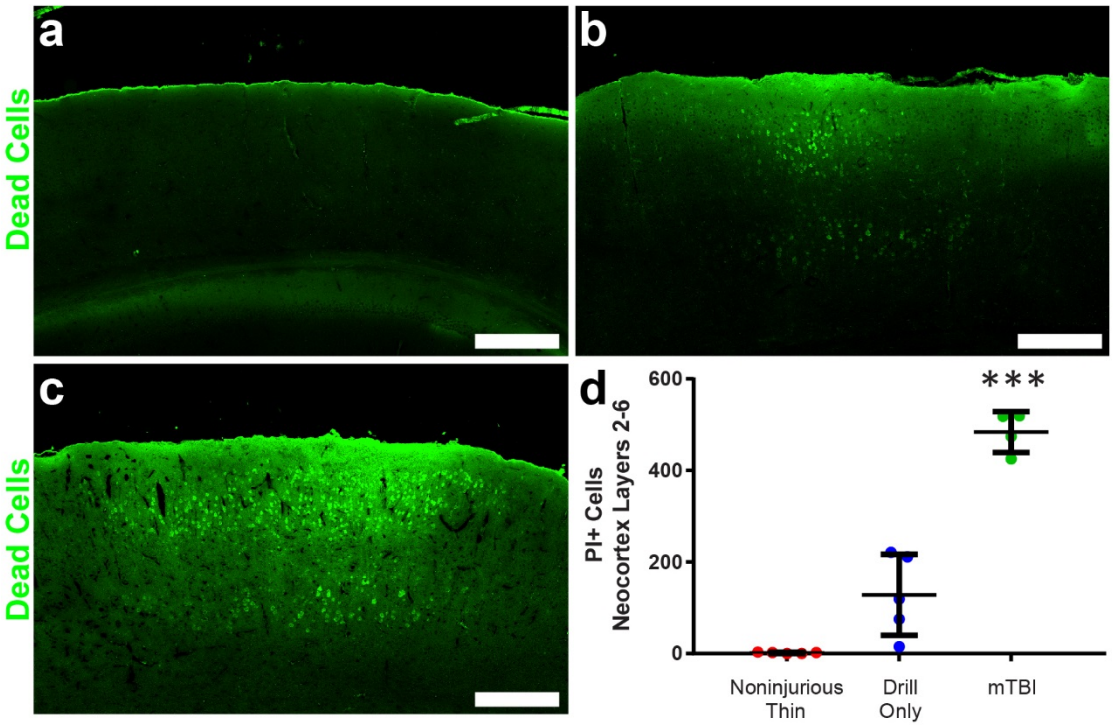


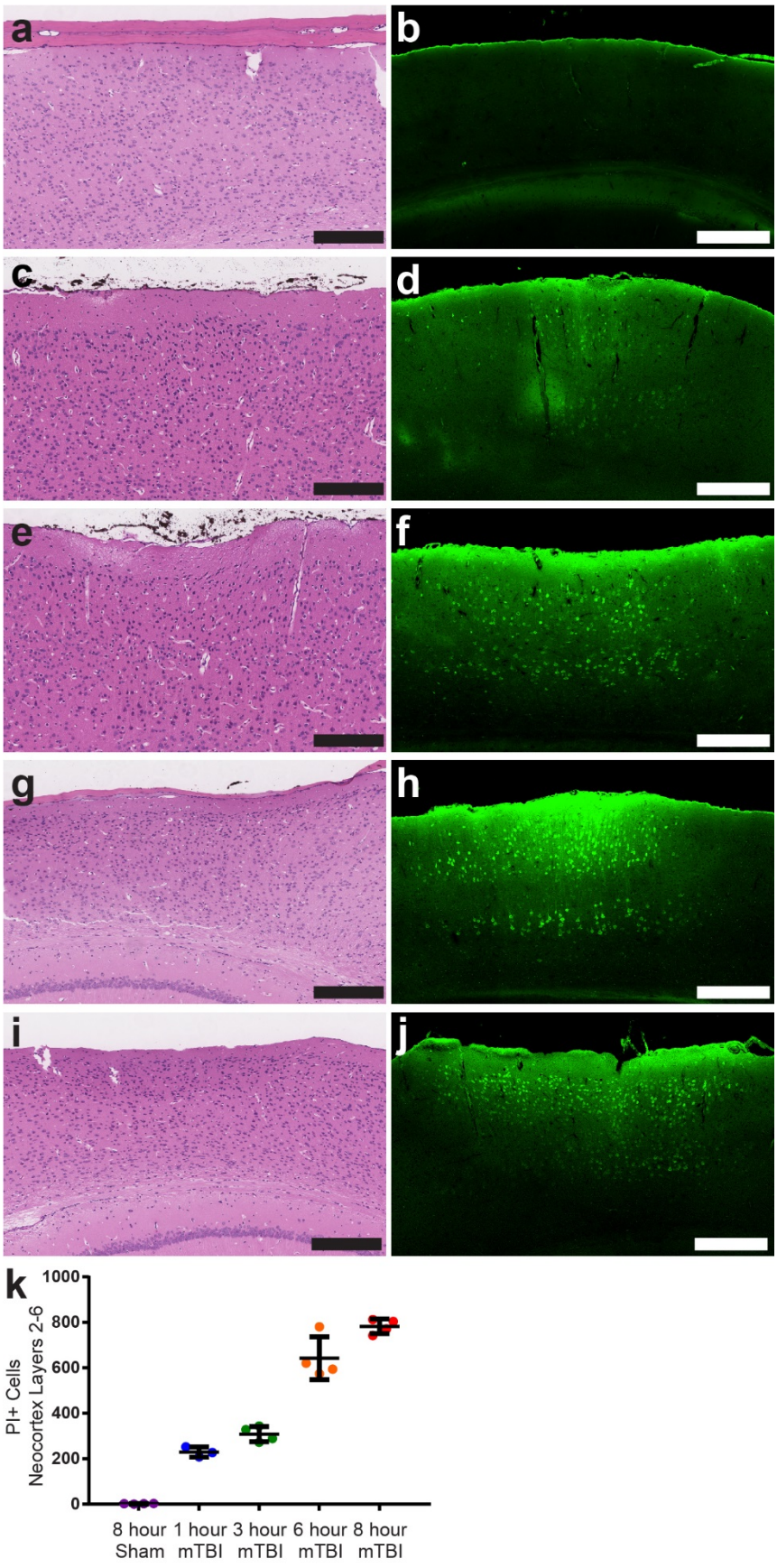
Figure 1. Compression induces most neocortical cell death in a model of mTBI**. (a-d)** Wild type male C57Bl/6J mice were subjected to (**a**) non-injurious skull thinning preparation, (**b**) rapid drilling or (**c**) mTBI, all resulting in a skull approximately 30 μm in thickness. Neocortices from uninjured, thinned mice had fewer than 5 dead cells per mouse, while neocortices from drilled and mTBI mice had means of 128 and 484 dead cells per mouse, respectively (**d**). mTBI mice had statistically significantly higher cell death in the neocortex compared to other groups. Graph in (**d**) represents a single experiment with n = 5 animals per group for each experimental condition. Scale bars, 250 μm. *** $p \leq 0.001$; one-way ANOVA.

layers, we collected samples for descriptive histopathology at one, three, six, and eight hours after induction of injury (**Fig. 2c-j**). All mice were incubated with transcranial aCSF for the duration of anesthesia. For comparison, we collected similar mice maintained under anesthesia for 8 hours with intact skulls that were incubated with aCSF to demonstrate normal neocortical anatomy with minimal PI+ dead cells (**Fig. 2a-b**). The skull in Fig. 2a is representative of those found in all male mice in subsequent studies prior to mTBI. In hematoxylin and eosin (H&E)-stained sections at all time points after injury, dead cells are pyknotic, shrunken and dense with dark hematoxylin staining (**Fig. 2c,e,g,i**). In comparable PI-labeled sections, dead cells are brightly PI+ (**Fig. 2d,f,h,j**). The number of PI+ dead cells increases over time in all neocortical layers, and the lesion spreads laterally in both directions from the initial compression point. Interestingly, layer IV is relatively spared until approximately 8 hours after injury. Deeper layers (V and VI) have few dead cells until later time points, when the band of dead cells are nearly as wide as those in more superficial layers. Dead cells were not detected in the ipsilateral hippocampus or deeper structures, nor were they detected in the contralateral neocortex at any examined time point (not shown).

Conclusions

We concluded that transcranially incubated PI is an excellent method by which to quantitatively assess meningeal and neocortical cell death using TPLSM and histopathology. Using this new assay, we determined that the majority of neocortical cell death induced in our model is caused by compression rather than

Figure 2. Transcranial propidium iodide (PI) reveals increasing dead cells in the neocortex after mTBI. **(a-b)** Naïve mice with normal skulls were collected following 8 hours of anesthesia and transcranial incubation with aCSF, showing normal neocortical anatomy in (**a**) H&E sections and (**b**) minimal to zero dead cells in PI-labeled sections. **(c-j)** Similar mice subjected to mTBI were collected at 1 hour (**c**,**d**), 3 hours (**e**,**f**), 6 hours (**g**,**h**), and 8 hours (**i**,**j**) following injury. Dead cells appear pyknotic (dense and shrunken with strong hematoxylin staining) on H&E, and label brightly with PI. Over time, the number of dead cells increases in all neocortical layers and the lesion spreads laterally from the initial compression point. Interestingly, neocortical layer IV is initially spared. (**k**) As early as 1 hour post-mTBI, PI+ dead cells are increased compared to mice with sham surgery that were maintained under anesthesia for 8 hours. The number of dead cells increases over time through 8 hours post-mTBI. n = 4 for sham, 3 hour, 6 hour, and 8 hour groups. n = 3 for 1 hour group. Scale bars in (**a**-**f**), 200 μm. Scale bars in (**g-j**), 250 μm.


a
b
c
d
e
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g
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i
j
k
PI+ Cells
Neocortex Layers 2-6
1000
800
600
400
200
0
8 hour Sham
1 hour mTBI
3 hour mTBI
6 hour mTBI
8 hour mTBI

drilling, and that the number of dead cells in the neocortex increases over time from 1 to 8 hours following injury.

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CHAPTER 4

Transcranial A2A Agonism Induces a Neuroprotective State That Resists the Damaging Effects of Mild Traumatic Brain Injury

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Introduction

Traumatic brain injury (TBI) is a complex, heterogenous condition affecting diverse populations worldwide, including almost 2 million people in the United States annually. Young and elderly individuals are at increased risk, as are individuals in certain occupations including members of the armed forces and professional athletes [1-4]. Mild TBI (mTBI) is the most common type and frequently results in potentially long-lasting symptoms including headaches, memory loss and mood alterations. TBI has been linked to chronic neurodegenerative diseases including Alzheimer's disease and Chronic Traumatic Encephalopathy[5]. Despite recognition as a significant public health concern, efficacious clinical treatments have been elusive and current standard clinical recommendations are few. There is substantial need for investigation of pathogenic mechanisms and novel clinical approaches to TBI[6-8].

There are numerous animal models that allow study of the complicated pathology and varying severity of TBI[9,10]. Our lab has established a murine model of focal, compressive and closed-skull mTBI to study molecular pathogenesis. In the hours following mTBI, initial mechanical injury leads to meningeal cell death, glia limitans damage and release of reactive oxygen species (ROS). These contribute to ongoing cell death in the neocortical brain parenchyma[11], with other groups describing further mechanisms contributing to cell death, including release of chemokines and cytokines[12] and alterations in normal glutamate concentrations[13]. In addition to deleterious consequences, early events trigger responses by resident microglia and peripherally-derived immune cells that are driven by purinergic

signaling[11]. Rapid release of adenosine following TBI to modulate inflammation and excitotoxicity has been previously reported[14].

Adenosine is produced from breakdown of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) via hydrolysis. Signaling via myriad intracellular pathways occurs through interaction with four receptors (A1R, A2AR, A2BR, and A3R)[15]. Adenosine receptors are expressed on peripheral bone marrow-derived cells and all central nervous system (CNS) resident cells examined[16,17]. Currently, there is significant debate in the literature regarding the role of adenosine signaling in TBI and neurodegenerative diseases. A2AR and A2BR signaling is considered a modulator of purinergic inflammatory responses in numerous organs including the brain[18]. The A2AR has been implicated in Alzheimer's and Parkinson's diseases, and there is evidence from some animal models of these diseases and ischemic stroke that antagonists to this receptor might hold therapeutic promise[19,20]. Similarly, in the controlled cortical impact model of TBI, inhibition or genetic inactivation of the A2AR improves behavioral outcomes and decreases lesion size[21-23]. Contrasting evidence from models of mild spinal cord injury show that acute administration of A2AR agonists is beneficial, whereas antagonists provide benefit for chronic lesions[24]. These conflicting results may be due to differential effects of A2AR signaling at different environmental glutamate concentrations, with agonists beneficial at low glutamate concentrations[25]. The precise involvement of A2AR signaling on various resident and peripheral cell types following TBI is also currently unclear[26]. Given the important role of adenosine signaling in healthy CNS functions and the potential for the involvement of this signaling in pathologic processes[27], it is

important to understand the therapeutic potential of adenosine signaling-modulating compounds and the underlying molecular mechanisms in mTBI.

Here we report that agonists to the A2AR, but not the A2BR, A3R or A1R, protect the neocortex when administered transcranially following mTBI. Using a variety of intravital imaging and histopathology techniques, we show that this protective effect is specific to the A2AR on CNS resident cells and does not require peripheral bone marrow-derived cells. A2AR agonists are protective in two genetically-distinct mouse strains, and do not provide protection in global A2AR null mice. Further, this protective effect appears to impact all CNS resident cells but does not prevent cell death in the meninges. These agonists do not prevent damage to the glia limitans or decrease ROS following mTBI, and do not increase glutathione (GSH) or impact glutamate levels. Finally, we show that A2AR agonists protect the neocortex via signaling through protein kinase c δ (PKCδ) and ultimately carbonic anhydrase IX (CAIX), an enzyme that increases intracellular bicarbonate and allows maintenance of homeostatic pH despite acidification due to cellular injury.

Materials and Methods

Animals

All mice were housed under specific pathogen-free conditions on a 12-hour light/dark cycle with access to food and water *ad libitum*. All experiments were performed in compliance with protocols established by the Institutional Animal Care and Use Committee at the NIH. Male C57BL/6J (stock # 000664), Balb/cJ (stock # 000651) and CByJ.SJL(B6)-Ptprca/J (stock # 006584) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C;129S-*Adora2a*^{tm1Jfc}/J mice were initially

acquired from The Jackson Laboratory (stock # 010685) and then bred for population maintenance (originally developed and published by Chen et al.[28]). CX3CR1$^{gfp/+}$ mice were produced by crossing C57BL/6J mice with homozygous CX3CR1$^{gfp/gfp}$ mice in a contained breeding facility at the National Institutes of Health (NIH; Bethesda, MD). B6.129P2(Cg)-*Cx3cr1*$^{tm2.1(cre/ERT2)Litt}$/WganJ mice (stock # 021160) and C57BL/6-*Gt(ROSA)26Sor*$^{tm1(HBEGF)Awai}$/J mice (stock #007900) were initially acquired from The Jackson Laboratory and crossed to produce CX3CR1-Cre-ER x Rosa-DTR mice, which were maintained in a contained breeding facility at the NIH. All mice were 7-8 weeks of age at the time experiments were performed except for bone marrow chimera mice and microglia-depleted mice, which were 10 weeks old at the time of experimental procedures. Surgical anesthesia for all studies was achieved via combination injectable ketamine, xylazine and acepromazine (KXA). Induction doses of 85 mg/kg, 13 mg/kg, and 2 mg/kg, respectively, were combined in phosphate-buffered saline (PBS). Maintenance anesthesia consisted of subcutaneous injection of a similar anesthetic mix that was diluted 1:4 in sterile PBS. Mice were kept on a heating pad to maintain a body temperature of 37°C. Anesthesia was maintained for up to 12 hours total following induction.

Bone marrow chimeras

Bone marrow chimeras were generated by isolating bone marrow from Balb/cJ, CByJ.SJL(B6)-Ptprca/J and C;129S-*Adora2a*^{tm1Jfc}/J mice, suspending the strain-specific bone marrow samples in chilled saline, and administering 100 μL containing approximately 10 million whole bone marrow cells via intraperitoneal injection into mice prepared by via whole-body irradiation 6 hours prior to seeding.

Recipient mice were exposed to 815 rads to ablate endogenous bone marrow. The following chimeras were produced: CByJ.SJL(B6)-Ptprca/J (CD45.1-positive mouse) seeded with Balb/cJ bone marrow (CD45.2-positive marrow); Balb/cJ (Cd45.2-positive mouse) seeded with CByJ.SJL(B6)-Ptprca/J bone marrow (CD45.1-positive marrow); CByJ.SJL(B6)-Ptprca/J (CD45.1-positive mouse) seeded with C;129S-*Adora2a*^{tm1Jfc}/J bone marrow (CD45.2-positive, A2A$^{-/-}$ marrow); C;129S-*Adora2a*^{tm1Jfc}/J (A2A$^{-/-}$ mouse) seeded with CByJ.SJL(B6)-Ptprca/J bone marrow (CD45.1-positive marrow); **(See Appendix, Table 2)**. Chimeras recovered for 8 weeks prior to any experimental procedures and were provided dietary supplementation as needed for recovery from irradiation. Blood samples were collected 1 week preceding transcranial drug studies and chimerism was confirmed by FACS detection of CD45.1 and CD45.2-positive cell populations.

Depletion of microglia in CX3CR1-Cre-ER Rosa-DTR mice

To induce expression of cre recombinase, CX3CR1-cre-ER Rosa-DTR mice were fed tamoxifen diet chow (Envigo, Huntingdon, United Kingdom) exclusively for four weeks following a three-day acclimation period in which tamoxifen diet chow was mixed with standard rodent diet chow. Mice received three intraperitoneal injections of 1 μg diphtheria toxin (DT; Sigma-Aldrich Corp. St. Louis, MO) on consecutive days with the final injection given the morning of mTBI experiment to deplete microglia. Depletion was confirmed by immunohistochemical comparison to wildtype B6 controls using Iba-1 labeling (rabbit polyclonal; Wako Chemicals USA Inc., Richmond, VA).

Model of focal, compressive, mild traumatic brain injury (mTBI)

We previously described a focal, compressive, mTBI model[11]. Following induction of surgical anesthesia, the heads were shaved between the ears to just above the eyes. A vertical midline surgical incision was made using scissors and the skin overlying the skull was parted, revealing the underlying skull. A pair of pointed forceps was used to gently remove the periosteum covering the right parietal bone. A stainless-steel bracket with a 2-millimeter central hole was affixed to the center of the right parietal bone using surgical glue. An Ideal micro-drill (Braintree Scientific, Braintree, MA) with a 0.7-millimeter burr (Fine Science Tools, Foster City, CA) was used to drill a 1 millimeter diameter window into the skull in approximately 45-60 seconds. The skull comprising the window was drilled to a final thickness of approximately 30 micrometers and then compressed onto the underlying parenchyma 10 times using the blunt side of a round-tip, microophthalmic surgical blade (Surgistar, Knoxville, TN).

Transcranial propidium iodide cell death assay

To assess the lesion induced in the meninges and parenchyma following mTBI, we developed an assay using propidium iodide (PI, ThermoFisher Scientific, Waltham, MA) as an *in vivo* indicator of cell death. At the desired time point following mTBI, any transcranial treatment was removed from the skull and 200 microliters of 1.5 mM PI were applied to the skull and allowed to incubate for 45 minutes.

Assessment of meningeal cell death

After PI incubation, a single wash with artificial cerebrospinal fluid (aCSF) was followed by collection of a high-resolution z-stack with a total depth of 250

micrometers via two-photon laser scanning microscopy (TPLSM). These stacks were collected at a wavelength of 920 nanometers with offset and gain optimized for each experiment and then maintained at consistent levels for all animals that study. TPLSM z-stacks were assessed using Imaris (Bitplane, South Windsor, CT) image analysis software to quantify PI-positive dead cells in the meninges. 250-micrometer stacks included the skull at the center of the lesion, the meninges and approximately 50 micrometers of neocortical layer one. Matlab plugins embedded in Imaris were used to create a surface object representative of the skull and apply a dot object to each individual dead cell. A filter was then applied to exclude any dead cells overlapping with the skull and to include only dead cells within 30 micrometers below the skull (those within the meninges). These dots were then quantified to give the number of dead cells in the meninges at the center of the lesion.

Assessment of parenchymal cell death

Following TPLSM scanning for meningeal cell death, mice then underwent deep anesthesia via intraperitoneal (IP) injection of 3.8% chloral hydrate and transcardiac perfusion fixation with 4% paraformaldehyde (PFA). The head was removed from the body and the mandible and skin removed. Cleaned heads were placed in 4% PFA overnight for immersion fixation. Heads were then processed in one of two ways. Method 1: heads were dried and the lesion site marked with an industrial paint marker, then placed in 30% sucrose for storage and transport to Histoserv, Inc. (Germantown, MD). The heads were decalcified for 24 hours using a combination of formic acid and sodium hydroxide, then returned to sucrose for 24-48 hours prior to embedding. A histology technician trimmed the heads for coronal

sectioning to include the marked lesion and a small zone of skull on either side. Method 2: heads were dried and the brain carefully removed and placed into a 2 mm stainless steel brain sectioning mold (Zivic Instruments, Pittsburgh, PA). A 4 mm segment of brain containing the concave mTBI lesion was taken and placed in 30% sucrose for transport to Histoserv, Inc. the same day. A histology technician then embedded the brain segment. Slides were made by a combination of step- and serial-sectioning, beginning at the rostral-most portion of the marked lesion site. Steps were approximately 40 micrometers and serial sections were 8 micrometers thick to provide two examples at each step through the lesion. This resulted in 10-12 slides with two sections each per lesion.

Upon receipt of the unstained slides cut from the mTBI lesion, the slides were coverslipped with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) within 3 days. Slides were stored at -80°C until coverslipping. The slides were scanned using an Olympus Fluoview FV1200 laser scanning confocal microscopy system. The widest diameter of the thinnest skull was identified in these slides, and both serial sections representing the center of the lesion were collected for quantification. The number of PI-positive dead cells colocalizing with DAPI-positive nuclei in neocortical layers 2-6 were quantified using Imaris image analysis software. Dots were applied to DAPI-positive nuclei and PI-positive dead cells and two new "channels" produced from these dots. A colocalization algorithm available in Imaris was used to determine the number of dead cells in a scanned section.

Adenosine receptor agonists and antagonists

We applied the following agonists or antagonists directly to the skull bone in an aCSF vehicle 15 minutes following mTBI lesion induction: UK432097 (A2A agonist, 10 μM; initially provided by Kenneth Jacobson and subsequently purchased from Axon Medchem, Groningen, Netherlands); Regadenoson (A2A agonist, 725 μM; Santa Cruz Biotechnology, Dallas, TX); SCH442416 (A2A antagonist, 10 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); BAY 60-6583 (A2B agonist, 10 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); 2-Cl-IB-MECA (A3 agonist, 1 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); 2-Chloro-*N*-cyclopentyladenosine (A1 agonist, 2 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); MRS 1754 (A2B antagonist, 0.5 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); MRE 3008F20 (A3 antagonist, 1 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); MRS 1523 (A3 antagonist, 5 μM; Sigma-Aldrich Corp. St. Louis, MO); 8-cyclopentyl-1,3-dipropylxanthine (A1 antagonist, 1 μM; Tocris Bioscience, Avonmouth, Bristol, United Kingdom). All lyophilized drugs were initially solubilized in DMSO and stored at -20 or -80 degrees Celsius. The control vehicle for all experiments was therefore an appropriate concentration of DMSO in aCSF **(See Appendix, Table 3)**. The working concentration of each agonist or antagonist was determined proportionally based on the Ki and EC50 of each compound at the respective targeted receptor. A single-day dose titration was then performed with n = 5 per group for UK432097 at 5, 10, and 20 μm and compared to vehicle-treated control mice. This experiment indicated that 10 μm was the most efficacious concentration of those tested (**Fig. 1**).

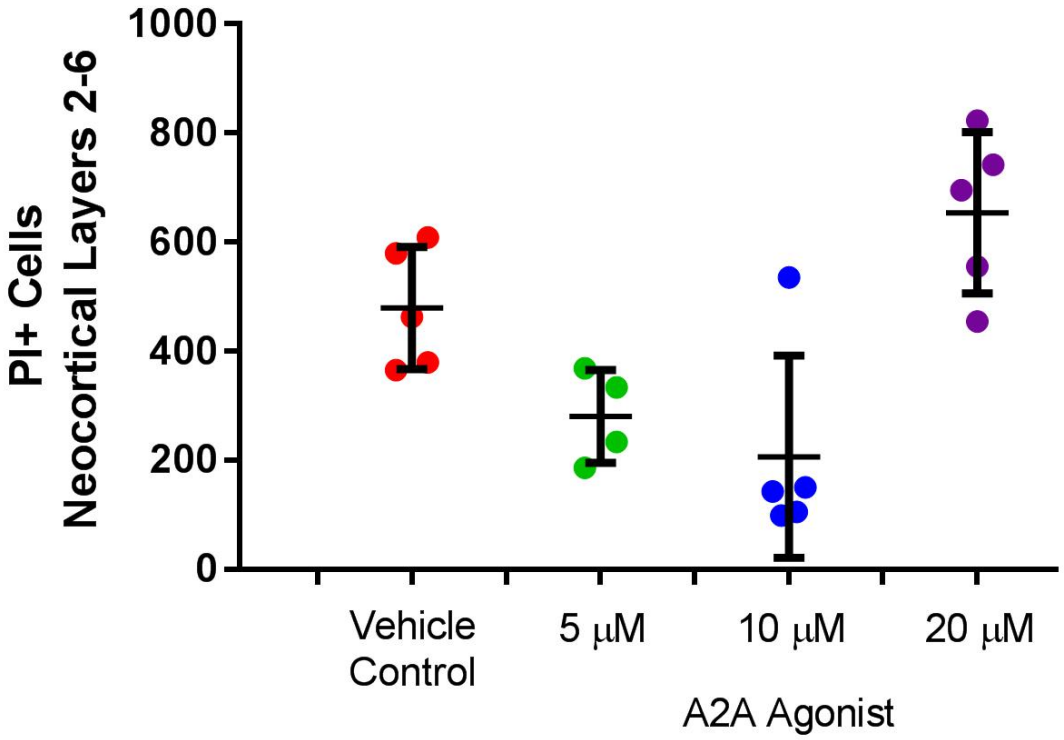


Figure 1. Single day dose titration with UK432097. Three doses of UK432097 were tested *in vivo* by transcranial administration in a single day to determine the working dose to be used in subsequent experiments. Despite a statistical outlier that had a lesion comparable to vehicle-treated controls, 10 μM was clearly the most efficacious transcranial dose and was selected as the concentration for all following experiments.

Pathway inhibition compounds

To assess *in vivo* mechanisms, the following compounds were administered transcranially in an aCSF vehicle with or without an A2AR agonist: Rottlerin (Protein Kinase Cδ/ε inhibitor; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); TAT-cyclo-CLLFVY (Hif-1α dimerization inhibitor; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); S4 (Carbonic anhydrase (CA) IX inhibitor; Tocris Bioscience, Avonmouth, Bristol, United Kingdom); **(See Appendix, Table 4)**.

Confocal microscopy

Three-dimensional images were collected using an Olympus Fluoview FV1200 laser scanning confocal microscopy system equipped with a 20x objective. Sequential scanning with 405, 488 and 559 nm laser lines was used to produce overlays with two to three colors.

Intravital two-photon laser scanning microscopy

Anesthetized mice were imaged using a Leica SP5 or SP8 two-photon imaging system (Leica Microsystems, Buffalo Grove, IL) equipped with an 8,000 Hz resonant scanner, one or two Mai Tai HP DeepSee lasers (Spectra-Physics, Santa Clara, CA) tuned to 915 or 920 nm, and an InSight laser (Spectra-Physics, Santa Clara, CA) tuned to 1050 nm. The SP5 system was equipped with a x20/1.0 NA immersion objective (Leica), a NDD4 external detector (Leica) and custom dichroic mirrors (Semrock, Rochester, NY) to allow separation of fluorescence emission. The SP8 system was additionally equipped with a IRAPO L x25/1.00 W immersion objective with a Leica motCORR motorized collar allowing software-adjusted color

correction and HyD NDD detectors (Leica). All *in vivo* imaging studies took place in a custom environmental chamber with temperature-controlled airflow.

Glia limitans leakage assay

To assess the permeability of the glia limitans following mTBI, mice were incubated with either DMSO-aCSF (10 μM) vehicle control or UK432097 (10 μM) for either 30 minutes prior to induction of mTBI or 15 minutes following compression injury. Sulforhodamine (SR) 101 (1 mM; Sigma-Aldrich Corp. St. Louis, MO) was applied to the skull 3 hours after injury and incubated transcranially for 15 minutes. This was followed by a 5 minute aCSF wash and immediate collection of a high-resolution, three-dimensional two-photon stack 150-200 μm in depth to allow visualization of the skull, meninges and superficial neocortical parenchyma at the mTBI location. Leakage through the glia limitans was determined by measurement of the sum of the fluorescent dye present at 30 μm or greater below the inner skull surface in the entire 1024 x 1024 stack. Imaris image analysis software and Matlab plugins were used to render the skull as a solid object, then measure the fluorescent sum at or deep to 30 μm below the lower bound of the skull object.

***In vivo* reactive oxygen species assay**

ROS levels were assessed in the meninges following mTBI by applying DMSO-aCSF (10 μM) vehicle control or UK432097 (10 μM) for either 30 minutes prior to injury or 15 minutes following mTBI. At 1 hour following mTBI, mice were scanned with only an aCSF for objective immersion to allow measurement of background fluorescence. Amplex Red Reagent (500 μM; ThermoFisher Scientific, Waltham, MA) was then applied transcranially and incubated for 10 minutes,

followed by a single aCSF wash and immediate collection of a high-resolution, three-dimensional two-photon stack 150 μm in depth to allow visualization of the skull, underlying meninges and superficial neocortex in the same location as the background prescan. ROS levels were quantified in Imaris with Matlab plugins to render the skull as a solid object, then the geometric mean fluorescent intensity of the Amplex Red Reagent beneath the lower surface of that object was measured across the entire 1024 x 1024 stack. Background was subtracted from the final mean fluorescent intensity measurement.

Quantification of jellyfish microglia

To determine the number of jellyfish microglia in CX3CR1$^{gfp/+}$ mice treated with either vehicle control or A2AR agonists, a single high-resolution field of view was imaged by TPLSM for a total of 6 hours in each mouse with n = 12 per treatment group. One vehicle control and one agonist-treated mouse were imaged on each experimental day. The number of jellyfish microglia in the field of view was counted by eye in Imaris.

Quantification of honeycomb microglia process length

To determine the effect of A2AR agonists on honeycomb microglia in CX3CR1$^{gfp/+}$ mice, a single high-resolution field of view was imaged by TPLSM for a total of 6 hours in each vehicle- or agonist-treated mouse. As previously published[11], the process length per honeycomb microglia was measured. Briefly, a dot was applied to each honeycomb microglia soma in Imaris. A random number generator and a grid were used to randomly select 10 microglia per field of view (mouse) for analysis. The filament function was used to seed points along the full length of each

process originating at the soma of a selected microglia. The total length of these filament processes was recorded.

Glutathione and glutathione disulfide detection assay

The amount of glutathione (GSH) and glutathione disulfide (GSSG) in microdissected neocortical tissue from naïve, vehicle control-treated and A2A agonist-treated mice was measured using a fluorometric detection assay kit with an internal standard control (BioVision Inc., Milpitas, CA). The protocol provided with the kit for small tissue samples was followed. Microdissections of the mTBI lesions were accomplished via 1.5 mm diameter, sterile, disposable biopsy punches with plungers (Integra Miltex, Plainsboro, NJ) to eject tissue into collection buffer. Biopsy punches were inserted to the depth required to collect only neocortical tissue. Homogenized and preserved samples were frozen at -80 degrees Celsius for up to one week following collection before processing. Samples were collected at the same time of day for all experiments to account for the influence of circadian rhythm on GSH levels[29].

Glutamate detection assay

The amount of glutamate in microdissected neocortical tissue from naïve, vehicle control-treated and A2A agonist-treated mice was measured using a fluorometric detection assay kit with an internal standard control (Abcam, Cambridge, MA). Microdissections of the mTBI lesions were accomplished via 1.5 mm diameter, sterile, disposable biopsy punches with plungers (Integra Miltex, Plainsboro, NJ) to eject tissue into ice-cold Mammalian Cell Lysis Buffer (Abcam, Cambridge, MA) per included instructions for tissue samples. Tissues were

homogenized in 10-15 passes using a glass Kimble Dounce tissue grinder with 1 mL capacity (Kimble Chase, Vineland, NJ) and processed in the same day per kit instructions.

Immunohistochemistry

Proportion of PI-positive cells represented by neurons. Coronal brain sections from vehicle control-treated and A2A agonist-treated mice previously selected from the center of the mTBI lesion via confocal microscopy were labeled with an Alexafluor 488-conjugated, monoclonal NeuN antibody (1:300 in a 0.1% Triton X-100 solution in phosphate-buffered saline; Abcam, Cambridge, MA) to detect neurons. Slides were prepared by gentle coverslip removal and washing in fresh PBS 3 times for 3 minutes to remove residual mounting media. Sections were incubated in Background Buster and Fc Block (Innovex Biosciences, Richmond, CA) for 1 hour, followed by overnight primary conjugated antibody incubation at 4 degrees Celsius. The sections were washed in PBS 3 times for 3 minutes each, then coverslipped with Vectashield and scanned by confocal microscopy. The proportion of PI-positive cells that were neurons was quantified by colocalization of NeuN, PI and DAPI-positive signals in neocortical layers 2-6 in Imaris. Dots were applied to DAPI-positive nuclei, NeuN-positive cells and PI-positive dead cells and three new "channels" produced from these dots. A colocalization algorithm available in Imaris was used to determine the number of dead cells that were NeuN+ neurons in a scanned section.

Microglia depletion confirmation via Iba-1 labeling. Coronal brain sections from vehicle control-treated and A2A agonist-treated mice depleted of microglia that

were previously selected from the center of the mTBI lesion via confocal microscopy were labeled with rabbit polyclonal Iba-1 (1:400 in a 0.1% Triton X-100 solution in phosphate-buffered saline; Wako Chemicals USA Inc., Richmond, VA) to detect microglia. B6 immunohistochemistry controls that were not treated with DT but otherwise handled in an identical manner to vehicle control-treated animals were included for comparison. Slides were prepared by gentle coverslip removal and washing in fresh PBS 3 times for 3 minutes to remove residual mounting media. Sections were incubated in Background Buster and Fc Block (Innovex Biosciences, Richmond, CA) for 1 hour, followed by overnight primary antibody incubation at 4 degrees Celsius. The sections were washed in PBS 3 times for 3 minutes each, the labeled for 6 hours with anti-rabbit secondary antibody tagged with Alexa 488 fluorophore (1:500 in a 0.1% Triton X-100 solution in phosphate-buffered saline). Following 3 additional PBS washes, the slides were coverslipped using Vectashield.

Group sizes and statistical analyses

Initial group sizes of minimum n = 6 were used for all experiments to ensure reproducibility and allow detection of statistical differences. All experiments were reproduced at least once. Animals were excluded from analyses only when mortality was attributed to anesthesia. Mice were randomized prior to assignment to experimental groups. Additionally, in experiments in which mTBI mice were treated with vehicle or an adenosine receptor agonist or antagonist, mTBI lesions were induced in an alternating manner such that two animals from the same experimental group were not compressed in succession without an intervening animal from the other group. Statistical significance ($p < 0.05$) was determined via Student's t-test for

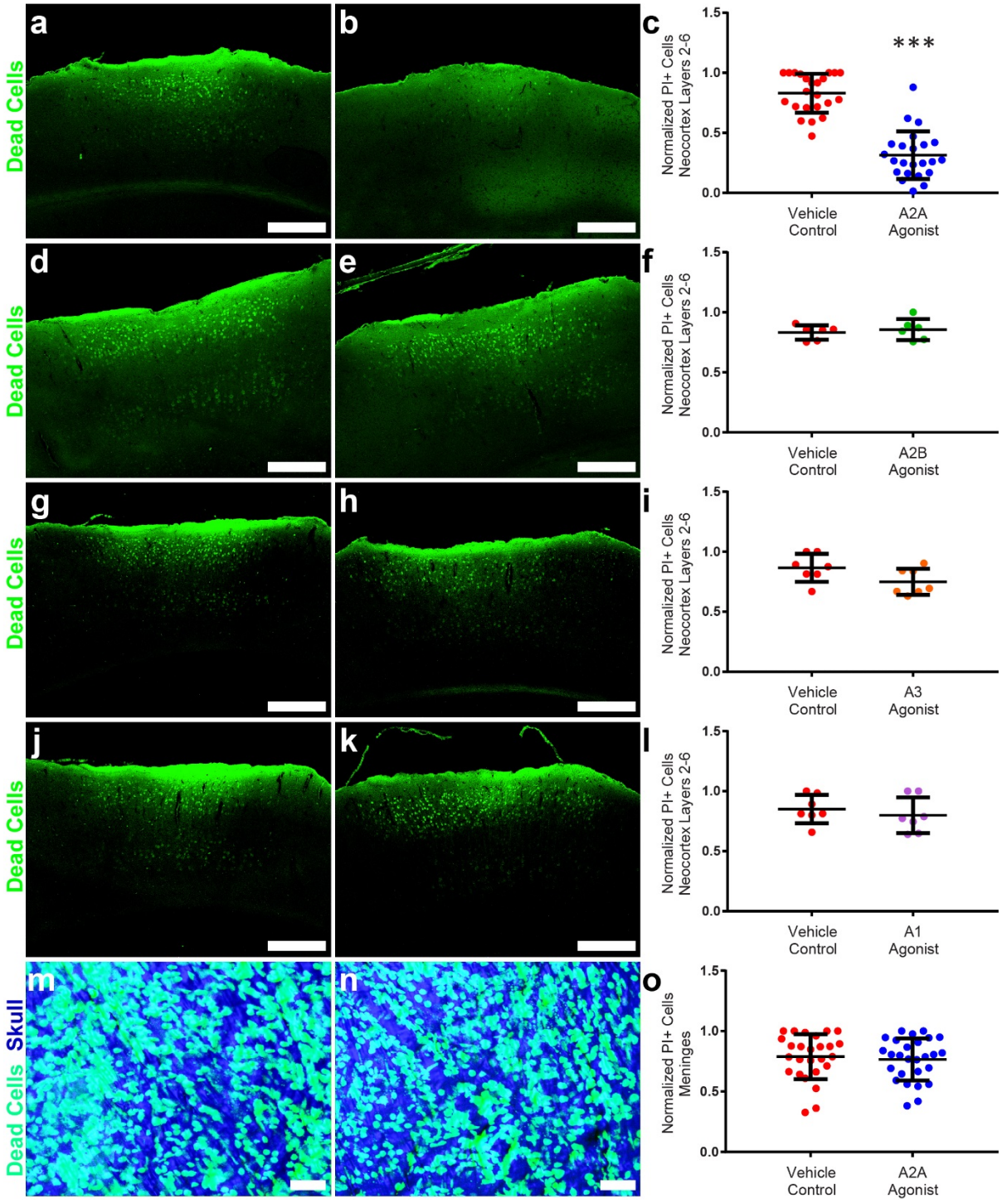
experiments comparing two groups, and one-way analysis of variance (ANOVA) with Holm-Sidak post-test for experiments comparing more than two groups. ANOVA on ranks or a rank sum test was used for non-parametric data. All statistical analyses were performed in SigmaPlot 11 (Systat Software Inc., San Jose, CA). Graphs were composed in Graphpad Prism 7 (Graphpad Software Inc., La Jolla, CA) **(See Appendix, Table 5)**.

Results

Transcranial application of an A2A adenosine receptor agonist protects the neocortex following mTBI

To determine the effect of agonizing adenosine receptors on the neocortical lesion produced by mTBI, we administered an agonist targeting each of the four adenosine receptors (A2AR, A2BR, A3R, and A1R at 10 μm, 10 μm, 1 μm, and 2 μm, respectively) via the transcranial route 15 minutes after mTBI (**Fig. 2a-l)**. Incubations were maintained for 8 hours following mTBI, at which time PI was applied transcranially to label dead cells. Treatment with the A2AR agonist UK432097 decreased the number of PI+ dead cells in the neocortical parenchyma by approximately 63% compared to vehicle-treated control mice (**Fig. 2a-c**; n = 25 from 6 independent experiments, normalized mean of 0.31 in A2AR agonist-treated mice compared to 0.83 in vehicle control-treated mice). In contrast, transcranial application of an agonist to the A2BR, A3R, or A1R, respectively, did not protect the neocortex 8 hours following mTBI (**Fig. 2d-l**; n = 8 from 2 independent experiments per agonist). Interestingly, treatment with an A2AR agonist did not decrease the number of PI+ dead cells in the meninges 8 hours following mTBI in the same

Figure 2. Transcranial application of an A2AR agonist protects the neocortex, but not the meninges, following mTBI. **(a-c)** The A2A adenosine receptor agonist UK432097 (**b**) decreases the number of PI+ dead cells (green) in the neocortex through 8 hours after mTBI compared to vehicle-treated controls (**a**). Graph in (**c**) represents 6 independent experiments with n = 4 or more animals per group, *** $p \leq 0.001$, unpaired t-test. **(d-l)** Transcranial application of agonists to the A2B ($p = 0.592$), A3 ($p = 0.076$) or A1 ($p = 0.484$) adenosine receptors does not decrease the number of PI+ dead cells in the neocortex 8 hours following mTBI. Graphs in (**f**), (**i**) and (**l**) represent 2 independent experiments with n = 4 animals per group, unpaired t-tests. Scale bars, 250 μm. **(m-o)** UK432097 does not protect the meninges following mTBI ($p = 0.575$). Scale bars, 50 μm. Meningeal data represented in graph in (**o**) correspond to the neocortical data from animals represented in graph (**c**); Mann-Whitney Rank Sum test.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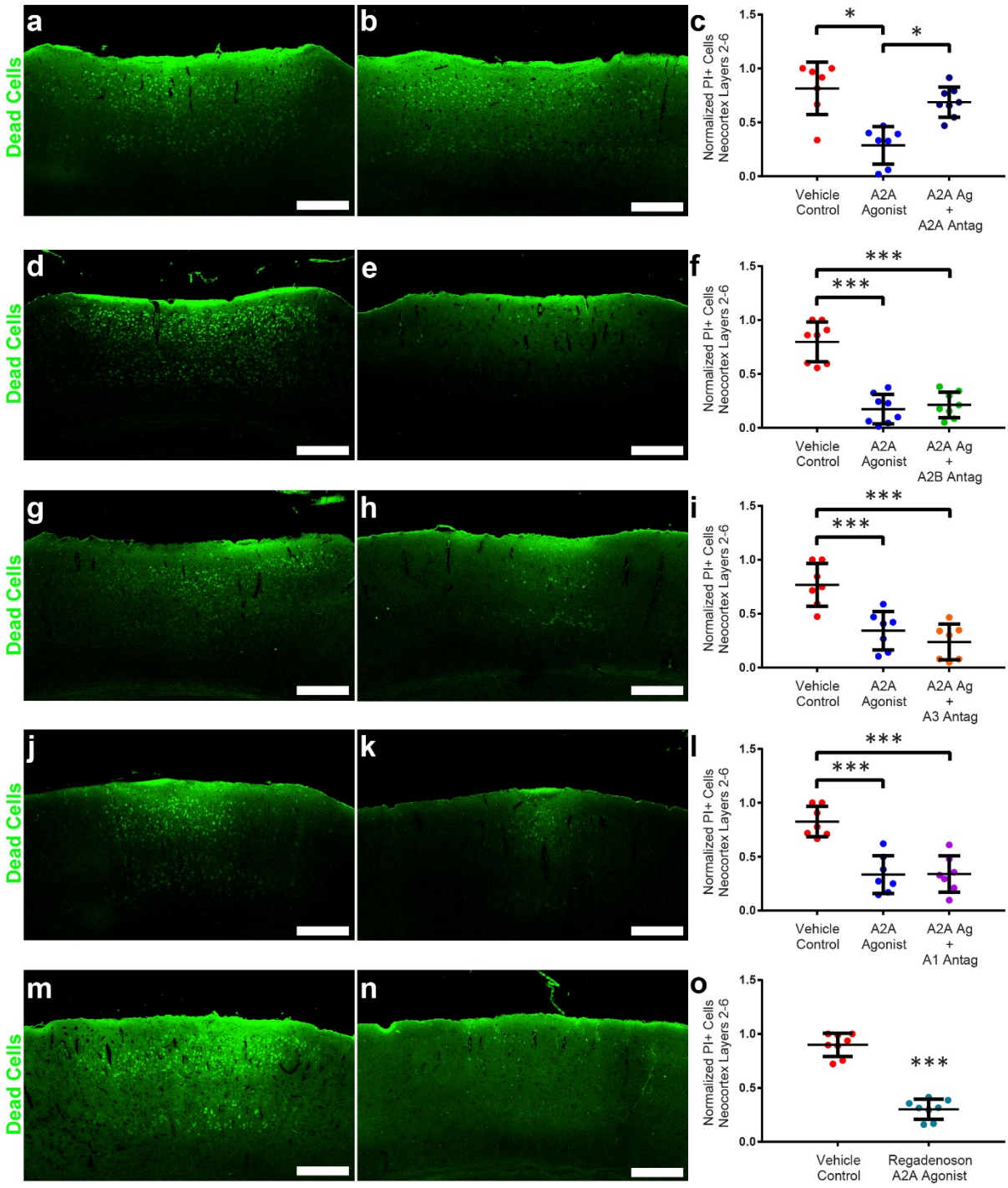
animals in which the parenchyma was protected (**Fig. 2m-o**; n = 25), suggested that parenchymal protection occurs via a mechanism that cannot prevent meningeal cell death following mTBI.

Neocortical protection following mTBI is specific to the A2A adenosine receptor

We next tested whether the neocortical protection resulting from transcranial treatment with an A2AR agonist was specific for that adenosine receptor. Mice were pretreated with a transcranial application of a highly selective A2AR antagonist for 30 minutes prior to induction of mTBI. A combination treatment of A2AR agonist and antagonist was subsequently applied transcranially 15 minutes after mTBI, and incubation was maintained to 8 hours after injury. The number of PI+ dead cells in the neocortices of mice receiving both the A2AR agonist and antagonist were compared to mice receiving transcranial pretreatment with aCSF followed by either vehicle control or A2AR agonist alone. Pretreatment blockade of the A2AR eliminated the protective effect of treatment with an A2AR agonist (**Fig. 3a-c**; n = 8). Treatment with the same A2AR antagonist alone did not protect the neocortex after mTBI (**Fig. 4a-c**; n = 7). Similar cotreatment experiments with antagonists to the A2BR, A3R, and A1R (**Fig. 3d-f**, **3g-i**, **3j-l**, respectively) did not prevent the protective effect of treatment with an A2AR agonist. This strongly suggests that the neocortical protection we observed was due specifically to signaling via the A2AR.

To further investigate receptor specificity, we next treated mice with Regadenoson, a more selective, FDA-approved A2AR agonist, via transcranial administration at 15 minutes after mTBI. These incubations were maintained for 6

Figure 3. The protective effect of transcranial A2AR agonists is blocked by co-application of an inhibitor to the A2A, but not the A2B, A1 or A3, adenosine receptors following mTBI. **(a-c)** Transcranial application of the A2AR inhibitor SCH442416 eliminates the protective effect of UK432097 in the neocortex (**b**), resulting in a lesion composed of PI+ dead cells (green) comparable to vehicle-treated controls (**a**) 8 hours after mTBI ($p > 0.05$). UK432097 continues to exert protection when applied with antagonists to the **(d-f)** A2B, **(g-i)** A3 and **(j-l)** A1 receptors, with no difference between groups receiving A2AR agonist alone or cotreatments ($p = 0.605$, 0.292 and 0.953, respectively). **(m-o)** The FDA-approved A2AR agonist Regadenoson also significantly decreases the PI+ dead cells in the neocortex following mTBI. Graphs in (**c**), (**f**), (**i**), (**l**), and (**o**) represent 2 independent experiments with $n = 4$ animals per group for each experimental condition. Scale bars, 250 μm. * $p \leq 0.05$, *** $p \leq 0.001$; one-way ANOVA except data in (**o**) – Mann-Whitney Rank Sum test.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Regadenoson A2A Agonist

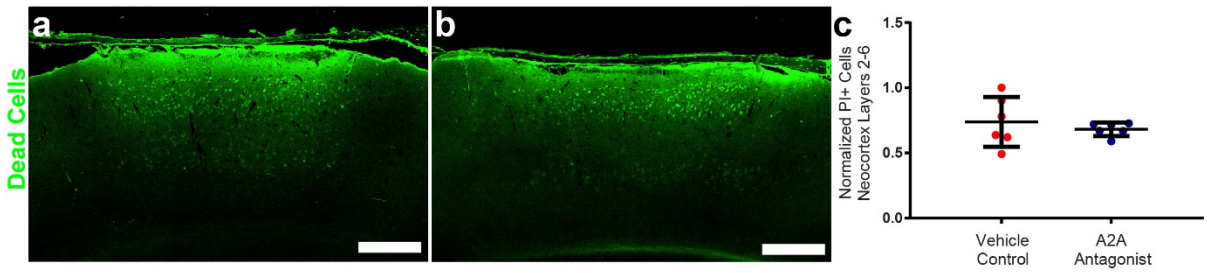


Figure 4. Transcranial application of an A2AR antagonist does not protect the neocortex following mTBI. **(a-c)** Transcranial application of the A2AR antagonist SCH442416 (**b**) results in a lesion composed of PI+ dead cells (green) comparable to vehicle-treated controls (**a**) 8 hours after mTBI ($p > 0.05$). Graph in (**c**) represents 2 independent experiments with $n = 4$ and $n = 3$, respectively, animals per group. Scale bars, 250 μm. Unpaired t-test.

hours after injury, followed by PI incubation. Regadenoson decreased PI+ dead cells in the neocortex by approximately 70%, like UK432097, when compared to vehicle-treated controls (**Fig. 3m-o**; n = 8). Regadenoson also did not impact cell death in the meninges (**Fig. 5a-c**; n = 12), suggesting that the mechanism through which A2AR agonists protect the parenchyma does not prevent cell death in the meninges. In summary, transcranial application of A2AR agonists protect the neocortex in a receptor-specific manner.

A2AR null mice are not protected by transcranial A2AR agonist application following mTBI

In preparation for treating C;129S-*Adora2a*^{tm1Jfc}/J (global A2AR null mice) with Regadenoson, we first determined whether agonizing the A2AR would protect the neocortices of Balb/cJ mice (**Fig. 6a-c**; n = 10), the principle background strain for A2AR null mice. Compared to vehicle control-treated Balb/cJ mice (**Fig. 6a**), transcranial application of Regadenoson significantly decreased the number of PI+ dead cells in the neocortices of Balb/cJ mice (**Fig. 6b**, a 70% decrease), recapitulating the results we observed in C57Bl/6J mice (see **Fig. 2a-c**). In contrast, transcranial treatment with Regadenoson did not prevent cell death in the neocortices of global A2AR null mice compared to controls (**Fig. 6d-f**; n =8). We concluded from these results that protection of the neocortex following treatment with an A2AR agonist requires the presence of the A2AR receptor, further substantiating our conclusion that this is a receptor-specific mechanism.

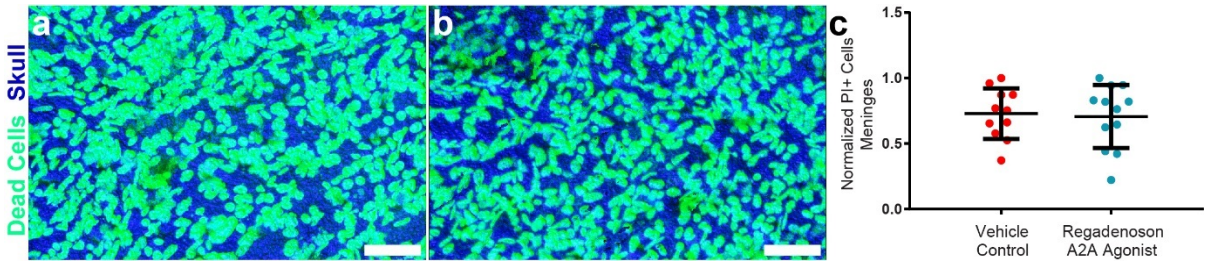


Figure 5. Regadenoson does not protect the meninges following mTBI. **(a-c)** PI+ dead cells are comparable in vehicle-treated (**a**) and Regadenoson-treated (**b**) mice 6 hours after mTBI ($p = 0.811$). Graph in (**c**) represents 2 independent experiments with $n = 6$ per experimental group. Scale bars, 50 μm. Unpaired t-test.

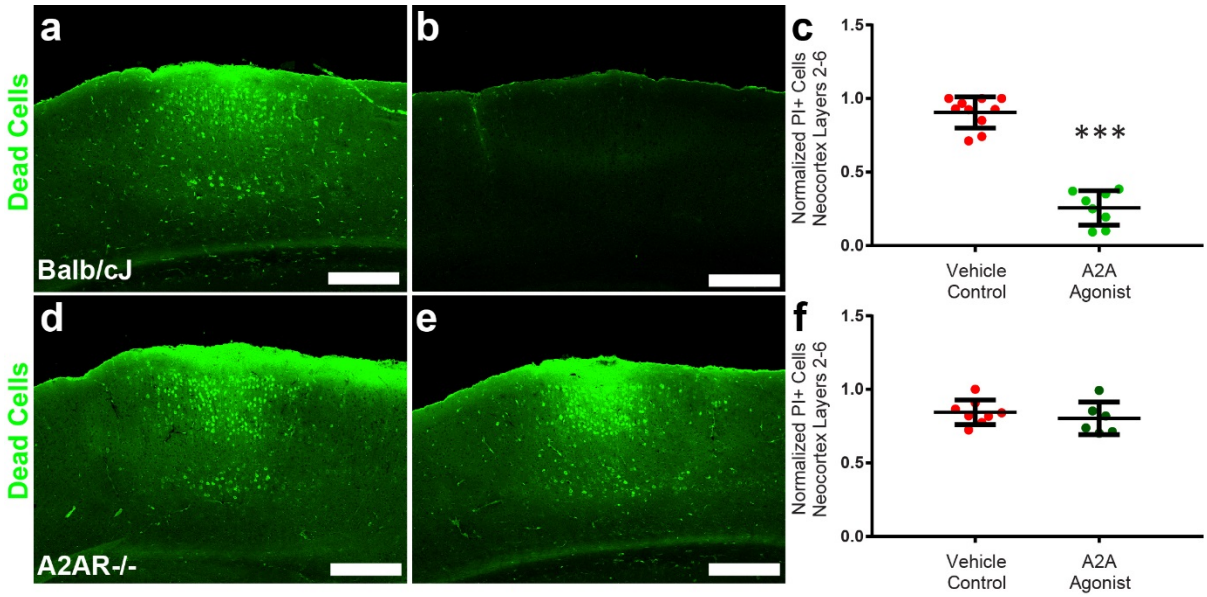


Figure 6. Transcranial application of A2AR agonists protects the neocortices of Balb/cJ mice, but not A2AR$^{-/-}$ mice. **(a-c)** The neocortices of Balb/cJ mice comparable the C57Bl/6 mice used in previous experiments are also protected following mTBI when treated transcranially with A2AR agonists, with a significant decrease in PI+ dead cells (green) compared to vehicle-treated controls. **(d-f)** In contrast, mice globally lacking the A2A adenosine receptor are not protected following mTBI ($p = 0.446$). Graph in (**c**) represents 2 independent experiments with n = 5 animals each. Graph in (**f**) represents 2 independent experiments with n = 4 animals each Scale bars, 250 μm. *** $p \leq 0.001$, Mann-Whitney Rank Sum test or unpaired t-test.

A2AR agonists protect the neocortex by acting directly on CNS resident cells

We next sought to determine whether A2AR agonists protect the neocortex via action on cells derived from the peripheral bone marrow or by acting directly on CNS resident cells. We irradiated Balb/cJ and global A2AR null mice, followed by transfer of bone marrow derived from donor mice to produce the following bone marrow chimeras: wild type Balb/cJ mice with congenic (Cd45.1+) bone marrow (**WT-WT**); Balb/cJ congenic mice (Cd45.1+ bone marrow) with A2AR null bone marrow (**KO BM**); global A2AR null mice with congenic (Cd45.1+) wild type bone marrow (**KO Brain**). Use of congenic donor and recipient animals allowed us to confirm chimerism by flow cytometric analysis prior to treatment studies. All experiments included vehicle control- and Regadenoson-treated WT-WT groups (**Fig. 7a,b** and **Fig. 7e,f**). Mice with A2AR-competent brains but A2AR null bone marrow (**KO BM**; **Fig. 7c**) responded to transcranial application of Regadenoson in a manner comparable to WT-WT mice, with a significant decrease in PI+ dead cells following mTBI (**Fig. 7d**). In contrast, global A2AR null mice with receptor-competent bone marrow (**KO Brain**; **Fig. 7g**) did not respond to transcranial treatment with Regadenoson, and had lesions comparable to WT-WT vehicle control-treated animals (**Fig. 7h**). These experiments demonstrate that protection of the neocortex by transcranial A2AR agonists requires the presence of the A2AR on CNS resident cells, and response to treatment is not impacted by peripheral, bone marrow-derived cells.

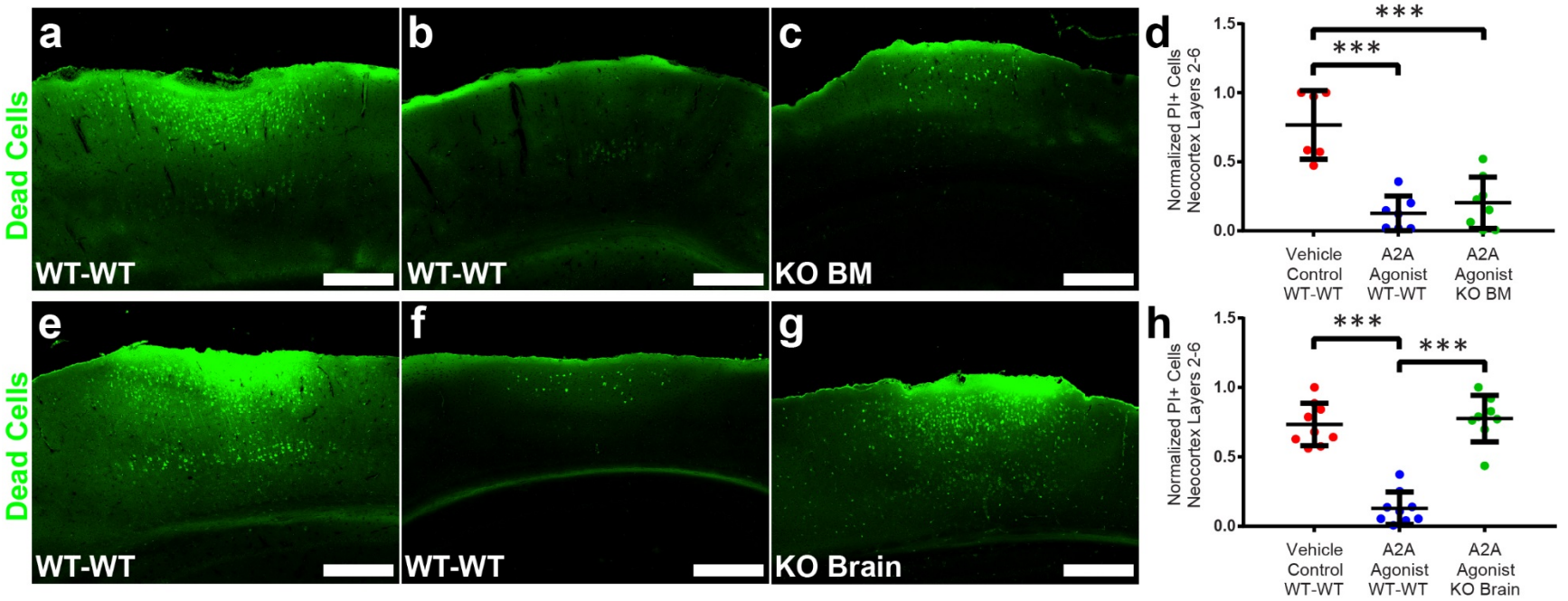


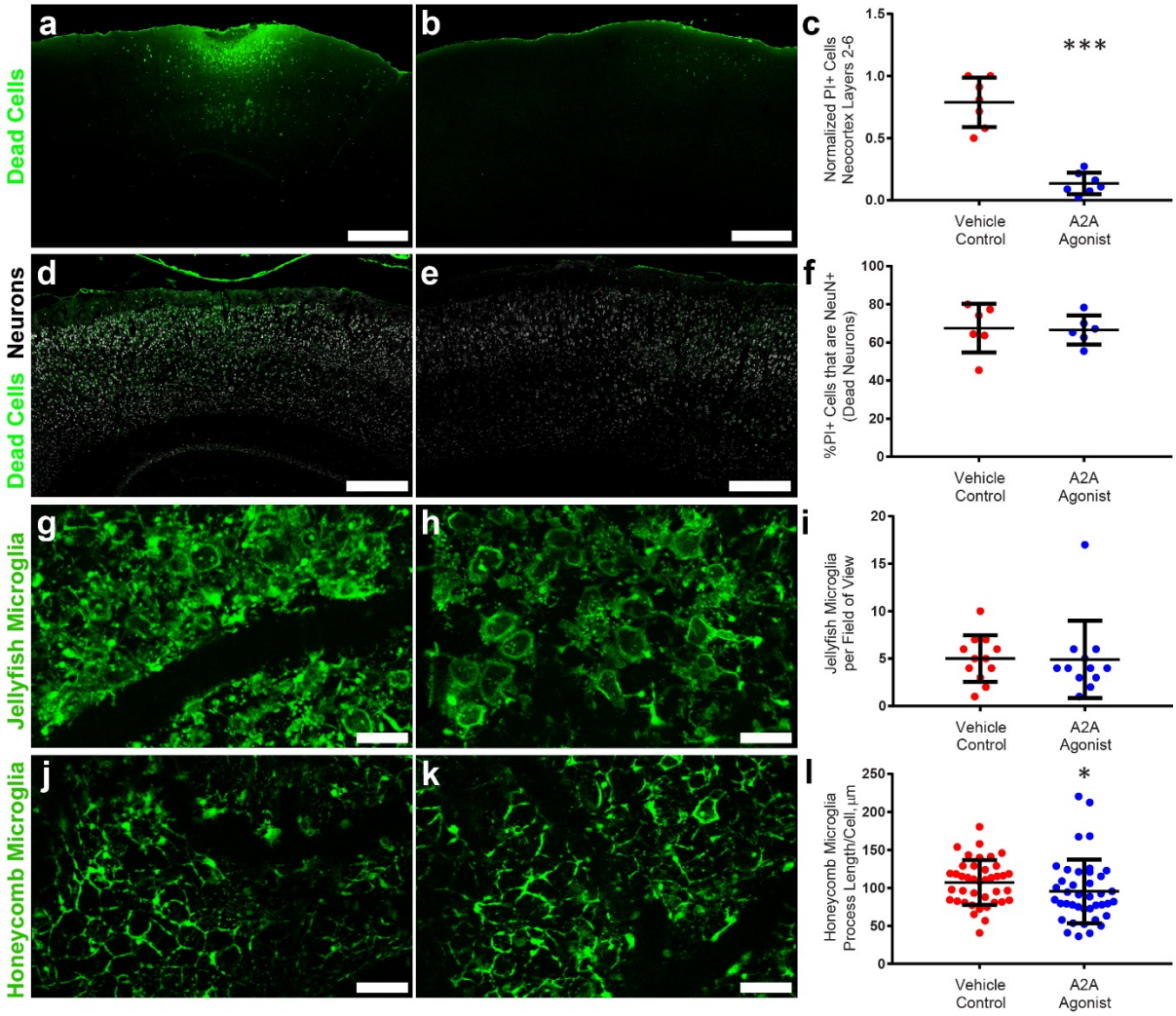
Figure 7. A2AR agonists act directly on CNS resident cells to protect the neocortex following mTBI. **(a-d)** Chimeras with A2AR-competent brains and A2AR-null bone marrow (KO BM) or **(e-h)** A2AR-null brains and A2AR-competent bone marrow (KO Brain) were treated transcranially with A2AR agonists and compared to similarly irradiated appropriate vehicle- and agonist-treated controls (WT-WT, left and right respectively). Animals in which CNS resident cells express the A2AR had significantly fewer PI+ dead cells (green) following mTBI (**c**), while those in which CNS resident cells lack the A2AR were not protected compared to vehicle-treated controls (**g**, $p = 0.556$). WT-WT mice treated with the A2AR agonist were not different from similarly treated KO BM mice (**d**, $p = 0.443$). Graphs in (**d**) and (**h**) represent 2 independent experiments for each experimental condition. Graph in (**d**), n = 3 for Vehicle WT-WT per experiment; n = 3 and 4 for A2A Agonist WT-WT, respectively; n = 4 for A2A Agonist KO BM per experiment. Graph in (**h**), n = 4 and 5 for Vehicle WT-WT, respectively; n = 4 and 5 for A2A Agonist WT-WT, respectively; n = 4 for A2A Agonist KO Brain per experiment. Scale bars, 250 μm. *** $p \leq 0.001$, one-way ANOVA.

Neocortical protection by A2AR agonists does not require microglia or preferentially protect neurons

To begin to understand the mechanism by which transcranial treatment with A2AR agonists protects the neocortex, we asked if microglia or neurons were more significantly affected than other CNS resident cell types. We first determined if microglia are necessary for A2AR agonists to protect the neocortex by utilizing mice with tamoxifen-driven expression of diphtheria toxin receptor (DTR) under the CX3CR1 promoter. After feeding tamoxifen diet chow for 30 consecutive days, only CX3CR1-positive cells such as microglia and peripheral monocytes express DTR. Microglia were depleted by injection of diphtheria toxin for three consecutive days, and a transcranial A2AR agonist treatment study was executed immediately following the final injection. Compared to vehicle-treated control animals (**Fig. 8a**,), those receiving transcranial Regadenoson (**Fig. 8b**,) had significantly fewer PI+ dead cells in the parenchyma (**Fig. 8c**; 82% decrease). We therefore concluded that microglia are not required for transcranial A2AR agonists to protect the neocortex.

We next tested whether Regadenoson affects neurons preferentially. We labeled tissue sections at PI+ mTBI lesion centers from vehicle control- and Regadenoson-treated mice with NeuN and quantified the number of dead neurons in neocortical layers 2 through 6. The mean percentage of PI+ dead cells that were also NeuN+ was 67% and 66%, respectively, in control- and Regadenoson-treated mice (**Fig. 8d-f**). This result indicates that agonizing the A2AR does not prevent loss of neurons over other cell types, and suggests that the mechanism of protection is not neuron-specific. As with other transcranial treatment experiments, Regadenoson

Figure 8. A2AR agonists do not preferentially protect a single cell type following mTBI. **(a-c)** Microglia were depleted using tamoxifen-inducible diphtheria toxin (DT) receptor mice fed with tamoxifen chow for 30 days, followed by 3 days of intraperitoneal injections of DT. A2AR agonists protect the neocortex following mTBI in the absence of microglia (**b**), with a significant decrease in PI+ dead cells (green) compared to similarly-depleted vehicle control mice (**a**). Scale bars, 250 μm. ***, $p \leq 0.001$. **(d-f)** Transcranial treatment with A2AR agonists does not preferentially protect neurons (NeuN+, white) following mTBI. The percentage of PI+-NeuN+ cells (dead neurons, green and white) in the neocortex is not statistically different in vehicle- or A2AR agonist-treated mice ($p = 0.874$). Scale bars, 500 μm. Graphs in (**c**) and (**f**) represent 2 independent experiments of n = 4 animals per group. **(g-l)** *In vivo* imaging of the meninges and superficial neocortex of CX3CR1$^{+/gfp}$ mice reveals that transcranially-applied A2AR agonists minimally change the behavior or stereotyped morphological changes of microglia. There is no statistical difference in the number of jellyfish microglia in A2AR agonist-treated mice (**h**) compared to vehicle controls (**g**) following mTBI ($p = 0.397$). In contrast, A2AR agonists decrease the process length per cell of honeycomb microglia (**k**) compared to vehicle controls (**j**). Graphs in (**i**) and (**l**) represent 6 independent imaging experiments with n = 2 mice each. Each dot in (**i**) is the total jellyfish microglia in the field of view collected from a single mouse. Each dot in (**l**) is the process length from one randomly-selected honeycomb microglia. Scale bars, 100 μm. * $p \leq 0.05$, Mann-Whitney Rank sum test or unpaired t-test.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decreased the number of PI+ dead cells in the neocortices by approximately 52% compared to vehicle-treated controls.

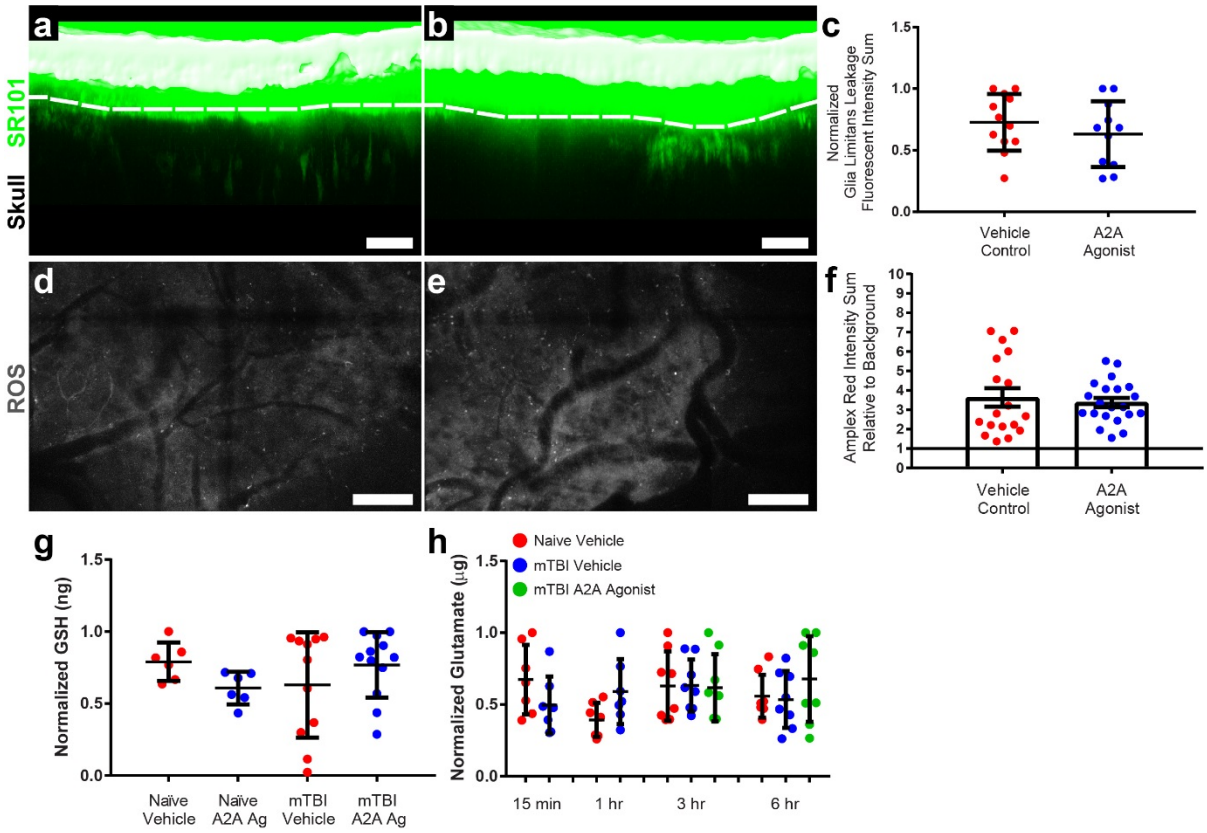
In vivo imaging of CX3CR1gfp/+ mice was performed following mTBI to assess whether agonizing the A2AR changes the morphology or response of microglia. We quantified the number of jellyfish microglia in control- and Regadenoson-treated mice (**Fig. 8g-i**) and found no difference between these groups, with means of 5 and 4.9, respectively ($p = 0.397$). We additionally measured the length of the processes per honeycomb microglia (**Fig. 8j-l**). In this case, we found a statistically significant difference between the treatment groups ($p = 0.034$). Processes were shorter in A2AR agonist-treated mice (mean 95 μm per cell compared to 107 μm in control-treated mice).

A2AR agonists do not protect the glia limitans, decrease extracellular ROS, increase glutathione, or alter glutamate

We tested whether agonizing the A2AR impacts very early mediators of post-mTBI pathology previously identified in our laboratory, including the glia limitans and ROS. To assess the integrity of the glia limitans, we passed SR101 transcranially and measured the mean fluorescent intensity of the dye in the superficial neocortex. Compared to vehicle-treated controls (**Fig. 9a**) A2AR agonist treatment (**Fig. 9b**) did not prevent leakage through the glia limitans (**Fig. 9c**).

ROS are detectable in the meninges and superficial neocortex acutely following mTBI by transcranial application of Amplex Red, which fluoresces in the presence of ROS. Vehicle control-treated (**Fig. 9d**) and A2AR agonist-treated (**Fig. 9e**) mice have comparable Amplex Red intensity sums relative to the background

Figure 9. A2AR agonists do not impact glia limitans integrity or reactive oxygen species (ROS). **(a-c)** SR101 (green) passed through the skull (white) fills the meninges and leaks through the glia limitans (white dotted line) in vehicle control-treated (left) and A2AR agonist-treated mice 3 hours after mTBI. The mean fluorescent intensity of SR101 detected in the parenchyma below the glia limitans is similar regardless of treatment (**c**, $p = 0.369$, unpaired t-test). **(d-f)** Amplex Red (white) fluoresces in the presence of ROS in the meninges of vehicle control-treated (left) and A2AR agonist-treated (right). The sum of the fluorescence detected by high resolution two-photon laser scanning microscopy one hour following mTBI is similar regardless of treatment (**f**, $p = 0.746$, Mann-Whitney Rank Sum test). **(g)** Glutathione is not depleted following mTBI in mice treated transcranially with A2AR agonist compared to those treated with vehicle control. There is not a statistical difference between naïve mice and those receiving mTBI, regardless of treatment ($p = 0.997$ for naïve vs. mTBI; $p = 0.809$ for vehicle vs. A2AR agonist; two-way ANOVA). **(h)** The concentration of glutamate is not statistically different at 15 minutes, one hour, 3 hours, or 6 hours regardless of whether the mice are naïve or receive a mTBI. Additionally, A2AR agonists do not alter glutamate levels at 3 or 6 hours after mTBI compared to naïve or mTBI mice receiving vehicle control ($p = 0.7$, two-way ANOVA). The only significant difference detected was between naïve mice at 15 minutes and one hour ($p = 0.016$, two-way ANOVA). Graph in (**c**) represents 4 independent experiments of $n = 3$ animals per group. Scale bar in (**a-b**), 50 μm. Scale bar in (**d-e**), 100 μm. Graph in (**f**) represents 3 independent experiments of $n = 6$ animals per group. Graph in (**g**) represents 2 independent experiments of $n = 3$ for naïve mice treated with vehicle and A2A agonist, and 3 independent experiments of $n = 4$ for mice receiving an mTBI followed by treatment with vehicle or A2A agonist.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fluorescence (**Fig. 9f**), indicating that agonizing the A2AR does not decrease ROS in the meninges.

We measured the glutathione (GSH) in naïve (n = 6 per treatment) and mTBI (n = 12 per treatment) mice treated with either vehicle control or A2AR agonist to determine if A2AR agonists increased GSH and therefore might make cells less susceptible to ROS-induced damage. There was no effect of naïve vs. mTBI status by two-way ANOVA ($p = 0.997$), and transcranial treatment with A2AR agonists did not result in an increase of GSH compared to vehicle control-treated mice (**Fig. 9g**; $p = 0.809$). However, GSH was also not depleted following mTBI when A2AR agonist-treated mice are compared to vehicle-treated control mice (normalized means 0.77 vs. 0.63, respectively).

The glutamate concentration present in naïve mice or those receiving a mTBI was compared 15 minutes, one hour, 3 hours, and 6 hours following injury. Animals with mTBI that were treated with an A2AR agonist were compared to these groups at 3 and 6 hours following injury (**Fig. 9h**). No statistical differences were detected between naïve and mTBI groups at any time point. Transcranial A2AR agonist treatment did not result in glutamate concentrations statistically different from either naïve or mTBI animals at 3 and 6 hours following mTBI ($p = 0.131$). The only statistically significant difference detected is that between naïve groups at 15 minutes and one hour ($p = 0.016$).

Inhibiting protein kinase c (PKC) δ prevents neocortical protection by A2AR agonists

We assessed whether PKCδ, a common downstream signaling mediator of the A2AR, is involved in the mechanism through which A2AR agonists protect the neocortex (**Fig. 10a-e**). Vehicle control- and Regadenoson-treated mice (**Fig. 10a,b**) were compared to mice treated with the PKCδ inhibitor Rottlerin alone (**Fig. 10c**) or a combination of Rottlerin and Regadenoson (**Fig. 10d**). Rottlerin alone did not result in a lesion statistically different from vehicle controls ($p = 0.154$). Transcranial application of Rottlerin eliminated the protective effect of Regadenoson (**Fig. 10e**, $p = 0.118$), suggesting involvement of PKCδ in the signaling pathway leading to decreased neocortical cell death after mTBI.

A2AR agonists protect the neocortex via a signaling pathway culminating in carbonic anhydrase IX

Hif-1α and carbonic anhydrase (CA) IX are involved in maintenance of cellular homeostasis and the response to cellular injury. To determine if these downstream mediators of A2AR signaling might be involved in the protective effect we observed with transcranial A2AR agonist application, we performed separate cotreatment experiments with inhibitors to each effector. We first compared the Hif-1α inhibitor TAT-cyclo-CLLFVY alone (**Fig. 10h**) to vehicle-treated (**Fig.10f**), A2AR agonist-treated (**Fig. 10g**) and cotreatment of TAT-cyclo-CLLFVY with Regadenoson (**Fig. 10i**). Treatment with Regadenoson alone significantly reduced the number of PI+ dead cells in the neocortex compared to both vehicle and TAT-cyclo-CLLFVY (**Fig. 10j**; $p \leq 0.01$). Inhibiting Hif-1α results in a lesion comparable to that in vehicle-

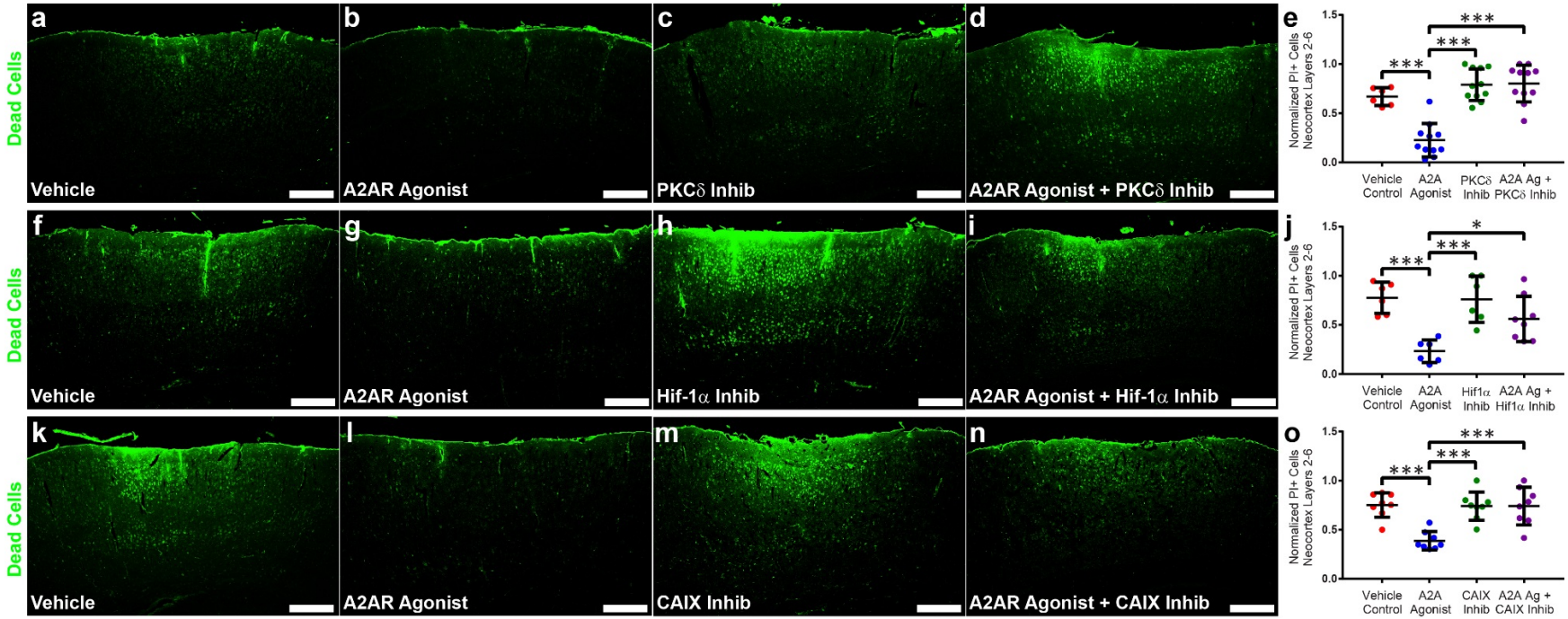

Figure 10. A2AR agonists protect the neocortex through Carbonic Anhydrase IX (CAIX) via Protein Kinase C delta (PKCδ) signalizing pathway. **(a-e)** Cotreatment of mice following mTBI with an A2AR agonist and Rottlerin (**d**), an inhibitor of PKCδ, results in PI+ dead cells (green) comparable to vehicle-treated controls (**a**, p = 0.118) and mice treated with Rottlerin alone (**c**, p = 0.862). **(f-j)** In contrast, cotreatment with an A2AR agonist and TAT-cyclo-CLLFV, a Hif-1α inhibitor, produces a trend of decreased neocortical protection that is not statistically different from vehicle (**f**)-, A2AR agonist (**g**)- or Hif-1α inhibitor (**h**)-treated mice (p = 0.259, 0.029 and 0.28, respectively). **(k-o)** Finally, cotreatment with an inhibitor to CAIX eliminates the protective effect of an A2AR agonist (**n**), resulting in a lesion comparable to both vehicle control (**k**, p = 0.878) and CAIX inhibitor alone (**m**, p = 0.991). In all experiments, A2AR agonist alone significantly decreased PI+ dead cells in the neocortex (**b**, **g**, **l**). Graph in (**e**) represents 3 independent experiments with n = 4 for A2A Agonist, PKCδ inhibitor and combination in each experiment. n = 3 for vehicle control from one experimental replicate and n = 2 for the second and third replicates. Graphs in (**j**) and (**o**) represent 2 independent experiments each with n = 4 per group except for total n = 6 for vehicle control group in (**j**). Scale bar, 250 μm. ** $p < 0.01$, *** $p \leq 0.001$, one-way ANOVA.

treated mice ($p = 0.96$). Cotreatment resulted in an effect that was not statistically different from vehicle ($p = 0.259$), Regadenoson ($p = 0.029$) or TAT-cyclo-CLLFVY alone ($p = 0.28$). We concluded from these results that while Hif-1α may contribute to the protective mechanism of transcranial treatment with A2AR agonists following mTBI, blocking Hif-1α is insufficient to statistically eliminate this protective effect.

We then compared transcranial application of S4, an inhibitor of CAIX, alone (**Fig. 10m**) to vehicle (**Fig. 10k**), Regadenoson alone (**Fig. 10l**) and cotreatment with S4 and Regadenoson (**Fig. 10n**). As with all previous experiments assessing PI+ dead cells, Regadenoson significantly protected the neocortex compared to vehicle and S4 ($p \leq 0.001$). Vehicle- and S4-treated mice had similar lesions ($p = 0.87$). S4 prevented neocortical protection by Regadenoson, resulting in a lesion statistically different from the A2AR agonist-only group ($p \leq 0.001$) and similar to both vehicle- ($p = 0.878$) and S4- ($p = 0.991$) treated groups (**Fig. 10o**). We therefore concluded that A2AR agonists decreased dead cells in the neocortex following mTBI through a signaling pathway ending in CAIX.

Conclusions

We sought to transcranially interrogate adenosine signaling following mTBI and uncovered a very early pathogenic mechanism resulting in neocortical cell death that is ameliorated by agonizing the A2AR. To date, we have demonstrated neuroprotection by transcranial A2AR agonists in over 120 mice subjected to our mTBI model, and made several important observations. The first of these is that this agonist-induced neuroprotective effect is specific to the A2AR, as demonstrated by our agonist-antagonist cotreatment studies, replication with a second selective

agonist (Regadenoson) and lack of protective effect in A2AR global null mice. Our observations may at first appear to contrast with the findings of other groups in that they report protection upon treatment with A2AR antagonists or inactivation of the A2AR[21,23,26]. However, others have shown that local glutamate levels at the time of treatment determine whether agonists or antagonists are protective following injury[25]. A temporal difference in treatment efficacy of agonists or antagonists has been demonstrated in blunt spinal cord trauma models[24,30,31], with early application of agonists providing benefit. Deficiency of the A2AR or treatment with antagonists most frequently show promise in moderate or severe models of TBI[21,32], models of chronic brain or spinal injury[22,32], or models of chronic neurodegenerative diseases such as Alzheimer's and Parkinson's diseases[20,24]. Moreover, replication of the protective effect of A2AR agonists in two genetically divergent mouse strains (C57Bl/6J and Balb/cJ) provides further evidence of a robust protective mechanism in the neocortex provided treatment is initiated rapidly after injury. While we do not demonstrate a deleterious effect upon initiation of A2AR agonist treatment at 1 hour post-mTBI, the neuroprotective effect is eliminated by this delay in treatment (**Fig. 11a-c**). No change is observed in the effect of treatment with an A2AR antagonist when treatment is initiated at 1 hour post-mTBI (**Fig. 11d-f**).

Our data revealed that neither UK432097 nor Regadenoson treatment protected the meninges regardless of when we applied transcranial solutions, including pretreatment beginning 30 minutes prior to mTBI (**Fig. 12a-f**). Given that many TBI patients incur meningeal injuries that may contribute to ongoing post-injury symptoms[33], it is interesting that the mechanism through which A2AR agonists

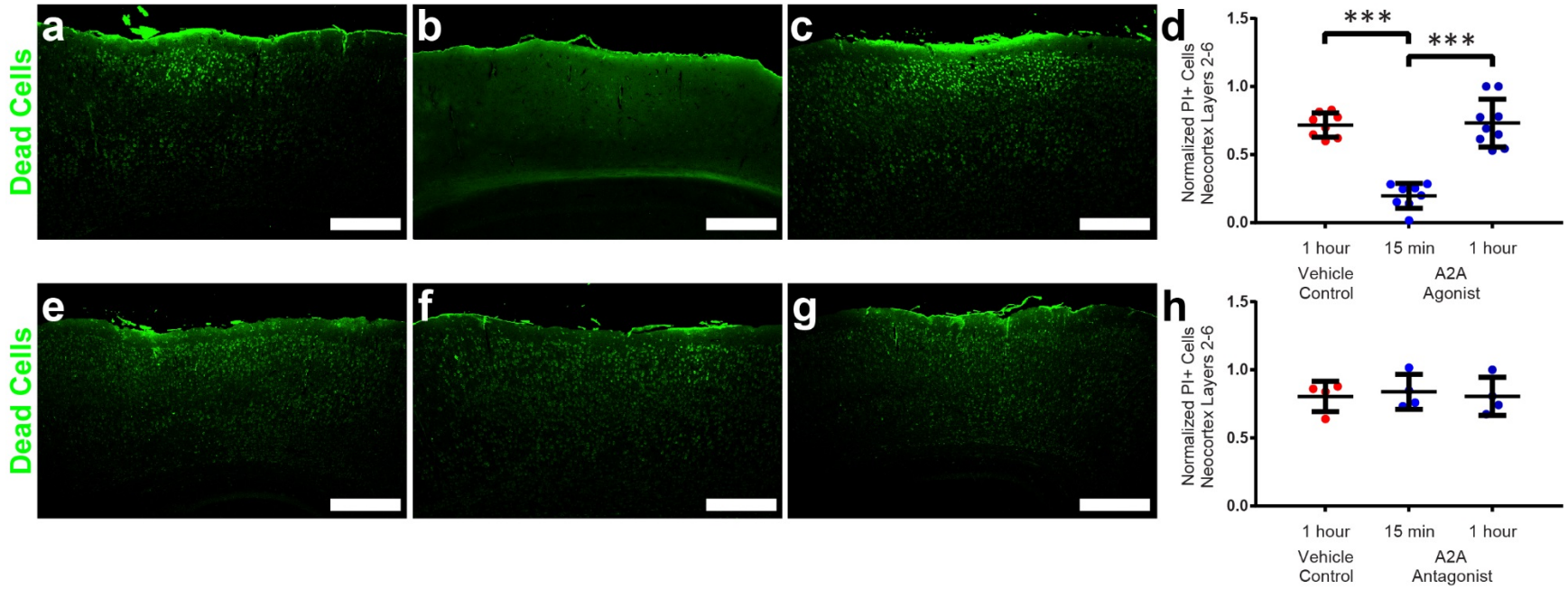

Figure 11. Transcranial application of an A2AR agonist is efficacious when applied acutely following mTBI. **(a-d)** Unlike the decrease in in neocortical cell death seen upon transcranial treatment with an A2AR agonist at 15 minutes post-mTBI (**b**), treatment with the same transcranial A2AR agonist 1 hour after injury eliminates the protective effect of the agonist (**c**; $p = 0.824$) and produces a lesion comparable to vehicle control-treated mice (**a**). A2AR agonist-treated mice incubated starting 15 minutes post-mTBI have statistically fewer PI+ dead cells (green) than the other groups. Graph in (**d**) represents 2 independent experiments each with n = 5 per group. Scale bars, 250 μm. *** $p \leq 0.001$, one-way ANOVA. **(e-h)** In contrast, an A2AR antagonist does not protect the neocortex regardless of time post-mTBI at which transcranial treatment is initiated (15 minutes post-mTBI, **f**; 1 hour post-mTBI, **g**), and produces a lesion comparable to vehicle-treated controls (**e**; $p = 0.91$). Graph in (**h**) represents 1 independent experiment with n = 4 per group. Scale bars, 250 μm. One-way ANOVA.

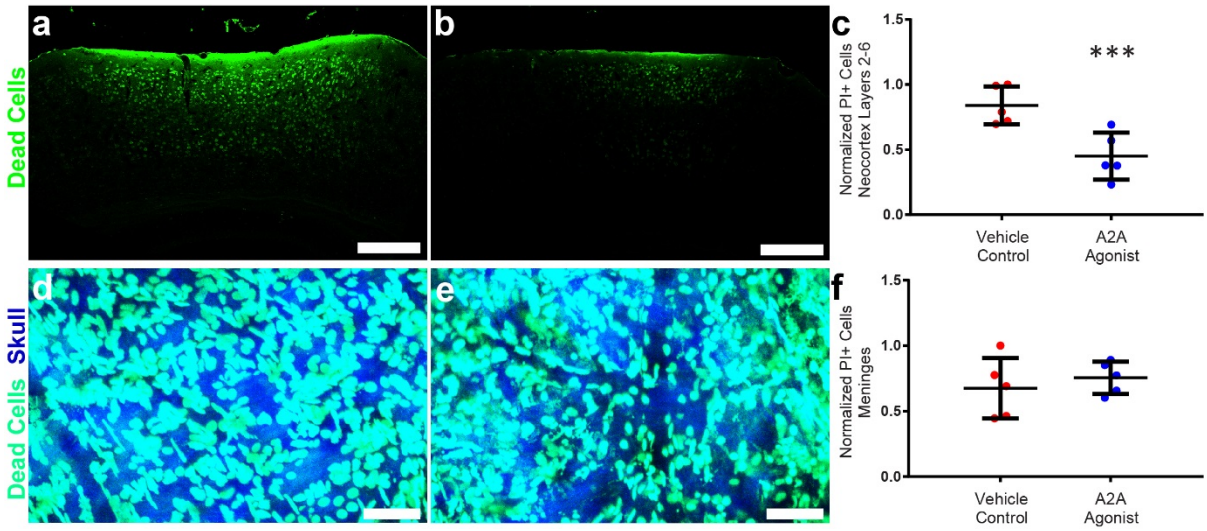

Figure 12. Transcranial application of an A2AR agonist prior to mTBI protects the neocortex, but not the meninges, following mTBI. **(a-c)** Application of an A2AR agonist 30 minutes prior to mTBI, followed by reapplication 15 minutes post-mTBI (**b**) decreases the number of PI+ dead cells (green) in the neocortex through 8 hours after mTBI compared to vehicle-treated controls (**a**). Scale bars, 250 μm. Graph in (**c**) represents 1 independent experiment with n = 6 animals per group, *** $p \leq 0.001$, unpaired t-test. **(d-f)** Pretreatment with an A2AR agonist does not protect the meninges following mTBI ($p = 0.513$). Scale bars, 50 μm. Meningeal data represented in graph in (**f**) correspond to the neocortical data from animals represented in graph (**c**); unpaired t-test.

protect the neocortex cannot prevent such an outcome in the meninges. This may be due to compressive injury resulting in cell death too rapidly for compensation, a difference in the anatomy and molecular pathogenesis driving early death of meningeal cells or absence of the protective pathway in meningeal cells. Our lab previously demonstrated that ROS drive early cell death in the meninges[11], and here we show that A2AR agonists do not impact ROS in the extracellular space. That large amounts of ROS are still detectable in the meninges despite treatment, coupled with the rapid onset of death in the meninges, likely accounts for the observed lack of protection.

Our treatment studies in bone marrow chimeras provide strong evidence that A2AR agonists must act directly on CNS resident cells to exert protection by this mechanism in spite of widespread expression of the A2AR on bone marrow-derived cells[17]. We have previously shown that neutrophils infiltrate the meninges within 1-3 hours following mTBI[11], but that these cells have little effect in the parenchyma and therefore were less likely to be the source of the neuroprotective mechanism in the neocortex. Macrophages also infiltrate the meninges but do not arrive until close to 12 hours following mTBI[34], eliminating these as the responsible cell population in this mechanism. Given the impact of irradiation on microglia[35], we also performed treatment experiments in which we depleted microglia. These experiments provided evidence that any replacement of microglia by peripheral immune cells did not impact the results of the chimera treatment experiments, and showed that A2AR agonists do not require normal numbers of microglia to decrease cell death in the neocortex. Additionally, we detected no change in the number of jellyfish microglia

following mTBI, a morphological and behavioral change resulting from purinergic signaling following the death of glia limitans astrocytes[11]. We did detect a statistically significant difference in the length of the processes on a honeycomb microglia, another morphological change we observe by 1 hour after mTBI that is caused by injury to glia limitans astrocytes [11]. This likely reflects overall decreased cell death due to treatment with A2AR agonists, rather than a direct impact on microglia.

We investigated whether A2AR agonists preferentially protect neurons, which are affected significantly by excitotoxic mechanisms[36] and may result from acute increases in glutamate following brain injury in human patients[37]. We found that there is no difference in the percentage of dead cells represented by neurons regardless of treatment in our model. This suggested that the A2AR agonist mechanism must protect multiple CNS resident cell types and indicated that the mechanism was likely generalized to multiple cell types. These results also made it less likely that control of glutamate levels and abatement of excitotoxicity-induced neuronal death was the mechanism of A2AR agonist-induced protection, and indeed we detected no difference in levels of glutamate with transcranial agonist application. Our results are consistent with previously published work that reported efficacy of A2AR agonists only with relatively low extracellular glutamate[25]. We detect a trend of relatively decreased glutamate 15 minutes following mTBI, our transcranial application start time, and relatively increased glutamate one hour following mTBI, when we see decreased efficacy of transcranial A2AR agonists.

We tested the integrity of the glia limitans and the ROS in the meninges to address mechanisms which might be generalized to multiple cells and that we and

other groups have previously shown to drive pathogenic changes following mTBI[11,38-41], and indeed one of the more promising therapeutic interventions to date targets oxidative mediators[42]. We found no statistical difference between vehicle and A2AR agonist groups in any of these parameters. Lack of protection of the glia limitans substantiates our data showing minimal change in microglial behavior following mTBI, as those changes are a direct result of damage to the glia limitans. Similarly, we would predict protection of the meninges upon agonizing the A2AR if the mechanism involved reduction of ROS, but we do not detect a decrease via intravital imaging, complementing our cell death assay results. An imbalance between ROS and response elements has been demonstrated following moderate TBI, including decreased GSH[43,44], suggesting that neuroprotection might be achieved by alleviating loss of antioxidant capacity. When we assessed whether treatment was increasing GSH in the neocortex following mTBI, we detected no difference in vehicle- or A2AR agonist-treated groups. This indicated that these compounds do not increase GSH and therefore do not increase a principle mechanism of cellular resistance to ROS. However, this also suggests that GSH is not depleted following mTBI in the presence of A2AR agonists, and we therefore surmised that the protective mechanism likely involved maintenance of cellular homeostasis despite an oxidative environment and in the absence of alterations in GSH.

Our data identified a protective mechanism that, upon agonizing the A2AR, signals through PKCδ and Hif-1α and culminates in CAIX, a transmembrane enzyme that converts carbon dioxide to bicarbonate to maintain homeostatic intracellular pH.

An inhibitor to any of these critical steps eliminates the protective effect of a transcranial A2AR agonist when applied simultaneously. This pathway was previously described in a model of hypoxic liver injury[45], but to our knowledge this is the first description of A2AR agonist-mediated upregulation of this pathway in a model of mTBI. Other groups have described neuroprotection upon treatment with an activator of Hif-1α[46], though much like studies of A2AR agonists and antagonists, contrasting evidence suggests beneficial effect with inhibitor treatment[47]. Our results clearly show that CAIX is critical for acute protection of the neocortex by A2AR agonists following mTBI, and that blocking activity of CAIX completely prevents this decrease in neocortical cell death. Intracellular acidification has been implicated in TBI and ischemic stroke[48] and has been hypothesized to contribute to neurodegenerative diseases[49] and secondary mediators of CNS cell death following TBI, ischemic stroke and epileptic seizures[50]. Alterations in intracellular pH can occur rapidly in CNS cells[51] and therefore could be altered by an early intervention. Changes in the extracellular environment that could contribute to intracellular pH alterations have been demonstrated following TBI and are associated with negative outcomes in human patients[52]. Maintenance of homeostatic pH via this pathway has been shown to be critical in prevention of other pathogenic processes resulting in cell death and tissue degeneration[53], suggesting we have uncovered a robust early neuroprotective mechanism that is likely active in preserving multiple CNS resident cells after mTBI.

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CHAPTER 5

Future Directions and Final Conclusions

Future studies should first focus on utilizing cell type-specific A2AR null animals to determine the precise effect of transcranial A2AR agonist application on neurons, astrocytes, microglia, and oligodendrocytes in both naïve mice and mice subjected to our acute mTBI model. In the context of previously published information about this pathway[1], our results strongly suggest that this mechanism is likely active in and protecting multiple cell types rather than acting through a specific resident cell to protect the neocortex. Our data demonstrate that microglia are not required for protection of the neocortex via this mechanism. Cell-type specific A2AR null animals are required to confirm this hypothesis and should target neurons, astrocytes, microglia, and oligodendrocytes. Time constraints precluded inclusion of such experiments in this body of work, but they represent the next logical step in substantiating the mechanism of neocortical protection we have outlined. While we demonstrate that even pretreatment with A2AR agonists does not prevent meningeal cell death in our model, we currently have little information about the effects of agonizing this receptor on individual cell types in the meninges, though one can likely infer immune suppressive effects on macrophages and other immune cells. Information about the effects of agonizing or antagonizing the A2AR on each of these cells types will also benefit the field of purinergic pharmacology, increasing our understanding of adenosine signaling in the brain and providing insights into potential off-target effects when attempting to influence signaling therapeutically. This latter point is particularly important as the field increasingly investigates A2AR

antagonists as potential treatments for neurodegenerative diseases[2-4]. Additionally, elucidation of the signaling changes that cause a temporal shift of A2AR agonism from beneficial to deleterious could shed further light on the molecular pathogenesis of cell death following TBI.

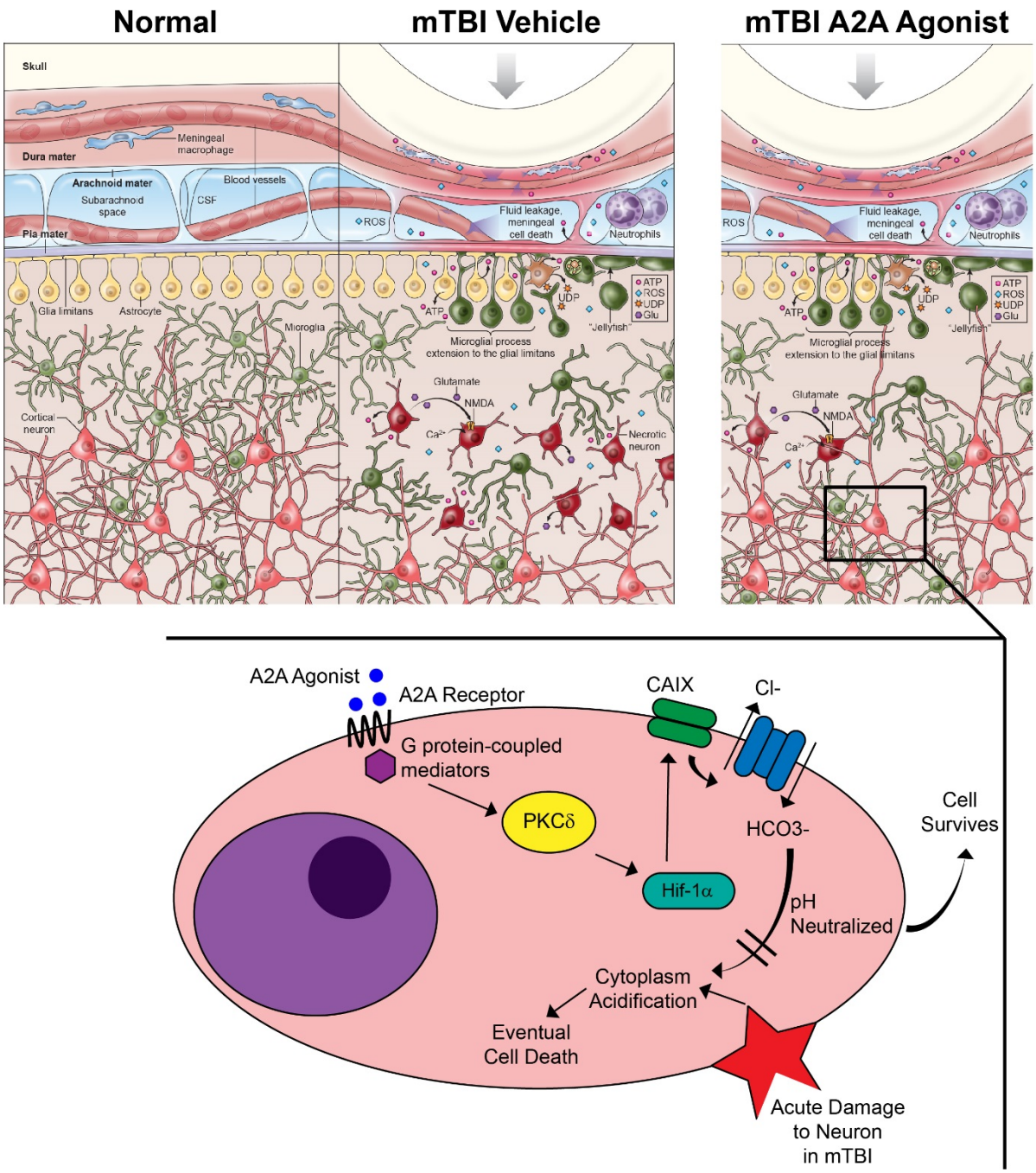
Development of therapies to address both the acute and chronic effects of TBI has been significant challenge to the medical community, necessitating creative approaches and discovery of pathogenic mechanisms. Lessons about the power of cellular pH are found in oncology, where scientists and physicians are investigating carbonic anhydrase inhibitors as adjunct therapy to enhance chemotherapeutic drugs. Numerous types of cancer express high levels of CAIX, which has been shown to impart a survival benefit to neoplastic cells by pH control, frequently rendering oxidative chemotherapeutics ineffective[5]. The mechanism of neocortical protection we describe in our mTBI model represents a novel pathogenic pathway that might be targeted for very early intervention following brain injuries. Development of field-stable methods to deliver adenosine ligands transcranially in humans could remove one barrier to addressing cell death in the brain acutely, preventing ongoing loss of neurons and perhaps preserving the critical brain functions that underlie many long-term sequela of TBI[6]. Work should also be undertaken to determine whether these compounds can be given peripherally at doses high enough to reproduce our neocortical protection results yet remain safe for the cardiovascular system.

An important area that is not addressed in our work is the effect of A2AR agonism on mTBI in female mice. Several groups have reported sex-based

differences in post-TBI outcomes[7,8], and thus reproduction of our work in female mice should be undertaken. We also do not assess whether agonizing the A2AR in young and old mice imparts the same neocortical protection. Children and older individuals are at increased risk of TBI[9] and therefore represent populations that could benefit from therapies based on our work. Assessment of A2AR-driven neocortical protection in these additional groups of mice is important in the context of preclinical drug investigation, and could provide further evidence of therapeutic potential if our positive results generalize regardless of age or sex. Reproduction of our work in other murine models of TBI, particularly those inducing more severe damage than our focal compressive model, could determine if our mechanism is at work in many types of brain injury or only in relatively mild TBI. The addition of transcranial A2AR agonist application in more laboratory animal species would also provide additional data for preclinical therapeutic development. Recent advances in positron emission tomography have allowed imaging of A2AR by radioligand detection, revealing A2AR expression outside the striatum with degenerative and inflammatory neuropathology[10]. To our knowledge, this technology has not yet been applied in TBI patients but could potentially provide another diagnostic and prognostic modality with which to provide better clinical information. A2AR agonists have also been successfully tagged with fluorophores, allowing monitoring of cellular redistribution following ligand binding[11]. This technology could be coupled with preclinical investigations based on our work, both to assess changes in adenosine receptors following TBI in laboratory animals but also potentially to monitor response to acute transcranial A2AR agonism.

In conclusion, we demonstrate that A2AR agonists applied transcranially shortly after mTBI ameliorate neocortical cell death via a signaling pathway linked to maintenance of homeostatic intracellular pH in a mouse model (**Fig. 1**). Further investigation of pathogenesis in models of mTBI are needed to inform development of novel therapies, and our work provides another pathway for additional study in preclinical models and a potentially fruitful new way to approach early intervention following mTBI. To our knowledge, this is the first description of an important role for this signaling pathway in a model of TBI of any severity, and is the second description of the successful use of transcranial compound delivery to address CNS pathology following mTBI in this model[12].

Figure 1. Transcranial A2A Agonism Induces a Neuroprotective State. Despite an inability to alter the cascade of events in the meninges following mTBI, A2A agonists are highly protective in the underlying neocortical parenchyma, preventing most cell death seen in vehicle-treated animals. Following binding to the A2A receptor, an intracellular signaling cascade activates protein kinase c δ (PKCδ), increasing signaling through Hif-1α and upregulating the activity of carbonic anhydrase IX (CAIX). CAIX activates the exchange of chloride ions (Cl-) for bicarbonate (HCO3-), which move into the cytoplasm and neutralize intracellular pH, preventing the eventual cell death that would otherwise follow acidification resulting from injury. Upper panels adapted from Corps et al. *JAMA Neurol* (2015)[13].


Normal
mTBI Vehicle
mTBI A2A Agonist
Skull
Dura mater
Meningeal macrophage
Arachnoid mater
Subarachnoid space
Blood vessels
CSF
Pia mater
ROS
Fluid leakage, meningeal cell death
Neutrophils
ATP
ROS
UDP
Glu
Glia limitans
Astrocyte
Microglia
Microglial process extension to the glial limitans
"Jellyfish"
Glutamate
NMDA
Ca2+
Cortical neuron
Necrotic neuron
A2A Agonist
A2A Receptor
G protein-coupled mediators
PKCδ
CAIX
Cl-
HCO3-
Hif-1α
pH Neutralized
Cell Survives
Cytoplasm Acidification
Eventual Cell Death
Acute Damage to Neuron in mTBI

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APPENDIX

Table 1. Outcomes of Targeting Adenosine Receptors in Sterile CNS Injuries

Receptor	Cerebral Ischemia (Stroke) Models	Effect/Outcome	Ch. 2 References
A1	Global ischemia model in gerbils Middle cerebral artery ischemia mouse and rat models	Decreased susceptibility to oxidative injury Decreased excitotoxicity susceptibility Prevents astrocyte apoptosis	27, 87, 111
A2B	Middle cerebral artery ischemia mouse model	TNF-α–mediated astrocytosis	87
A3	Middle cerebral artery ischemia mouse and rat models	Decreased synaptic activity – lower excitotoxicity susceptibility Microglial activation Low agonist concentrations: promotes survival, resistance to oxidation in astrocytes; better functional outcome (these effects reverse at high agonist concentrations)	29, 87

Table 1 (continued).

Receptor	Cerebral Ischemia (Stroke) Models	Effect/Outcome	Ch. 2 References
A2A	Global ischemia model in gerbils Middle cerebral artery ischemia mouse and rat models	Decreased susceptibility to oxidative injury Reactive astrocytosis upon activation Upregulated on microglia Decreased/increased proinflammatory cytokine production by microglia (reported in same model by different groups) Activation and inactivation reported protective Reduced glutamate release upon inhibition after ischemia Pretreatment with agonists highly protective very acutely	27, 87, 111, 112, 113, 114

Table 1 (continued).

Receptor	Spinal Cord and Traumatic Brain Injury Models	Effect/Outcome	Ch. 2 References
A1	Controlled Cortical Impact *In vitro* rat hippocampus neuron injury model Controlled Cortical Impact Multiple models	Activation: decreased microglial proliferation Agonist: decreased cell death Deficiency: post-traumatic epilepsy Relatively broadly protective, similar to ischemia models	87, 116, 117, 118
A2B	n/a	Increased Interleukin-6 production by astrocytes upon agonist treatment in naïve striatum	115
A3	Controlled Cortical Impact TBI	Similar to ischemia models but minimally studied	87

Table 1 (continued).

Receptor	Spinal Cord and Traumatic Brain Injury Models	Effect/Outcome	Ch. 2 References
A2A	Blast TBI, Controlled Cortical TBI in rodents	Antagonism/Genetic KO: lesion improvement, behavioral improvement at chronic time points	119, 120, 121, 122, 123, 124, 125, 126, 127, 128
	Spinal Cord Impact in rodents	Early agonism, late antagonism: lesion and functional improvement	
	SCI	Agonism: decreased MAPK signaling in oligodendrocytes	
	Controlled Cortical Impact TBI	Genetic KO in peripheral immune cells: lesion improvement	
	Spinal Cord Impact	Global genetic KO with receptor-competent bone marrow: produces lesion different from wild type	
		Genetic KO in spinal cord: protective; activation on peripheral immune cells: lesion improvement	

Table 2. Bone Marrow Chimera Groups for Treatment Studies. Graphical representation of the data described herein is found in **Ch. 4 Fig. 5d and 5h**.

Bone Marrow Donor	Bone Marrow Recipient	Resulting Phenotype	Location of Data	Outcome of Treatment
Balb/cJ CD45.2+	CByJ.SJL(B6)-Ptprca/J CD45.1+	WT-WT	Vehicle **Ch. 4 Fig. 5a**	Control
CByJ.SJL(B6)-Ptprca/J CD45.1+	Balb/cJ CD45.2+	WT-WT	A2AR Agonist **Ch. 4 Fig. 5b**	Neuroprotection
C;129S-*Adora2a*^{tm1Jfc}/J CD45.2+ A2A KO	CByJ.SJL(B6)-Ptprca/J CD45.1+	KO BM	A2AR Agonist **Ch. 4 Fig. 5c**	Neuroprotection
CByJ.SJL(B6)-Ptprca/J CD45.1+	Balb/cJ CD45.2+	WT-WT	Vehicle **Ch. 4 Fig. 5e**	Control
Balb/cJ CD45.2+	CByJ.SJL(B6)-Ptprca/J CD45.1+	WT-WT	A2AR Agonist **Ch. 4 Fig. 5f**	Neuroprotection
CByJ.SJL(B6)-Ptprca/J CD45.1+	C;129S-*Adora2a*^{tm1Jfc}/J CD45.2+ A2A KO	KO Brain	A2AR Agonist **Ch. 4 Fig. 5g**	Comparable to Control

Table 3. Adenosine Receptor Agonists and Antagonists

Compound Name	Target Receptor	Effect of Binding	MW	Ki at Target	Experimental Dose
UK432097	A2A	Agonist	777.89	4 nM	10 μM
BAY 60-6583	A2B	Agonist	379.44	4 nM	10 μM
2-Cl-IB-MECA	A3	Agonist	544.74	0.33 nM	1 μM
2-Chloro-*N*-cyclopentyladenosine	A1	Agonist	378.82	0.8 nM	2 μM
Regadenoson	A2A	Agonist	390.35	290 nM	275 μM
SCH442416	A2A	Antagonist	389.42	4 nM	10 μM
MRS 1754	A2B	Antagonist	486.52	1.97 nM	0.5 μM
MRE 3008F20	A3	Antagonist	399.55	18.9 nM	5 μM
8-cyclopentyl-1,3-dipropylxanthine	A1	Antagonist	304.39	10 nM	1 μM

Table 4. Pathway Inhibition Compounds. Target binding for each compound is expressed either as the binding affinity of the inhibitor for the target (Ki), or as the relative ability of the compound to inhibit the target (IC_{50}). Ki is an intrinsic property of an inhibitor, while IC_{50} is relative to assay conditions. Ki is therefore used when available from the compound manufacturer.

Compound Name	Target	Effect of Binding	MW	Target Binding	Experimental Dose
Rottlerin	Protein Kinase C δ/ε	Inhibition	516.54	IC_{50} = 3-6 μM	50 μM
TAT-cyclo-CLLFVY	Hif-1α Dimerization	Inhibition	2559.1	IC_{50} = 1.3 μM	390.76 μM
S4	Carbonic Anhydrase IX	Inhibition	339.88	Ki = 7 nM	1 μM

Table 5. Statistical Tests, Outcomes and Significance for All Experiments

Experiment	Statistical Test	Result	p value(s)	Location of Data
Source of Neocortical Cell Death	One-way ANOVA	mTBI different from drilling and non-injurious prep	**$p \leq 0.001$**	Chapter 3 Figure 2
A2A Agonist (parenchyma)	Unpaired t-test	A2A agonist different from vehicle in parenchyma	**$p \leq 0.001$**	Chapter 4 Figure 1
A2B Agonist	Unpaired t-test	A2B agonist similar to vehicle	$p = 0.592$	Chapter 4 Figure 1
A3 Agonist	Unpaired t-test	A3 agonist similar to vehicle	$p = 0.076$	Chapter 4 Figure 1
A1 Agonist	Unpaired t-test	A1 agonist similar to vehicle	$p = 0.484$	Chapter 4 Figure 1
A2A Agonist (meninges)	Mann-Whitney Rank Sum (normality fail)	A2A agonist similar to vehicle in meninges	$p = 0.575$	Chapter 4 Figure 1
A2A agonist + A2A antagonist	Kruskal-Wallis One-way ANOVA on Ranks (normality fail)	Agonist + antagonist group similar to vehicle	Vehicle: $p \leq 0.05$ Agonist: $p > 0.05$	Chapter 4 Figure 2
A2A agonist + A2B antagonist	One-way ANOVA	Agonist + antagonist group different from vehicle, similar to agonist alone	Vehicle: $p = 0.605$ Agonist: **$p \leq 0.001$**	Chapter 4 Figure 2
A2A agonist + A3 antagonist	One-way ANOVA	Agonist + antagonist group different from vehicle, similar to agonist alone	Vehicle: $p = 0.292$ Agonist: **$p \leq 0.001$**	Chapter 4 Figure 2
A2A agonist + A1 antagonist	One-way ANOVA	Agonist + antagonist group different from vehicle, similar to agonist alone	Vehicle: $p = 0.953$ Agonist: **$p \leq 0.001$**	Chapter 4 Figure 2
Regadenoson	Mann-Whitney Rank Sum (normality fail)	Regadenoson different from vehicle in parenchyma	**$p \leq 0.001$**	Chapter 4 Figure 2

Table 5 (continued).

Experiment	Statistical Test	Result	p value(s)	Location of Data
A2A Antagonist (parenchyma)	Mann-Whitney Rank Sum (Equal Variance fail)	Antagonist similar to vehicle	p = 0.818	Chapter 4 Figure 3
Balb/cJ A2A agonist	Mann-Whitney Rank Sum (normality fail)	A2A agonist different from vehicle	**$p \leq 0.001$**	Chapter 4 Figure 4
A2A Null A2A agonist	Unpaired t-test	A2A agonist similar to vehicle	p = 0.446	Chapter 4 Figure 4
BM Chimera: KO BM, WT Brain A2A agonist	Two-way ANOVA	No effect of chimerism; all A2A agonist groups different from vehicle	Vehicle vs. KO BM: **$p \leq 0.001$** Agonist vs. KO BM: p = 0.443	Chapter 4 Figure 5
BM Chimera: WT BM, KO Brain A2A agonist	Two-way ANOVA	No effect of chimerism; A2A agonist in KO Brain mice similar to vehicle	Vehicle vs. KO Brain: p = 0.556 Agonist vs. KO Brain: **$p \leq 0.001$**	Chapter 4 Figure 5
Microglia Depletion A2A agonist	Mann-Whitney Rank Sum (Equal Variance fail)	A2A agonist different from vehicle in microglia-depleted mice	**$p \leq 0.001$**	Chapter 4 Figure 6
PI NeuN %	Unpaired t-test	Proportion of A2A agonist PI+ NeuN+ cells similar to proportion of vehicle PI+ NeuN+ cells	p = 0.874	Chapter 4 Figure 6

Table 5 (continued).

Experiment	Statistical Test	Result	p value(s)	Location of Data
Microglia Jellyfish	Mann-Whitney Rank Sum (normality fail)	Number of jellyfish in A2A agonist similar to number of jellyfish in vehicle	p = 0.397	Chapter 4 Figure 6
Microglia Honeycombs	Mann-Whitney Rank Sum (normality fail)	Length of A2A agonist honeycomb processes different from vehicle honeycomb process length	p = 0.034	Chapter 4 Figure 6
Glia Limitans Leakage	Unpaired t-test	SR101 fluorescence is similar in A2A agonist and vehicle	p = 0.369	Chapter 4 Figure 7
Amplex Red ROS	Mann-Whitney Rank Sum (normality fail)	Amplex Red fluorescence similar in A2A agonist and vehicle	p = 0.746	Chapter 4 Figure 7
GSH (ng)	Two-way ANOVA	No effect of naïve vs. mTBI; A2A agonist similar to vehicle	p = 0.809	Chapter 4 Figure 7
Glutamate (μg)	Two-way ANOVA or ANOVA on Ranks (Equal Variance fail)	Effect of time in naïve mice 15 min vs. 1 hour; no effect of naïve vs. mTBI at any time point; A2A agonist similar to vehicle	Naïve 15 min vs. 1 hr: **p = 0.016** Naïve vs. mTBI vehicle, all time points: p = 0.7 Vehicle vs. Agonist 15 min: p = 0.159 1 hour: p = 0.064 3 hours: p = 0.929 6 hours: p = 0.432	Chapter 4 Figure 7

Table 5 (continued).

Experiment	Statistical Test	Result	p value(s)	Location of Data
A2A agonist + PKC δ Inhibitor	One-way ANOVA	A2A agonist different from all other groups, Agonist + Inhibitor similar to vehicle and Inhibitor alone	Agonist vs. other groups: **$p \leq 0.001$** Vehicle vs. Co-treatment: $p = 0.118$ Vehicle vs. Inhibitor: $p = 0.154$ Inhibitor vs. Co-treatment: $p = 0.862$	Chapter 4 Figure 8
A2A agonist + Hif-1α Inhibitor	One-way ANOVA	A2A agonist different from all other groups, Agonist + Inhibitor similar to vehicle and Inhibitor alone	Agonist vs. other groups: **$p \leq 0.006$** Vehicle vs. Co-treatment: $p = 0.259$ Vehicle vs. Inhibitor: $p = 0.960$ Inhibitor vs. Co-treatment: $p = 0.280$	Chapter 4 Figure 8

Table 5 (continued).

Experiment	Statistical Test	Result	p value(s)	Location of Data
A2A agonist + CAIX Inhibitor	One-way ANOVA	A2A agonist different from all other groups, Agonist + Inhibitor similar to vehicle and Inhibitor alone	Agonist vs. other groups: **$p \leq 0.001$** Vehicle vs. Co-treatment: $p = 0.878$ Vehicle vs. Inhibitor: $p = 0.870$ Inhibitor vs. Co-treatment: $p = 0.991$	Chapter 4 Figure 8
A2A agonist 15 min vs. 1 hour post-mTBI	One-way ANOVA	Early agonist different from other groups; late treatment with agonist similar to vehicle	Early Agonist vs. other groups: **$p \leq 0.001$** Vehicle vs. late Agonist: $p = 0.824$	Chapter 5 Figure 1
A2A antagonist 15 min vs. 1 hour post-mTBI	One-way ANOVA	All groups similar	$p = 0.910$	Chapter 5 Figure 1
Pre-treatment A2A agonist (parenchyma)	Unpaired t-test	A2A agonist different from vehicle in parenchyma	**$p \leq 0.001$**	Chapter 5 Figure 2
Pre-treatment A2A agonist (meninges)	Unpaired t-test	A2A agonist similar to vehicle in meninges	$p = 0.513$	Chapter 5 Figure 2