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Blood-brain barrier dysfunction as a physiological trigger of cellular senescence

By

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A dissertation submitted in partial satisfaction of the

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in

Molecular and Cell Biology

in the

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of the

University of California, Berkeley

Committee in charge:

Professor Daniela Kaufer, Co-Chair

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Abstract

Blood-brain barrier dysfunction as a physiological trigger of cellular senescence

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Daniela Kaufer, Co-Chair

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Blood-brain barrier dysfunction (BBBD) and accumulation of senescent astrocytes occur in the hippocampus during brain aging and contribute to cognitive decline, neural dysfunction, and neurodegeneration. In this work, I advance our understanding of the relationship between these events. In chapter one, I describe the vital role astrocytes play in maintaining brain homeostasis and the various functions they perform in the healthy brain. I also summarize known mechanisms by which astrocytes mediate neuropathology. Given the salience of transforming growth factor beta 1 (TGF β) signaling in aging phenotypes and neural dysfunction, I conclude that BBBD-induced TGF β hyperactivation should be explored as a potential mechanistic link between BBBD and senescence in the brain.

In chapter two, I test the hypothesis that chronic exposure to BBBD-associated serum albumin can induce stress-induced premature senescence (SIPS) in astrocytes via TGF β hyperactivation. To this end, I utilized an *in vivo* model of BBBD in which albumin was continuously infused into the lateral ventricles of adult mice. Prolonged albumin exposure significantly increased TGF β activation and the burden of senescent astrocytes in hippocampal tissue. Pharmacological inhibition of the type I TGF β receptor (TGF β R) ALK5 or conditional genetic knockdown of astrocytic TGF β R prior to albumin infusion was sufficient to prevent albumin-induced astrocyte senescence.

Since I observed TGF β -dependent astrocyte senescence in the albumin infusion model of BBBD, I sought to determine whether senescence was also implicated in the etiology of repetitive mild traumatic brain injury (rmTBI). In chapter 3, I utilized a novel closed-head injury model of rmTBI in which a single injury results in brief loss of consciousness and acute transient neurological symptoms. Five days of injuries resulted in diffuse BBBD and increased activation of TGF β signaling and senescence markers that were associated with behavioral disinhibition. rmTBI mice also experienced enduring deficits in learning and memory retention.

Together, these results establish a link between TGF β signaling activation and cellular senescence and suggest that BBBD can trigger these phenotypic changes. In chapter 4, I conclude by offering my perspectives on BBBD, TGF β signaling, and cellular senescence in neuropathology, and provide future directions for inquiry in the field.

Dedication

To everyone who invested in me and supported my development; I am eternally grateful.

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Chapter 1. Introduction

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1.1. Preface

Age-onset neurodegenerative disorders such as Alzheimer's disease (AD) and related dementias (ADRD) are debilitating conditions that progressively impair the memory, cognition, and daily functioning of afflicted individuals. As the average lifespan has increased in the west, so has the prevalence of symptomatic AD. An estimated 6.2 million adults aged 65 and older are living with Alzheimer's dementia, presenting a substantial societal burden [1]. Yet, despite decades of research, negligible progress has been made in developing effective disease-modifying treatments capable of halting or reversing AD progression and associated cognitive decline. The unsatisfactory clinical trial outcomes of experimental AD therapies indicate a need for a greater understanding of the age-related processes that drive neurodegenerative diseases.

In recent years, increased focus has been placed on understanding the role of astrocytes in driving such neurological disorders. As the most abundant cell type in the adult brain, astrocytes form a sophisticated tissue-wide signaling network responsible for maintaining brain homeostasis and regulating various brain activities. Consequently, there are many potential mechanisms by which astrocytes may contribute to neural deficits in the context of advanced age and disease. Two such mechanisms are blood-brain barrier dysfunction (BBBD) and cellular senescence. Both progressively increase during aging and are associated with increased risk of neurodegenerative disease, but the relationship between the two, if any, has not yet been established. Here, we discuss the causes and consequences of BBBD and senescence with respect to astrocytes and identify future areas of investigation in the field.

1.2. Astrocyte functions

Astrocytes are stellated glial cells that interface with nearly every functional element of the brain. They are the most abundant glial cells in the brain and can comprise up to 50% of the tissue volume in some regions [2]. Their branch-like processes surround neurons, axons, synapses, and blood vessels, and perform numerous functions that are essential for brain homeostasis and neural functioning [3]. As such, astrocytes play a pivotal role in preserving brain health, as well as driving pathogenic processes [4,5]. Our understanding of astrocyte functions is continually growing as researchers study this heterogeneous population of cells under different conditions, and it is increasingly evident that astrocytes perform unique functions depending on their temporal and regional location within the brain [6–9]. Here, we will briefly review the astrocyte functions related to neuronal support, immune modulation, and regulation of the blood-brain barrier (BBB), as three factors that go awry in aging and disease.

1.2.1. Neuronal support

At the macrostructural level, astrocytes help form a functional glial network that extends from the ependyma to the pial surface via gap junctions [10,11]. The perivascular feet of astrocytes associate with the parenchymal basal lamina to form a plexus called the glia limitans. This thin but

dense structure surrounds the pia mater, subpial space, and perivascular spaces, and plays an essential role in controlling the movement of substances from the blood or cerebrospinal fluid (CSF) into the brain parenchyma where neurons are located [12].

In addition to their role in barrier function, astrocytes maintain the optimal conditions required for neurotransmission within the cerebral microenvironment. To transmit a signal, neurotransmitters are released from an axon terminal into the synaptic cleft, where they interact with post-synaptic receptors. Ending the transmission requires neurotransmitter uptake from the synaptic cleft by neurons and astrocytes. While neurons primarily uptake the inhibitory transmitter gamma-amino butyric acid (GABA), astrocytes are responsible for the uptake and metabolism of the excitatory amino acid glutamate [13–15]. Additionally, the propagation of nerve impulses involves cellular depolarization, which causes local extracellular changes in ion concentration. Astrocytes contain ion channels, enzymes, and receptors that enable them to modify extracellular ion concentrations and pH following depolarization to restore the surrounding milieu to its resting state [16–19]. Astrocytes are also critical in removing harmful metabolites and waste products from the brain. They can directly metabolize some soluble waste products like ammonia; alternatively, they collect and shuttle unwanted metabolites and soluble proteins such as amyloid beta ($A\beta$) to the vasculature for elimination via the glymphatic system [15,20,21].

Besides maintaining the cerebral microenvironment, astrocytes also participate in neurotransmission and are critical in shaping the complex circuitry of the brain [22]. Astrocytes form tripartite complexes with presynaptic and postsynaptic nerve terminals whereby they help define synaptic connections. Astrocytes are the major source of extracellular matrix proteins, cell adhesion molecules, and neurotrophic factors in the central nervous system (CNS), which are essential in promoting neurite growth and elongation [23–27]. Additionally, astrocytes physically associate with neuronal synapses via perisynaptic astrocytic processes (PAPs) that regulate many aspects of their formation, maturation, and function [22,28]. For example, astrocytes regulate synaptogenesis via secretion of thrombospondins (TSP) [29] and TGF β 1 [30,31], and can specifically control the maturation and plasticity of certain circuits via secretion of Hevin and SPARC [32,33].

1.2.2. Immune modulation

Compared to other tissues, the brain is a relatively immune-privileged site because it lacks a significant resident lymphoid population, and the BBB substantially restricts entry of circulating immune cells into the CNS. Astrocytes are not immune cells *per se*, but are capable of many immune functions, including phagocytosis, antigen presentation, and facilitating immune cell trafficking [34,35]. As such, astrocytes may produce pro- or anti-inflammatory cytokines such as IL-1 β , IL-6, TNF, IL-10, IL-27, and TGF- β in response to disease, stress, or injury [34,36]. Within the CNS, they communicate bidirectionally with microglia to coordinate a defense response [37]. Along with microglia, astrocytes phagocytose neuronal material including synapses, apoptotic neurons, and degenerating axons, as well as toxic proteins such as $A\beta$ plaques in AD and α -synuclein in Parkinson's disease [38,39]. Consistent with their ability to present antigens, astrocytes express major histocompatibility complex (MHC) antigens that are upregulated in response to disease [40]. For example, high levels of astrocytic MHC-II were found in the brains of patients with Parkinson's disease (PD), which correlated with the load of pathological, phosphorylated alpha synuclein (α SYN) [41]. Notably, perivascular and infiltrated CD4 $^{+}$ T cells were surrounded by MHC-II expressing astrocytes, indicating astrocyte-T cell cross-talk in the PD

brain [41]. Astrocytic MHC-I is upregulated during aging, which appears to be protective since it is associated with preserved cognitive function [42].

Due to their proximity to blood vessels, astrocytes play a critical role in mediating reciprocal communication between CNS-resident cells and the immune system. Depending on the subsets involved, astrocytes respond to T cell signals to either boost or limit CNS inflammation. For instance, pathogenic Th17 cells signal to astrocytes via GM-CSF and IL-17 to boost neurotoxic astrogliosis [43–45]; yet, FOXP3+ regulatory T cells secrete amphiregulin to suppress astrogliosis and promote recovery after ischemic stroke [46]. Conversely, they can also produce chemokines such as CCL2, CXCL10, and CXCL12 involved in leukocyte recruitment into the CNS [47]. During aging, there is an increase in production of astrocytic CXCL10, which serves as a chemoattractant for peripheral immune cells and aids in T cell adhesion to endothelial cells [48]. In summary, astrocytes are critical regulators of immune responses in the CNS, as they may promote or dampen neuronal damage and inflammation depending on the context and stimuli.

1.2.3. Blood-brain barrier regulation and maintenance

The BBB is a highly selective semipermeable barrier that restricts entry of blood cells and plasma components into the brain parenchyma, facilitates influx of essential nutrients, and mediates efflux of neurotoxic products. Together, these tasks maintain an optimal environment for neuronal survival and function. The anatomical BBB consists of a continuous monolayer of endothelial cells (ECs) connected by tight junctions (TJ) and adherens junctions (containing claudin, occludin, and zonula occludens proteins). TJ proteins restrict paracellular permeability and also segregate the apical and basal domains of the cell membrane, which enables endothelial polarization [49]. Regulation of the BBB is accomplished by the neurovascular unit (NVU), a multicellular unit that functionally connects the brain and the cerebral vasculature. The NVU is composed of specialized ECs, pericytes, and perivascular astrocytes, whose end-feet sheath all cerebral blood vessels [50].

In addition to the paracellular pathway, there are several transcellular pathways across the BBB that are carefully regulated by the NVU. Due to large surface area of lipophilic membranes in ECs, small gaseous molecules such as O₂ and CO₂ and small lipid-soluble agents can diffuse freely through the endothelium. Specialized EC transporter proteins such as glucose transporter 1 (GLUT1) and L-type amino acid transporter 1 (LAT1) supply the brain with glucose and amino acids, respectively. Additional transporters exist that supply the brain with nucleosides, nucleobases, and other substances [51]. Some transporters such as P-glycoprotein (Pgp) are energy-dependent and act as efflux transporters for neurotoxic molecules [52]. Other proteins such as insulin and transferrin are taken up by ECs via receptor-mediated endocytosis and then transported across the BBB in vesicles in a process called receptor-mediated transcytosis (RMT) [53]. Native plasma proteins such as albumin are typically excluded from the adult healthy brain, but can cross the BBB via adsorptive-mediated transcytosis (AMT) under specific conditions [54]. AMT is also vesicle-mediated but involves nonspecific binding to the membrane surface charges before internalization and transport through EC cell bodies.

The perivascular end feet of astrocytes show several specialized features, including a high density of K⁺ transporters and aquaporin (AQP4) water channels, which are involved in ion recycling and brain volume regulation, respectively [55]. Through a combination of cell-cell interactions and soluble factors, perivascular astrocytes regulate the expression of TJ proteins and directly modify the transport properties of the cerebral endothelium [55–57]. Compared with ECs

cultured alone, ECs co-cultured with astrocytes or astrocyte-conditioned media exhibit increased TJ formation, transporter expression, and overall improved barrier function [58]. Subsequent studies have identified the molecular mechanisms responsible for astrocytic regulation of the BBB. For example, astrocyte secretion of factors such as angiopoietin 1 (ANG1) and sonic hedgehog (SHH) cause ECs to upregulate TJ proteins, thus enhancing barrier tightness [59–61].

Beyond BBB regulation, a recent study showed that astrocytes contribute a necessary and nonredundant function in adult BBB maintenance [62]. The study used a genetic DTA ablation system to conditionally and selectively ablate astrocytes from the adult mouse brain and observed significant BBBD, indicated by leakage of Cadaverine (~900 Da) and fibrinogen (340 kDa) into the parenchyma. Blood vessels within the regions of astrocyte loss had lower expression of the TJ protein zonula occludens-1 (ZO-1), while expression of the endothelial transporter GLUT1 remained undisturbed. BBBD persisted for several weeks following ablation, suggesting a lack of barrier repair [62].

Other roles of astrocytes in BBB function have been studied in the context of disease. For example, the E4 variant of apolipoprotein E (APOE), the main susceptibility gene for AD, leads to accelerated BBBD and cognitive decline in humans and animals [63,64]. APOE is primarily synthesized and secreted in the CNS by astrocytes and is required for BBB formation and maintenance [65]. A recent study used allele-specific knock-in mice of the human E4, E3, and E2 APOE variants and showed that humanized APOE4, but not APOE2 or APOE3, mice exhibited BBBD, increased matrix metalloproteinases-9 (MMP9), impaired TJs, and reduced astrocyte end-foot coverage of blood vessels [66]. This provides a seminal example of how astrocyte dysfunction can directly lead to BBBD and subsequent neurological disease. Additional mechanisms by which astrocytes contribute to BBBD in aging and disease continue to be explored.

1.3. Mechanisms of astrocyte-mediated neuropathology in the aging brain

Brain aging consists of a set of physiological and cellular changes that result in reduced neural function. Salient changes associated with aging include significant decreases in certain neuronal populations, dendritic spines, axonal arborization, post-synaptic density, and cortical volume [67]. Together, these physiological changes result in cognitive impairment and memory loss. Given the many diverse functions astrocytes perform in the brain, it's logical that astrocyte dysfunction would result in neural deficits and neuropathology. Thus, there is substantial interest in understanding the precise role of astrocytes in age-related neural dysfunction, especially in neurodegenerative diseases. Here, we will discuss two potentially related mechanisms by which astrocytes contribute to neuropathology in aging: blood-brain barrier dysfunction (BBBD) and cellular senescence.

1.3.1. TGF β hyperactivation following blood-brain barrier dysfunction (BBBD)

One of the physiological consequences of advanced age is the progressive decline in NVU function, which results in increased BBB permeability and leakage of blood-borne molecules into the neural microenvironment. Clinical studies have shown that BBBD is prevalent in aging individuals [68–70] and is correlated with neurodegenerative diseases, especially ADRD [71–74]. The BBBD phenotype is multifaceted and adversely impacts nearly every element of the brain. The endothelium in BBBD is characterized by focal degeneration [75], decreased expression of TJ proteins [76,77], increased EC pinocytosis [78,79], and decreased expression of GLUT1 [80] and

Pgp [81] transporter proteins. Decreased GLUT1 expression in BBBD impairs influx of glucose, and decreased Pgp expression impairs efflux of neurotoxic molecules such as A β , allowing them to accumulate in the brain tissue where they contribute to inflammation and neural degeneration [81–83]. Pericytes and astrocytes have reduced vascular coverage in BBBD, and astrocytes exhibit downregulation of AQP4, the protein primarily responsible for end-foot adhesion and polarization at the vascular wall [84–88]. Leakage of neurotoxic blood-derived proteins into the parenchyma triggers microglial activation and astrogliosis as an injury response, contributing to neuroinflammation [89–91]. In the short term, this inflammation triggers microglia to migrate to damaged vessels and maintain the BBB via expression of the TJ protein Claudin-5; however, during sustained inflammation, microglia phagocytose astrocytic end-feet, further impairing BBB integrity [92]. In addition to leakage caused by loss of BBB integrity, recent evidence suggests there is a global age-related shift in BBB transcytosis mechanisms that further increases the influx of neurotoxic proteins such as albumin, fibrinogen, and autoantibodies into the aging brain [88]. Specifically, the aging BBB exhibits diminished RMT transport and increased non-specific transport of macromolecules compared to young brains. Regardless of the mechanism of entry, the dysregulated presence of serum proteins causes astrogliosis and neuroinflammation, and the dysfunctional phenotype of NVU components in BBBD creates a hostile environment for neurons. As a result, white matter, neuronal axons, and synapses are compromised in BBBD, leading to cognitive impairment [75].

The hippocampus, the brain region's center for learning and memory, appears to be especially vulnerable to age-related BBBD. Hippocampal BBBD is associated with mild cognitive impairment (MCI) and occurs before tissue atrophy and dementia in AD [68,93]. Notably, hippocampal neural hyperexcitability is also an early biomarker of MCI in humans that precedes dementia [94,95] and is associated with disease progression in rodent AD models [96,97]. These findings suggest there is a relationship between BBBD and neural hyperexcitability in aging. Indeed, a recent study from our group identified astrocytic TGF β signaling as a mechanistic link between BBBD and hyperexcitability in the aging hippocampus and established how these factors contribute to cognitive impairment [70,98]. This study was based on the observation that the context and symptomatology of age-related neural dysfunction resemble those observed in traumatic brain injury (TBI). Both aging and TBI involve a period of BBBD followed by secondary neural dysfunction and are associated with increased risk of neurodegenerative disease and dementia [71,99]. In both cases, the neural dysfunction is characterized by excitation-inhibition (E/I) imbalance, hippocampal and cortical circuit hyperexcitability, and cognitive deficits [70,98].

TBI causes sudden and pronounced BBBD which can persist for months to years after the initial injury [100–102]. BBBD enables blood-derived serum albumin and fibrinogen molecules to extravasate into the brain, where they elicit a robust inflammatory response mediated by astrocytic TGF β signaling [103–105]. Albumin binds to the type II TGF β receptor (TGF β R) on astrocytes and activates the canonical ALK5 type I TGF β R after receptor dimerization. This results in phosphorylation of the downstream effector protein SMAD2 (pSMAD2) and subsequent activation of pro-inflammatory and epileptogenic transcriptional programs [106–108]. Molecular hallmarks of this altered astrocytic phenotype are upregulation of the cytoskeletal intermediate filaments GFAP and vimentin as well as S100 calcium-binding proteins. The astrocytic inflammatory program involves production of cytokines such as interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1; CCL2), and matrix metalloproteinase 9 (MMP-9), which degrades critical extracellular structures and destabilizes perineuronal nets (PNNs) around inhibitory interneurons [103,107]. The astrocytic epileptogenic program features

upregulation of excitatory glutamate receptors as well as downregulation of voltage-gated potassium channels, plasticity-associated NMDA receptors, and genes involved in inhibitory GABAergic transmission [103,107]. Notably, albumin-induced TGF β activation itself upregulates astrocytic expression of TGF β and its receptors, resulting in a positive feedback loop that can cause the altered phenotype to persist [70,106]. In this state, the normal homeostatic neurotransmitter recycling and ion buffering functions of astrocytes are impaired, leading to disrupted neural communication. Remodeling of neural circuits occurs over time, which causes network hyperexcitability, E/I imbalance, and cognitive deficits [104,108–111]. Furthermore, astrocytic secretion of TGF β increases neuronal expression of c1q [112], a complement protein which and mediates synapse elimination [113] and increases with age in the CNS [114].

In the case of aging, the onset of BBBD is more gradual than in TBI, but also temporally constrained. Depending on an individual's lifestyle and risk factors, BBBD begins as early as middle age in the hippocampus and progresses in scope and severity into advanced age [68,70,72,73]. Interestingly, our study found that albumin-induced astrocytic TGF β activation is a mechanism underpinning key neural deficits in aging [70]. Immunohistochemistry (IHC) revealed that albumin begins accumulating in hippocampal astrocytes as early as 12 months ("middle age") in mice and is consistently elevated in aging up to end of life at ~2 years, indicating BBBD. Concurrent with the time course of BBBD, aged mice showed a progressive increase in the amount of pSMAD2 colocalized with albumin-positive hippocampal astrocytes, indicating BBBD-induced TGF β signaling activation. Additionally, Western blot analysis showed increased concentration of active TGF β 1 (a positive feedback output of the TGF β pathway) in the hippocampi of aged mice. Compared to young mice, aged mice exhibited increased seizure vulnerability and paroxysmal slow wave events (PSWEs), indicating increased neural hyperexcitability. Activation of TGF β signaling via serum albumin infusion into the cerebral ventricles of young mice was sufficient to elicit increased hyperexcitability, aberrant neural activity, and learning impairment as measured by the Morris Water Maze assay. Pharmacological inhibition of the ALK5 type I TGF β R with the small molecule drug IPW-5371 significantly reduced TGF β activation and seizure vulnerability in young mice infused with albumin. Importantly, conditional knockdown of the type II astrocytic TGF β R and systemic treatment with IPW were both able to reverse the BBBD-induced neuropathological changes observed in aged mice, providing further evidence for the mechanistic role of TGF β activation in driving these changes [70,98].

1.3.2. Cellular senescence

Another consequence of advanced age is the accumulation of senescent cells [115–120]. Cellular senescence is an important anti-cancer mechanism that occurs in replication-competent cell types. The senescence response permanently arrests cell growth in response to stresses that could promote malignant transformation [121]. There are two main types of senescence; replicative senescence is triggered by extreme telomere erosion caused by repeated cell division [122], while stress-induced premature senescence (SIPS) is triggered by sublethal levels of cellular stress in the absence of telomere erosion. A common trigger of SIPS is DNA damage, which can be caused by factors such as environmental toxins, viral infection, and ionizing radiation [123–125]. Other stressors can trigger SIPS by activating molecular pathways that are associated with cellular transformation. For example, epigenetic factors, mitochondrial dysfunction, disrupted nutrient signaling, chronic inflammation, oxidative stress, and proteotoxic stress can also cause SIPS [126]. The senescence response ultimately activates the p53/p21^{WAF1} and p16^{INK4A}/pRB

tumor suppressor pathways, which establish and maintain growth arrest [123,127–129]. The senescent phenotype is not precisely defined and differs among cell types and conditions, but is generally characterized by permanent cell cycle arrest, increased lysosomal mass, loss of nuclear integrity, decreased functional capacity, and expression of the senescence-associated secretory phenotype (SASP) [115,130–133].

Senescent cells accumulate with age in a variety of tissues and contribute to age-associated tissue dysfunction and disease. A seminal study in the field showed that senescent cells accumulate in a progeroid mouse model of accelerated aging, and that elimination of senescent cells via a drug-inducible transgene extends lifespan and prevents the onset of aging phenotypes including cataracts, sarcopenia, and subcutaneous fat loss [134]. There are two known mechanisms by which senescent cells drive tissue aging and dysfunction. The first is through loss of function, in which senescent cells have reduced ability to perform their vital activities within the tissue. The second is through the SASP, in which senescent cells secrete inflammatory cytokines, chemokines, growth factors, and proteases to communicate cellular damage to the surrounding cells [126,135,136]. Many SASP factors are considered beneficial in limited quantities because they stimulate tissue repair and increase immune surveillance; however, their abundance in aging tissue has been shown to impair tissue structure and function by disrupting intercellular signaling and maintaining a pro-inflammatory environment [135,137–140].

While normally quiescent in the adult brain, astrocytes maintain their ability to replicate and thus are susceptible to SIPS [141]. Astrocytic SIPS has been observed in response to oxidative stress [142], proteasome inhibition [142], HIV infection [143], methamphetamine [143], A β oligomers [144], and the environmental toxin paraquat [145]. Human and animal studies have demonstrated that senescent cells accumulate in the aging CNS and are associated with neuroinflammation and neurodegeneration [146–150]. In human brain samples, the prevalence of senescent astrocytes in the brain was shown to increase with age, with a significantly higher burden observed in the cortices of AD patients compared to age-matched controls [144]. Recent studies in animal models have demonstrated a causal relationship between glial senescence and neurodegeneration. Accumulation of senescent astrocytes and microglia were found in the cortices and hippocampi of a mouse model of tau-dependent pathology, and genetic ablation of these cells reduced tau deposition and prevented neuronal degeneration [151]. Similarly, senescent astrocytes were found in abundance in the substantia nigra of a mouse model of paraquat-induced Parkinson's disease, and their selective genetic ablation was sufficient to reduce nigral dopaminergic cell loss and improve motor function [145]. Notably, novel pharmacological agents have been developed that can selectively eliminate senescent cells, and are being explored in clinical trials for the treatment of age-related diseases [152–154]. These findings indicate that senescent astrocytes actively participate in driving age-associated neural dysfunction and pathology, including neurodegeneration, and can be leveraged as a therapeutic target.

Like other senescent cell types, senescent astrocytes appear to drive neural dysfunction via both loss of function and their SASP. Given the important role of astrocytes in supporting brain homeostasis and maintaining the neural microenvironment, senescence-associated loss of astrocyte function is especially detrimental to neuronal survival and performance [155]. Indeed, substantial impairments have been reported in several astrocyte functions, including impaired wound healing ability, phagocytic uptake, metabolic function, and neuroprotective capacity [156,157]. Further, downregulation of potassium and glutamate transporters in senescent astrocytes promotes neurotoxicity in cortical neurons [158]. As a heterogeneous population, it is likely that other specialized astrocyte functions are also impaired that have not yet been identified.

Additionally, the astrocytic SASP participates in driving pathological changes in the aging brain by generating a chronic inflammatory environment. Senescent astrocytes secrete inflammatory and proteolytic SASP factors such as IL-6, IL-8, IL-1 β , MMP-3, and MMP-9 [144,145,151,159,160]. Interestingly, these factors are elevated in the cerebrospinal fluid (CSF) and serum of AD patients, suggesting that senescence-associated inflammation accompany and may contribute to AD progression [161,162]. In particular, IL-6 is a salient SASP component that is a biomarker of AD [163] and whose overexpression drives neurodegeneration in AD models [164–166].

1.4. Summary

As detailed above, a growing body of evidence suggests that both BBBB and astrocyte senescence progressively increase in the aging brain and are independently associated with increased risk of neurodegenerative disease, but the relationship between the two has not been established. Given the salience of TGF β signaling activation in aging phenotypes and neural dysfunction, BBBB-induced TGF β hyperactivation should be explored as a potential mechanistic link between BBBB and senescence in the aging brain.

Chapter 2. Blood-brain barrier dysfunction promotes astrocyte senescence through albumin-induced TGF β signaling activation

Adapted from: Preininger, M.K., Zaytseva, D., Lin, J.M., Kaufer, D. (2022) Blood-brain barrier dysfunction promotes astrocyte senescence through TGF β signaling activation. Manuscript in preparation.

2.1. Introduction

Advanced age is a primary risk factor for neurodegenerative disorders that impair cognitive function such as dementia and Alzheimer's disease (AD) and related dementia (ADRD). Yet, the mechanisms by which advanced age facilitates neuropathology remain elusive. One hypothesis suggests that an age-related increase in physiological and environmental stress induces stress-induced premature senescence (SIPS) in astrocytes, which in turn contributes to the neuronal dysfunction and neuroinflammation associated with age-onset neurodegenerative disorders [142,144,147,167–169]. Cellular senescence is a process that occurs in response to exhausted replication or sublethal levels of cellular stress [126]. The senescent phenotype is generally characterized by cell cycle arrest, increased lysosomal mass, loss of nuclear integrity, decreased functional capacity, and expression of a senescent-associated secretory phenotype (SASP) [130,132]. The prevalence of senescent astrocytes in the brain increases with age, with a significantly higher burden observed in the cortices of patients with AD compared to age-matched controls [144]. Recent evidence from mouse models of tau pathology [151] and Parkinson's Disease [145] support the senescence hypothesis, linking increased astrocyte senescence with neuroinflammation and neurodegeneration. These studies demonstrate that periodically clearing senescent glial cells from the nervous system with targeted interventions can prevent the initiation and progression of disease. While the role of these cells in disease continues to be explored, it is important to identify key physiological and environmental triggers of astrocyte senescence.

One potential physiological trigger of astrocyte senescence is age-associated BBBD and subsequent TGF β signaling activation. BBBD enables blood-borne proteins such as serum albumin [70,103,106,108] and fibrinogen [105] to extravasate into the brain parenchyma, where they elicit a robust inflammatory response by activating canonical TGF β signaling. In astrocytes, albumin binds to the type II TGF β R, which dimerizes and subsequently activates the type I TGF β R ALK5 before undergoing receptor-mediated endocytosis. This leads to the phosphorylation of the downstream effector protein SMAD2 and subsequent activation of pro-inflammatory and epileptogenic transcriptional programs [103,104,106,107]. Additionally, albumin-induced TGF β signaling activation upregulates astrocytic expression of TGF β and its receptors, resulting in a positive feedback loop that can lead to chronic neurological symptoms [106]. Increased BBBD and concomitant albumin leakage has been documented in aging and negatively impacts brain health and function [70,72,170,171]. BBBD in the hippocampus, the brain's center for learning and memory, is especially consequential. BBBD has been observed in the hippocampus of individuals with mild cognitive impairment (MCI) [68,172] and occurs before hippocampal atrophy in AD [74,173], suggesting hippocampal BBBD may be critical early step towards neurodegeneration [174].

Notably, studies have demonstrated that TGF β activation mediates senescence in various cell types *in vitro*, including fibroblasts [175–177], keratinocytes [178], mesenchymal stem cells (MSCs) [179], glioblastoma cells [180], cardiomyocytes [181], and lung epithelial cells [182].

Also, TGF β 1 as a SASP component was found to be an important mediator of paracrine senescence *in vivo* [183]. Thus, we hypothesized that BBBD-induced TGF β hyperactivation in the brain may play a role in promoting astrocyte senescence. While previous studies have identified the effects of BBBD on astrocyte activation and neurological impairment [70,103,106,107], this is the first study to explore its effects on astrocyte senescence. In this study, we sought evidence for a paradigm that integrates BBBD, TGF β hyperactivation, and senescence—three factors that are consistently associated with age-related neurological disorders. Specifically, we tested the hypothesis that prolonged exposure to the blood serum-derived protein albumin promotes hippocampal astrocyte senescence in a TGF β -dependent manner.

2.2. Materials and methods

Animal care

All rodent procedures were approved by and performed in accordance with UC Berkeley's Institutional Animal Care and Use Committees. Animals were housed under pathogen-free conditions with a 12-hour light and 12-hour dark cycle with food and water available *ad libitum*.

Astrocyte isolation and cell culture

Primary rat hippocampal astrocytes were isolated from P8 Sprague Dawley rat pups (Charles River Laboratories) and cultured under serum-free conditions following an established immunopanning protocol [184]. In brief, brain tissues were mechanically and enzymatically dissociated to generate a single-cell suspension, which was incubated successively on antibody-coated plates to subtract microglia, endothelial cells, and oligodendrocyte-lineage cells. After negative selection, astrocytes were positively selected by incubation on an Itgb5-coated plate. Isolated astrocytes were subsequently cultured in a chemically-defined, serum-free medium (50% neurobasal, 50% DMEM, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 1 mM sodium pyruvate, 292 μ g ml⁻¹ L-glutamine, SATO supplement, 5 μ g ml⁻¹ N-acetyl cysteine) supplemented with 5 ng ml⁻¹ HBEGF as previously described in detail [184].

Immunocytochemistry of cultured astrocytes

Adherent cells were rinsed with cold PBS and fixed with 2% paraformaldehyde solution for 10-15 minutes at room temperature (RT), permeabilized with ice-cold 100% ethanol for 5 min, rinsed again with PBS, and blocked overnight at 4°C with 5% normal goat serum. Cells were incubated for 2 hours at RT with primary antibodies, then rinsed 3 times with PBS to remove excess antibody. Cells were incubated with fluorescently-conjugated secondary antibodies for 1 hour at RT in the dark. Cells were washed 3 more times with PBS. Nuclear counterstaining was performed by adding NucBlue Fixed Cell ReadyProbes Reagent (Thermo Fisher) to the secondary antibody solution. For more information on the antibodies used, refer to Supplementary Table 1 and Supplementary Table 2. Images were acquired and exported using an Echo Revolve (Discover Echo).

Flow cytometry assays

Single cell suspensions of cells harvested from tissue culture were stained using a standard procedure. Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with ice-cold 100% EtOH for 3 minutes, and blocked with 3% normal goat serum in PBS for 2 hours at RT. After blocking, cells were incubated with primary antibody or isotype

control for 1 hour at RT, washed with PBS, then incubated with fluorescently conjugated secondary antibody for 1 hour at RT in the dark. After incubation with secondary antibody, cells were washed 3 times with PBS. Fluorescent measurements were acquired using a LSR Fortessa flow cytometer (Becton Dickinson) and analyzed with FlowJo Analysis Software (Tree Star).

Gene expression analysis

Total RNA was extracted from astrocytes using Direct-zol RNA MiniPrep kit (Zymo Research) and gene expression was quantified via quantitative reverse transcription PCR (RT-qPCR). First-strand cDNA synthesis was performed from 1 µg of RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Primers were designed using the NCBI Primer-BLAST tool and supplied by Integrated DNA Technologies. UCSC Genome Browser In-Silico PCR tool was used to confirm the specificity of primer sequences. PCR products were amplified using a CFX96 Real-Time PCR System and threshold cycles were measured using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Dissociation curve analysis was performed to verify single products for each reaction. For each sample, reactions were performed in triplicate on 96 well plates (Bio-Rad). Mean threshold cycles were normalized to the *Hprt* housekeeping gene and relative changes in mRNA levels were quantified using the $2^{-\Delta\Delta CT}$ method [185]. For a list of primer sequences, refer to Supplementary Table 3.

Cell culture experiments

Astrocytes were seeded on tissue culture dishes at a density of 10,000 cells per cm² and analyzed for senescence markers after 7 days of treatment. Cells were treated by adding either 10 nM TGFβ1 (PeproTech) or 0.2 mM albumin (Sigma-Aldrich) to fresh culture media every 48 hours. For TGFβ receptor inhibition, cells were pre-treated with either 1 µM SJN2511 (Tocris Biosciences) or DMSO (Sigma-Aldrich) control for 30 minutes prior to albumin treatment.

Senescence-associated β-galactosidase staining

SA-β-Gal staining was performed using a commercially available Senescence β-Galactosidase Staining Kit (Cell Signaling Technologies) according to the manufacturer's instructions. Quantification was performed by randomly selecting frames and manually counting positive cells using a Zeiss AxioImager microscope (Carl Zeiss) equipped with a Stereo Investigator system (MBF Biosciences). At least 200 cells were counted per sample. Representative images were captured at 20x using an Optronics Microfire A/R color camera.

Osmotic pump implants and IPW-5371 treatment

Osmotic pumps for intracerebroventricular (ICV) delivery of albumin were implanted in adult male C57BL/6 mice under 2% isoflurane anesthesia as previously described [108,186]. Using a stereotaxic frame, a hole was drilled through the skull at 0.5 mm caudal, 1 mm lateral to bregma, anterior to the hippocampus. A cannula was placed into the right lateral cerebral ventricle and fixed with surgical glue. Cannulas from Brain Infusion Kit 3 (ALZET) were attached to a Mini-Osmotic Pump Model 2001 (ALZET) filled with 200 µL of either 0.4 mM bovine serum albumin (Sigma-Aldrich) solution or artificial cerebrospinal fluid (aCSF; 129 mM NaCl, 21 mM NaHCO₃, 1.25 mM NaH₂PO₄, 1.8 mM MgSO₄, 1.6 mM CaCl₂, 3 mM KCl, and 10 mM glucose) and implanted subcutaneously in the right flank. Pumps infused at a rate of 1 µL per hour for 7 days. At day 7, the mice were anesthetized again for pump removal; wounds were closed and fixed

with surgical glue. For the IPW-5371-treated cohort, mice received 20 mg/kg of IPW-5371 via intraperitoneal (i.p.) injection daily for 7 days.

Genetic knockdown of astrocytic TGF β signaling in transgenic mice

Generation and characterization of the triple transgenic TGF β R2 knockdown mice (*a*TGF β R2/KD) used in this study have been previously described in detail [70]. Briefly, the mice were bred to express tamoxifen activated Cre recombinase (CreERT) under the astrocytic promoter glial high affinity glutamate transporter (GLAST) with a floxed exon 4 of *tgfr2* (fl), and a transgenic LacZ reporter gene inhibited by a floxed neomycin cassette. Tamoxifen thus induces activation of astrocytic CreERT resulting in a null TGF β R2 allele and LacZ reporter expression. The inducible Cre/lox system was activated by 5 days of i.p. tamoxifen injection (Sigma Aldrich; 160 mg/kg dissolved in corn oil). GLAST-CreERT; *tgfr2* (fl/+) heterozygotes were used as controls and received the same dosage of tamoxifen. Mice were weighed daily to ensure accurate dosage.

Western blot analysis

Mouse hippocampal tissues were manually homogenized, and protein lysates were extracted using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) including a protease (Calbiochem) and phosphatase inhibitor cocktail (Roche). Protein samples were run under reducing conditions. 20 μ g of protein lysate was mixed with 4x Laemmli buffer (Bio-Rad) containing 5% 2-mercaptoethanol (Sigma) and fractionated by SDS-PAGE using the Mini-PROTEAN Tetra System (Bio-Rad) and Mini-PROTEAN TGX Precast gels (4-20%, 15-well comb, 15 μ L/well). Each gel was loaded with 7 μ L of Chameleon Duo pre-stained protein ladder (LI-COR) for reference. Following separation, samples were transferred to a 0.45 μ m polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked for 1 hr at RT with Intercept TBS blocking buffer (LI-COR) and incubated overnight at 4°C with primary antibodies. Membranes were then washed 3 times for 10 mins with TBST (0.05% Tween-20 in TBS) and incubated with secondary antibodies in blocking buffer for 1 hr at RT in the dark. Membranes were washed 3 times for 10 mins with TBST and visualized using the Odyssey DLx Imaging System (LI-COR). Fluorescence analysis was performed in Image J (NIH). Blot values were normalized to GAPDH internal control and mean relative changes in protein levels were normalized to the control group. For details about the antibodies used, refer to Supplementary Table 1 and Supplementary Table 2.

Mouse tissue sampling and immunohistochemistry

Mice were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold, heparinized (10 units/mL) physiological saline for 10 minutes followed by 4% paraformaldehyde (PFA) in PBS. Mouse brains were removed and post-fixed in 4% PFA in PBS for 24 hours at 4°C, then cryoprotected by saturating with 30% sucrose in PBS. Brains were embedded in Tissue Plus O.C.T. compound (Scigen) and frozen at -80°C. Brains were later sliced on a CryoStar NX70 cryostat into 20 μ m coronal sections and mounted onto charged slides for immunostaining. Prior to staining, slides were treated for antigen retrieval by incubating them in 10 mM citrate buffer (pH 6.0) with 0.05% Tween-20 at 70°C for 20 minutes. For added permeabilization, slides were incubated with 0.01% Triton X-100 in Tris-buffered saline (TBS) for 10 minutes at RT. To prevent nonspecific binding, slides were incubated with blocking buffer composed of 10% normal donkey serum in TBS with 0.05% Tween-20 for 1 hour at RT. After

initial blocking, tissues were incubated overnight with primary antibodies diluted 1:200 in blocking buffer. The next day, slides were washed 3 times in TBS and subsequently incubated with fluorescent conjugated secondary antibodies diluted 1:800 in blocking buffer in the dark for 2 hours at RT. Nuclear counterstaining was performed by adding NucBlue Fixed Cell ReadyProbes Reagent (Invitrogen) to the secondary antibody solution. Slides were washed 3 times in TBS and mounted with ProLong Diamond (Invitrogen). For details about the antibodies used, refer to Supplementary Table 1 and Supplementary Table 2.

Lamin B1 quantification

Immunostained sections were imaged with a Nikon TiE inverted confocal microscope using a 100x oil objective. Z-stacks were acquired using 1 μ m steps for a total of 20 steps per region using NIS Elements imaging software (Nikon). Fluorescence intensity was quantified using ImageJ (NIH) as previously described [145]. Nuclei of GFAP+ and GFAP- cells were segmented within each Z-stack and mean gray value was measured in the channel corresponding to Lamin B1. At least 5 GFAP+ astrocytes and 5 GFAP- cells were sampled per section and at least 2 hippocampal sections were sampled per animal.

Statistical analysis

Statistics and graphs were performed using GraphPad Prism 8 software. For the *in vitro* time course experiment, a two-way ANOVA was used followed by Tukey's multiple comparisons test. For all other experiments, a one-way ANOVA was used followed by Dunnett's post-hoc test to compare treatment groups to the control group when a main effect was detected. For correlations, two-tailed linear regressions were performed to determine Person's r and p-value. Significance thresholds were set at *p < 0.05, **p < 0.01, ***p < 0.001.

2.3. Results

Albumin exposure activates TGF β signaling in cultured hippocampal astrocytes

To test the effects of albumin exposure on astrocytes *in vitro*, hippocampal astrocytes were isolated from postnatal day 8 rat pups, a time at which albumin is ostensibly excluded from the developing rat cortex [187]. The hippocampus was selected for analysis because it is especially vulnerable to age-associated BBBD [188]. To avoid the confounding effects of serum proteins, astrocytes were isolated and maintained in chemically-defined, serum-free media using a previously established protocol [189]. After isolation, cultures were expanded to passage 3 before being used for experiments. Cultures were 97% positive for the astrocytic marker glial fibrillary acidic protein (GFAP) as assessed via flow cytometry, and displayed typical astrocytic morphology with multiple branch-like processes when imaged via immunocytochemistry (Fig. 1A)

Previous studies have demonstrated that albumin binds to the type II TGF β R and activates canonical type I TGF β R ALK5, leading to the phosphorylation of the downstream effector protein SMAD2. Consequently, albumin exposure leads to increased levels of *Tgfb1* mRNA in astrocytes [103,104,106,190]. To determine the kinetics and dose-dependence of this response *in vitro*, astrocytes were treated with 0.2 mM of albumin and harvested after 1, 4, 8, and 24 hours of exposure for gene expression analysis via RT-qPCR. The albumin concentration used in this study is consistent with previous *in vitro* studies investigating its signaling effects on primary astrocytes and derived from *in vivo* transient hippocampal albumin measurements following BBBD

[103,191,192]. Treatment with 10 nM of TGF β 1 under the same conditions was also included as a previously validated positive control for TGF β signaling activation in astrocytes [106]. Astrocytes exhibited increased *Tgfb1* transcription in response to albumin treatment, reaching peak *Tgfb1* mRNA levels at 4 hours after exposure to albumin or TGF β 1 and remained significantly elevated in the albumin condition for at least 8 hours. Importantly, treatment with SJN2511, a selective small molecule TGF β R ALK5 inhibitor, blocked albumin induced upregulation of *Tgfb1* across all timepoints, with no significant difference observed from baseline (Fig. 1B).

Albumin-induced TGF β signaling increases expression of senescence markers in cultured astrocytes

To test whether albumin induces astrocyte senescence, we exposed cultures to 0.2 mM albumin for 7 days and analyzed them for cellular markers of senescence. A 7 day timepoint was selected in accordance with other *in vitro* protocols where prolonged exposure to a non-lethal stressor induced senescence in primary cells [143,145,180]. One common feature of senescent cells is increased lysosomal mass, which is measured by the detection of senescence associated β -galactosidase (SA- β -Gal) [115]. At day 7, albumin-treated astrocytes exhibited SA- β -Gal expression and flat morphology consistent with the senescent phenotype, whereas the control group exhibited no morphological changes (Fig. 1C). In the control group, only ~3% of the cells were SA- β -Gal positive at day 7. In albumin-treated cultures, a significant increase in the percentage of SA- β -Gal positive cells compared to control was observed, with a mean of 35.1% positive cells (Fig. 1C). TGF β R inhibition with SJN2511 prevented the albumin-induced increase in astrocyte senescence as detected by SA- β -Gal expression, limiting the percent of SA- β -Gal positive cells to a mean of 4.2% at day 7 with no significant difference from control (Fig. 1C).

Along with lysosomal changes, senescent cells exhibit changes in pathways related to cell cycle control and apoptosis that contribute to their survival after stressful conditions. Another marker of cellular senescence is the expression of p16^{INK4A}, a cyclin dependent kinase inhibitor that is upregulated upon cell cycle exit [193]. To test for expression of p16^{INK4A}, cells were assessed by flow cytometry at day 7. A detection gate was set to include single cells only, and the fluorescence threshold for p16^{INK4A} positivity was defined using a secondary-only negative control. In untreated control cells, a mean of ~10% of cells were detected as p16^{INK4A} positive. Consistent with the SA- β -Gal assay results, 7 days of albumin exposure significantly increased the percentage of p16^{INK4A} positive cells compared to control, with a mean of approximately 30% p16^{INK4A} positive cells detected in the albumin-treated condition (Fig. 1D). TGF β R inhibition with SJN2511 prevented the albumin-induced increase in astrocyte senescence. An average of 11.7% cells were detected as p16^{INK4A} positive in the SJN2511 treatment group, which was not significantly different from the control group (Fig. 1D).

Albumin-induced TGF β signaling increases expression of senescence-associated genes in cultured astrocytes

In addition to p16^{INK4A}, senescent cells often exhibit activation of the p21^{CIP1} pathway, associated with growth arrest [194], and BCL family proteins, which provide resistance to apoptosis [195]. Gene expression analysis via RT-qPCR after 7 days albumin exposure revealed increased mRNA levels of cyclin dependent kinase inhibitors 2a (*Cdkn2a*; p16^{INK4A}) and 1a (*Cdkn1a*; p21^{CIP1}) and the apoptosis regulators *Bcl2* (BCL-2), *Bcl2l1* (BCL-XL), and *Bcl2l2* (BCL-W) compared to control (Fig. 1E). Albumin-induced upregulation of these genes was prevented by pharmacological TGF β R inhibition with SJN2511 (Fig. 1E). These data support the hypothesis

that prolonged albumin exposure promotes expression of senescence-associated genes, including cell cycle and apoptosis inhibitors, in cultured hippocampal astrocytes in a TGF β -dependent manner.

Another feature of senescent cells is SASP expression, so we performed gene expression analysis on a subset of SASP factors that are associated with AD pathogenesis. Gene expression analysis via RT-qPCR after 7 days albumin exposure revealed increases in mRNA levels of genes encoding transforming growth factor beta 1 (*Tgfb1*; TGF β 1), interleukin 1 beta (*Il1b*; IL-1 β), interleukin 6 (*Il6*; IL-6), C-X-C motif chemokine ligand 10 (*Cxcl10*; CXCL10), monocyte chemoattractant protein-1 (*Ccl2*; MCP-1), and C-C motif chemokine ligand 5 (*Ccl5*; RANTES) (Fig. 1F). The albumin-induced activation of all measured SASP genes was prevented by TGF β R inhibition, with no significant differences observed between controls and SJN2511 treated cells (Fig. 1F). These data show that prolonged albumin exposure in cultured hippocampal astrocytes induces changes in gene expression of key components of both the SIPS and proinflammatory SASP phenotypes, and that these changes are dependent on activation of the TGF β R ALK5.

In vivo brain albumin infusion decreases nuclear membrane integrity in astrocytes, which is preventable by pharmacological TGF β R inhibition

To test whether exposure of the brain environment to albumin *in vivo* also leads to an accumulation of senescent astrocytes, we used an established mouse model of BBBD to simulate blood protein extravasation in the brain [70,98,108]. In this model, small osmotic pumps were implanted to continuously infuse 0.4 mM albumin or artificial cerebrospinal fluid (aCSF) directly into the right lateral ventricles of adult mice via for 7 days. The 0.4 mM albumin concentration was selected for intracerebroventricular (ICV) administration as it corresponds to normal albumin concentration in blood serum [196] and robustly triggers TGF β signaling in rodents [70,98,108]. aCSF is a biologically inert fluid in the brain parenchyma and is used as a sham control in this model.

To further evaluate the role of TGF β signaling in albumin-induced senescence, we tested the efficacy of IPW-5371, a small molecule TGF β R ALK5 inhibitor, in wild type adult mice receiving albumin infusions. IPW-5371 is an experimental drug with a promising clinical profile, including the ability to cross the BBB and a favorable PK/PD profile that enables once-per-day dosing (Rabender et al., 2016). IPW-5371 has been previously validated as a TGF β R ALK5 inhibitor in the context of BBBD in aged brains, where it was shown to significantly reduce levels of phosphorylated SMAD2 in the hippocampi of IPW-5371-treated mice compared to control mice [70].

In the IPW-5371 treatment cohort, mice received daily intraperitoneal injections (20 mg/kg) of IPW-5371 for 7 days following pump implant. On day 7, the pumps were removed, and the mice were allowed to recover. One week after pump removal, the mice were sacrificed, and the brains were harvested and prepared for immunohistochemistry (IHC) (Fig. 2A). Since commercially available p16^{INK4A} antibodies are not suitable for IHC in mice, we used nuclear Lamin B1 quantification to assess astrocyte senescence in mouse hippocampal tissue. Reduced Lamin B1 expression is detectable via IHC and is an established marker of cellular senescence [131,145]. Only the right hippocampus ipsilateral to the pump infusion was assayed for each section, specifically the hippocampal area of the dentate gyrus delimited by the granule cell layer and the end of the CA3 cell layer. In mice that received 7 days of albumin infusion, hippocampal astrocytes (GFAP+ cells) had significantly reduced Lamin B1 expression compared to control mice infused with aCSF (Fig. 2B, C). In hippocampal non-astrocytes (GFAP-), no significant

difference was detected in albumin-infused mouse tissues compared to control tissues (Fig. 2B, D). Importantly, albumin-induced astrocyte senescence was prevented by TGF β R inhibition with IPW-5371, with no significant differences observed between controls and IPW-5371-treated mice (Fig. 2B, C). These findings suggest that albumin-induced astrocyte senescence is mediated by TGF β signaling activation, and that astrocytes preferentially undergo senescence via this mechanism.

ICV albumin infusion increases hippocampal expression of senescence markers, which is preventable by astrocytic TGF β R knockdown

To test the causal role of TGF β signaling in albumin-induced astrocyte senescence, we used a previously established transgenic mouse line (*a*TGF β R2/KD) expressing inducible Cre recombinase under the astrocytic-specific glial high-affinity glutamate transporter (GLAST) promoter, which enables conditional knockdown (KD) of the floxed (fl) type II TGF β receptor (TGF β R2) specifically in astrocytes upon treatment with tamoxifen [70]. Tamoxifen induced efficient recombination in ~40% of hippocampal astrocytes and TGF β R protein levels were significantly ablated in hippocampal tissue samples, indicating successful genetic knockdown [70]. Selective knockdown of TGF β R2 alone is sufficient to inhibit TGF β signaling since membrane dimerization of type II and type I TGF β Rs is required for phosphorylation of the TGF β R ALK5 intracellular kinase domain and subsequent downstream signaling. Mice that were heterozygous for floxed TGF β R2 (fl/+) were used as controls. Using the same model as before, albumin or aCSF was infused directly into the right lateral ventricles of adult mice via osmotic pumps for 7 days. After 7 days of infusion, the pumps were removed, and the mice were allowed to recover. Two months after pump removal, the mice were sacrificed, and right hippocampi were harvested for protein extraction (Fig. 3A).

TGF β R2 fl/+ control mice that received ICV albumin infusion had significantly higher levels of TGF β signaling compared to mice that received aCSF infusion, as measured by levels of phosphorylated SMAD2 (pSMAD2) and the ratio of pSMAD2 to unphosphorylated SMAD2 (Fig. 3B). To explore an alternative senescence marker, we assessed activation of the p38 mitogen-activated protein kinase (p38MAPK) pathway, as indicated by its phosphorylated state (p-p38MAPK). Activation of p38MAPK is believed to play a causal role in cellular senescence following persistent low-level stresses [197] and is essential for SASP expression [198]. Therefore, p-p38MAPK has been used as an additional indicator of senescence in astrocytes [144,199] and neurons [200]. In albumin-treated TGF β R2 fl/+ mice, the levels of p38MAPK, p-p38MAPK, and the ratio of p-p38MAPK to p38MAPK were significantly higher compared to aCSF-treated controls (Fig. 3C). Notably, albumin infusion also significantly increased p16^{INK4A} expression in TGF β R2 fl/+ mice compared to controls (Fig. 3D). Mice with homozygous induced KD of TGF β R2 (fl/fl) in astrocytes were protected against albumin-induced increases in TGF β signaling and senescence marker expression (Fig. 3B-D). These results show that genetic KD of astrocytic TGF β signaling is sufficient to prevent albumin-induced senescence in these cells.

Regression analysis revealed there were positive correlations between the pSMAD2:SMAD2 ratio and p16^{INK4A} expression ($r = 0.54$, $p = 0.003$) (Fig. 3E) and the p-p38MAPK:p38MAPK ratio and p16^{INK4A} expression ($r = 0.60$, $p = 0.001$) (Fig. 3F) across all groups. Together, these data support a relationship between albumin-induced astrocytic TGF β signaling and elevated senescence pathways in the brain.

2.4. Discussion

Senescent astrocytes are present in aging brain tissue and play a role in neurological disease progression, but the specific triggers of age-related senescence remain elusive. In this study, we sought to test the hypothesis that the blood-derived protein albumin, which accumulates in the aging brain due to BBBD, can induce astrocyte senescence through TGF β hyperactivation. Using a serum-free, *in vitro* model, we discovered that one week of serum albumin exposure induced expression of senescence and SASP markers in isolated hippocampal astrocytes, and that pharmacological inhibition of the TGF β R ALK5 was able to prevent these phenotypic changes. Using an *in vivo* model of BBBD, we found that one week of continuous albumin infusion into the lateral ventricles of mice preferentially caused senescence in astrocytes, and that pharmacological TGF β R inhibition with IPW-5371 was able to prevent albumin-induced astrocyte senescence in mice. Using the same model, we found that 7 days of albumin infusion in control mice caused increased TGF β signaling (pSMAD2) and senescence marker expression (p16^{INK4A} and p-p38MAPK) in mouse hippocampi two months after pump removal. Senescence marker expression was TGF β -dependent since selective knockdown of astrocytic TGF β R prior to albumin infusion was sufficient to prevent these increases. Together, these results establish a link between TGF β hyperactivation and astrocyte senescence and suggest that prolonged albumin exposure due to BBBD can underlie these phenotypic changes.

The albumin-induced increase in transcripts encoding the AD-associated SASP factors IL-1 β , IL-6, CXCL10, MCP-1, and RANTES supports the link between senescent astrocytes and neurodegeneration. For instance, IL-1 β and IL-6 are both potent stimuli for leukocyte recruitment to the brain and have been implicated in neuroinflammation and neurofibrillary pathology in AD [163–165,201,202]. Chemokines serve a similar function in recruiting immune cells to the CNS during AD [203]. For instance, CXCL10 signals through the CXCR3 receptor to promote monocyte activation and migration [204]. CXCL10 is found in high concentrations in the brains of AD patients and activation of the CXCL10/CXCR3 axis has been shown to mediate development of AD-like pathology in mouse models [205]. In humans, peripheral levels of CXCL10 are elevated in older adults and negatively associated with working memory performance [206]. Overexpression of MCP-1 has been shown to enhance microgliosis, facilitate amyloid plaque formation, and accelerate cognitive decline in rodent models of AD [207,208]. Although its role in neuropathology is still being investigated, RANTES is upregulated in the cerebral microcirculation of AD patients and is also suspected to influence neuroinflammation [209].

Previous studies have demonstrated that senescent astrocytes have substantially reduced functional capacity compared to their normal counterparts. For example, senescent astrocytes exhibited impaired wound healing ability, phagocytic uptake, metabolic function, and neuroprotective capacity [156,157]. Among their numerous functions in the adult brain, astrocytes are required for BBB maintenance [62]. Therefore, it's possible that initial BBBD-induced astrocyte senescence establishes a positive feedback loop that results in progressive neurological decline. In this scenario, the accumulation of functionally impaired senescent astrocytes and secretion of pro-inflammatory SASP factors results in more BBBD and thus more TGF β -induced astrocyte senescence. Further studies should investigate how this process is regulated and explore potential strategies to prevent its onset.

While this study has identified blood-derived serum albumin as a potential physiological trigger of TGF β signaling activation and astrocyte senescence, it is unlikely to be the only activator of these pathways in the aging brain. Previous reports have identified additional age-related triggers of astrocyte senescence such as oxidative stress [142], proteotoxic stress [142], and

amyloid beta (A β) oligomers [144]. Interestingly, TGF β signaling is also implicated in these age-related senescence mechanisms, making our results consistent with past findings. In the case of oxidative stress, TGF β increases the production of reactive oxygen species (ROS) by impairing mitochondrial function and inducing NADPH oxidases; TGF β also suppresses the synthesis of antioxidant enzymes such as glutathione in various cell types, causing redox imbalance and promoting further oxidative stress [210–212]. In the case of A β pathology, TGF β overexpression increases astrocytic A β generation in transgenic mice [213–215], and TGF β levels are elevated in cortical astrocytes surrounding A β plaques in AD patients [216,217].

Given the salience of TGF β signaling activation in response to physiological triggers of senescence, this pathway should be explored as an attractive therapeutic target in the prevention of age-related neuropathology. Future studies should seek to elucidate the context in which various triggers promote senescence during aging and the extent of their respective contributions.

2.5. Figures

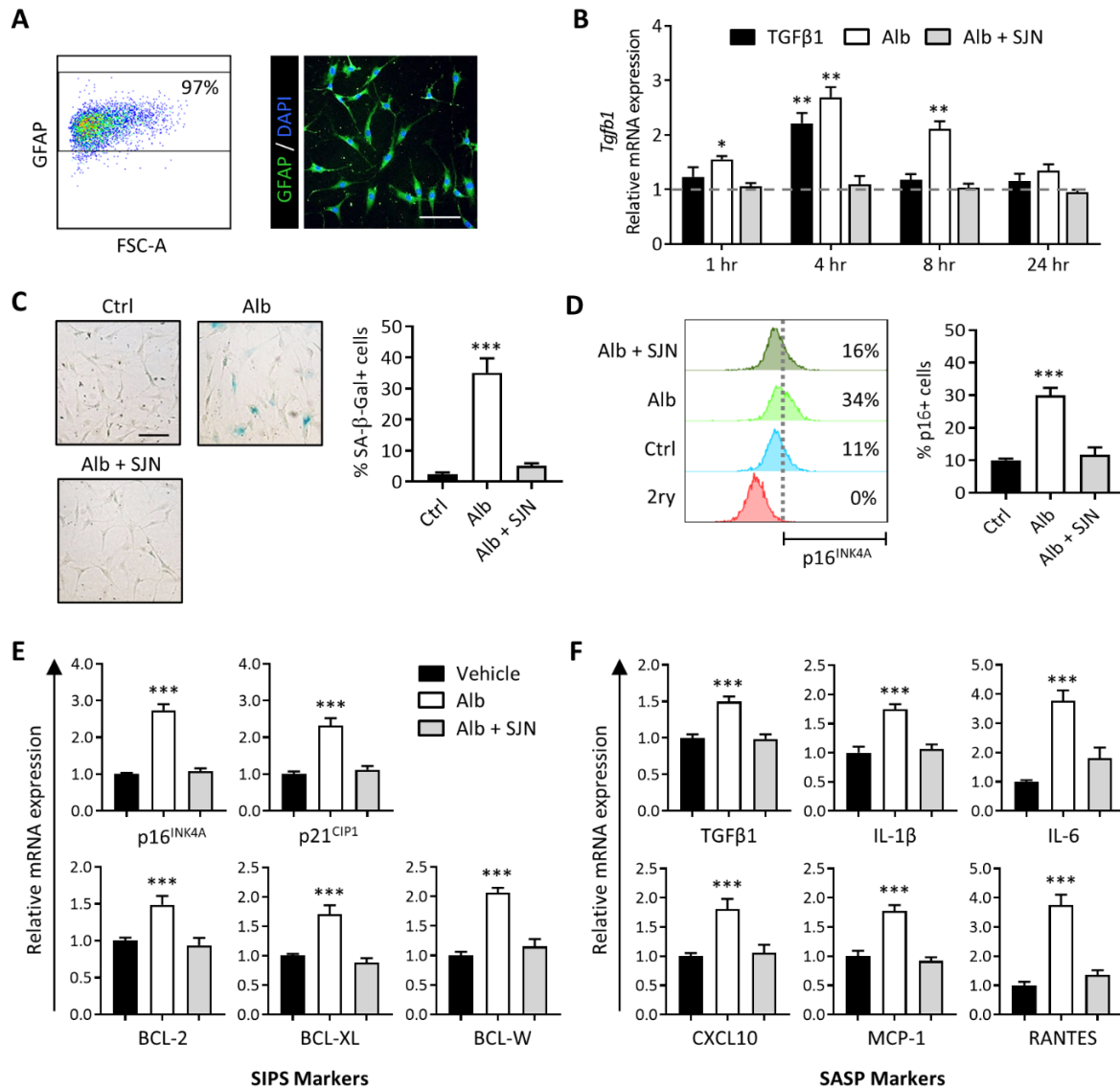


Figure 1. Albumin-induced TGFβ signaling activation induces astrocyte senescence in cultured hippocampal astrocytes. (A) Representative dot plot and microscopy image show astrocytes isolated from P8 rat hippocampi were 97% GFAP positive and exhibit typical morphology with multiple processes. (B) Bar graph shows relative *Tgfb1* mRNA expression levels against baseline (horizontal dotted line). Astrocytes exhibited a significant increase in *Tgfb1* transcription upon exposure to albumin, reaching maximum *Tgfb1* mRNA levels at 4 hours after exposure. Albumin-induced *Tgfb1* transcription was blocked by TGFβR inhibition with SJN2511. (C) Left: representative brightfield images show 7 days of albumin exposure induces senescence-associated morphological changes in astrocytes and increases SA-β-Gal expression. Right: Quantification via cell counting reveals a significant increase in the percentage of SA-β-Gal

positive cells in the albumin-treated cells compared to control, which was blocked by TGF β R inhibition with SJN2511. (D) Left: histograms depict the results of one representative flow cytometry experiment to illustrate the gating strategy applied for determining p16^{INK4A} positive cells. The bottom (red) histogram represents the isotype control used to set the fluorescence detection threshold (vertical dotted line). Cells with fluorescent values above the threshold were considered positive for p16^{INK4A}. Right: Summary data of cell counts from all experiments show that treatment with albumin induced significant increases in the percentage of p16^{INK4A} positive cells compared to control. SJN2511 treatment prevented the albumin-induced increase in p16^{INK4A} positive cells. (E) Gene expression analysis after 7 days of albumin exposure revealed increases in mRNA levels of genes encoding markers of stress-induced premature senescence (SIPS) and (F) genes encoding components of the senescence-associated secretory phenotype (SASP). Albumin-induced upregulation of these genes was prevented by TGF β R inhibition with SJN2511. Scale bar: 100 μ m. Bar graphs depict mean $\pm$ SEM of 3 independent experiments. Asterisks denote a significant difference from baseline *p < 0.05, **p < 0.01, ***p < 0.001.

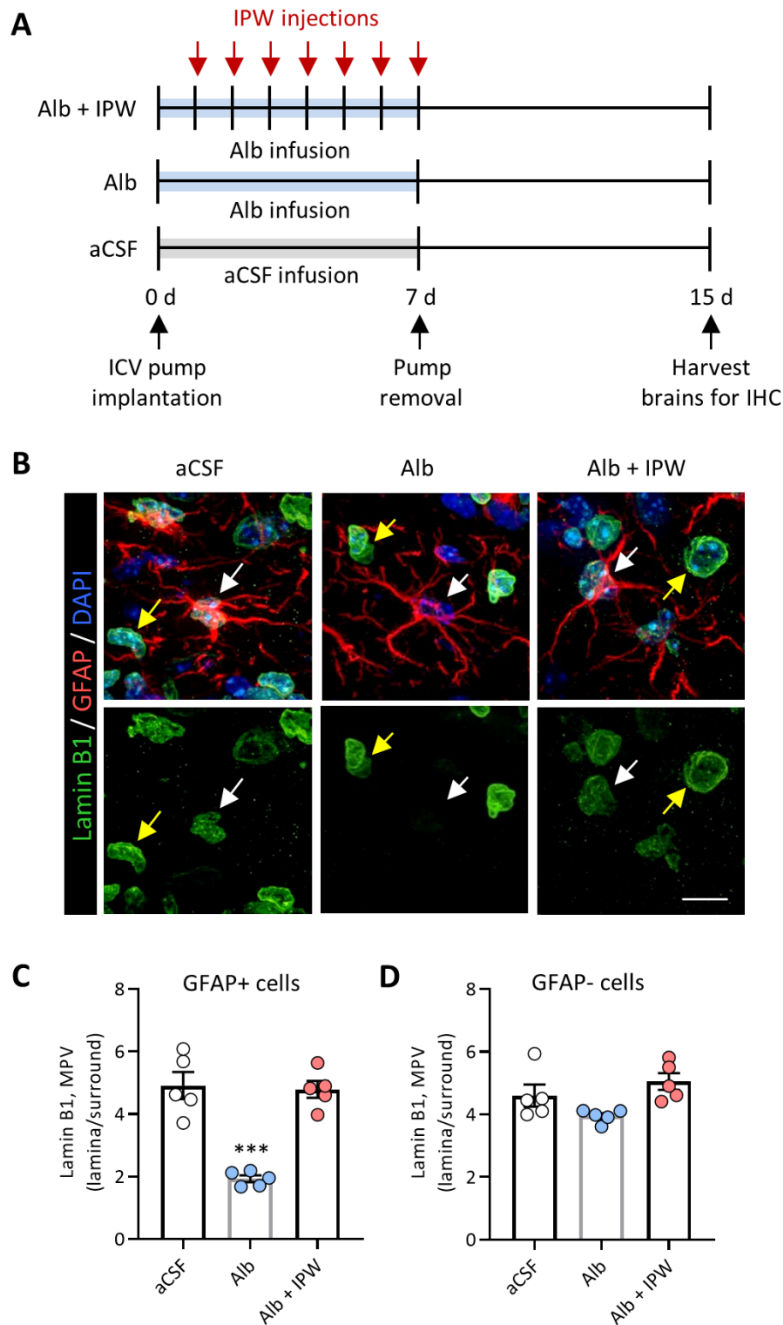


Figure 2. Pharmacological TGF β R inhibition prevents albumin-induced astrocyte senescence in a mouse model of BBBB. (A) Schematic of experimental design whereby mice were infused for 7 days with either aCSF or albumin before pump removal and 7 days of recovery. The control group received aCSF infusion (n=5), another group received albumin infusion only (n=5), and the treatment group received albumin infusion plus 7 days of daily IPW-5371 injections (n=5). Whole brains were extracted 7 days after pump removal and prepared for IHC. (B) Representative confocal Z-stack images of hippocampal sections immunostained for Lamin B1 (green), GFAP (red), and DAPI (blue). White arrows denote GFAP+ cells and yellow arrows

denote GFAP- cells. Images acquired at 100x magnification. (C) Quantification of Lamin B1 expression using mean pixel values (MPV) show that in albumin treated mice, GFAP+ astrocytes, but not GFAP- cells, have significantly decreased Lamin B1 levels compared to controls. Pharmacological TGF β R inhibition with IPW-5371 prevents albumin induced Lamin B1 deficits in GFAP+ astrocytes. Scale bar: 10 μ m. Bar graphs depict mean $\pm$ SEM of each experimental group. Asterisks denote a significant difference from control *p <0.05, **p<0.01, ***p<0.001.

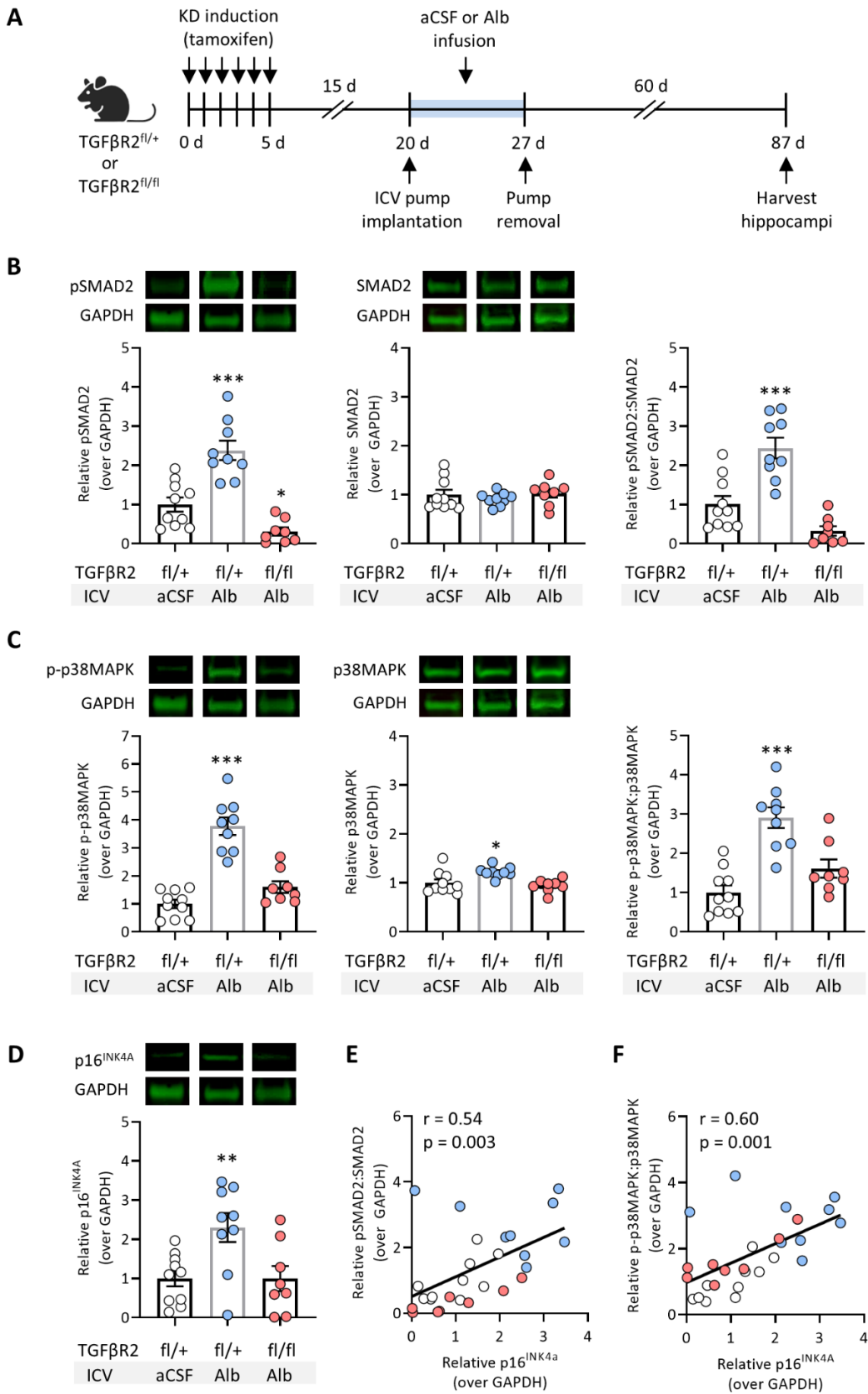


Figure 3. Astrocytic TGF β R knockdown prevents albumin-induced hippocampal senescence in a mouse model of BBBB. (A) Schematic of experimental design whereby ICV osmotic pumps were implanted into the right ventricles of adult transgenic mice. Mice heterozygous for floxed TGF β R2 (fl/+) were infused with aCSF (n=10) or albumin (n = 9), and mice with homozygous induced KD of TGF β R2 (fl/fl) were infused with albumin (n=8) for 7 days before pump removal and recovery. Hippocampal tissue was harvested 60 days after pump removal. Representative Western blot images and bar graphs show protein expression levels normalized to GAPDH of (B) pSMAD2, SMAD2, and the ratio of pSMAD2:SMAD2, (C) p-p38MAPK, p38MAPK, and the ratio of p-p38MAPK:p38MAPK, and (D) p16^{INK4A} in aCSF and albumin-treated mice. Regression analysis revealed positive correlations between the protein expression levels of (E) p16^{INK4A} and the ratio of pSMAD2:SMAD2, and (F) p16^{INK4A} and the ratio of p-p38MAPK:p38MAPK. Bar graphs depict mean $\pm$ SD of each experimental group. Asterisks denote a significant difference from control *p<0.05, **p<0.01, ***p<0.001.

Supplementary Table 1. Primary Antibodies

Target	Isotype	Supplier	Catalog #	Dilution	Application
GFAP	Goat IgG	Abcam	ab53554	1:500	FACS, ICC, IHC
Lamin B1	Rabbit IgG	Abcam	ab16048	1:200	IHC
p16 ^{INK4a}	Mouse IgG	Abcam	ab54210	1:500	FACS
p16 ^{INK4a}	Rabbit IgG	Assay Biotech	C0285	1:500	WB
SMAD2	Rabbit IgG	Cell Signaling	5339S	1:1000	WB
pSMAD2	Rabbit IgG	Millipore Sigma	AB3849-I	1:1000	WB
p38 MAPK	Rabbit IgG	Cell Signaling	9212S	1:1000	WB
p-p38 MAPK	Rabbit IgG	Cell Signaling	4511S	1:1000	WB
GAPDH	Rabbit IgG	Cell Signaling	2118S	1:1000	WB

FACS = flow cytometry; ICC = immunocytochemistry; IHC = immunohistochemistry; WB = Western blot

Supplementary Table 2. Secondary Antibodies

Conjugate	Host and Isotype	Supplier	Catalog #	Dilution	Application
Alexa 488	Donkey anti-rabbit IgG	Thermo Fisher	A-21206	1:800	IHC
Alexa 594	Donkey anti-goat IgG	Thermo Fisher	A-11058	1:800	IHC
Alexa 488	Goat anti-mouse IgG	Thermo Fisher	A-32723	1:500	FACS, ICC
IRDye 800CW	Goat anti-rabbit IgG	LI-COR	926-32211	1:10,000	WB

FACS = flow cytometry; ICC = immunocytochemistry; IHC = immunohistochemistry; WB = Western blot

Supplementary Table 3. Gene Primers for RT-qPCR

Gene	Primer Sequence
<i>Hprt</i>	5' – TCAGTCAACGGGGGACATAAA – 3' 3' – GGGGCTGTACTGCTTAACCAG – 5'
<i>Tgfb1</i>	5' – CAACCCAGGTCCTTCCTAAA – 3' 3' – GGAGAGCCCTGGATACCAAC – 5'
<i>Cdkn2a</i>	5' – AATCTCCGCGAGGAAAGC – 3' 3' – GTCTGCAGCGGACTCCAT – 5'
<i>Cdkn1a</i>	5' – ATCACCAGGATTGGACATGG – 3' 3' – GGTGTCAGAGTCTAGGGGA – 5'
<i>Bcl2l1</i>	5' – GCTGCATTGTTCCCGTAGAG – 3' 3' – GTTGGATGGCCACCTATCTG – 5'
<i>Bcl2</i>	5' – GGTCTTCAGAGACAGCCAGG – 3' 3' – GATCCAGGATAACGGAGGCT – 5'
<i>Bcl2l2</i>	5' – TCTAGTGGCTGACTTTGTAGGC – 3' 3' – GAAACCTGGGTGAAGCGTTG – 5'
<i>Ccl2</i>	5' – GCATCTGCCCTAAGGTCTTCA – 3' 3' – GTGGAAAAGGTAGTGGATGCATT – 5'
<i>Il1b</i>	5' – CACAGCAGCACATCAACAAG – 3' 3' – GTGCTCATGTCCTCATCCTG – 5'
<i>Ccl5</i>	5' – CCCTCACCATCATCCTCACT – 3' 3' – TCCTTCGAGTGACAAACACG – 5'
<i>Ccl20</i>	5' – TGTACGAGAGGCAACAGTCG – 3' 3' – TCTGCTCTTCCTTGCTTTGG – 5'
<i>Il6</i>	5' – GCTACCAAACCTGGATATAATCAGGA – 3' 3' – CCAGGTAGCTATGGTACTCCAGAA – 5'

Chapter 3: Repetitive mild traumatic brain injury induces blood-brain barrier dysfunction, cellular senescence, and neurobehavioral deficits in mice

Project contributions: This work was performed in collaboration with Lynn Yang, Allison Gleason, Aileen Zhang, and Brian Benyamini.

3.1. Introduction

Traumatic brain injury (TBI) refers to a condition of altered brain function caused by an external force to the head. TBI severity is classified as mild, moderate, or severe within 24 hours of the incident as measured by the Glasgow Coma Scale (GCS) [218]. The overwhelming majority (75-90%) of injuries are considered mild TBI (mTBI), and occur in the general population as a result of accidents and falls [219,220]. Yet, mounting evidence indicates that repetitive mild TBIs (rmTBIs), which are common in contact sports athletes, are a serious medical issue and are associated with an increased risk of developing chronic traumatic encephalopathy (CTE) [99,221,222] and dementia [223,224]. Patients with mTBI may experience loss of consciousness for under 30 minutes, alterations of consciousness for up to 24 hours, and post-traumatic amnesia for up to 24 hours [225]. mTBI leads to a variety of neurological symptoms immediately following injury including somatic symptoms (headaches, fatigue, nausea, balance problems) [226], cognitive symptoms (memory and attention deficits) [227], emotional symptoms (irritability, depression, anxiety) [228], and sleep disturbances [229].

Evidence from rodent models and human studies show that rmTBI triggers a slowly developing cascade that results in significant changes in brain metabolism, white matter integrity, functional connectivity, and cognitive function [230,231]. However, the cellular and molecular mechanisms underlying the short- and long-term symptoms involved in rmTBI are not completely understood. A recent rodent study of rmTBI showed that rats can be classified based on their susceptibility to neurological complications based on the levels of blood-brain barrier dysfunction (BBBD), transforming growth factor-beta (TGF β) signaling, and neuroinflammation, suggesting these mechanisms are involved in poor outcomes [102]. Given that albumin-induced TGF β activation was shown to contribute to stress-induced premature senescence (SIPS) in a mouse model of BBBD [232], we hypothesized that rmTBI-induced BBBD would increase TGF β signaling and senescence markers in the rmTBI brain, and that these increases would be correlated with neurological symptoms. In this study, we developed a closed-head impact injury model of rmTBI in mice to test this hypothesis.

3.2. Materials and methods

Animal care

All rodent procedures were approved by and performed in accordance with UC Berkeley's Institutional Animal Care and Use Committees. Adult male C57BL/6 mice were housed under pathogen-free conditions with a 12-hour light and 12-hour dark cycle with food and water available *ad libitum*.

Neurological Severity Scoring (NSS)

NSS was performed pre- and post-injury for each day of impacts, as well as 24 hours after the fifth and final day of impacts using methodology previously described in detail [99]. Post-

injury NSS was performed at least 30 minutes after awakening from anesthesia. In brief, it consists of 3 behavioral subtests: open-field, inverted wire mesh, and beam walk. Each 30-second subtest was scored on a 6-point scale (0-5), and a composite score was calculated by adding scores from the 3 subtests. Lower composite scores indicate increasing impairment [99]. Animals were handled for one week and received two days of NSS training prior to assessment. Each task was recorded, and videos were analyzed and scored according to objective criteria by blinded individuals. The arena was cleaned with 70% ethanol between subject trials. *Open-field*. The open field test was used to gauge exploratory behavior, motor function, and anxiety-like behavior. Mice were placed in the center of a 50 cm × 50 cm box and given 30 seconds to explore the arena. *Inverted wire mesh*. Mice were placed in the center of a 40 cm × 40 cm wire mesh with a smaller 20 cm × 20 cm square drawn on the wire, inverted by 180°, and held above the open field arena for 30 seconds. Mice were considered exiting the inner grid if a single paw grasped outside the 20 cm × 20 cm demarcated zone and considered to have traveled if all four paws moved from their initial hanging position. *Beam walk*. Animals were placed on one end of a metal beam and allowed 30 seconds to traverse the beam. Dark boxes were placed at both ends of the beam as an incentive for the animal to traverse the beam. If the animal traversed to one end of the beam, an experimenter flipped the mouse and redirected them to move to the opposite side of the beam.

Closed-head impact injury mouse model

A modified Marmarou weight-drop closed head model of TBI (120 mg, 110 cm height) [102,233–235] was used to induce mTBI with substantial rotation of the animal upon impact, as previously described [236]. Mice were pretreated with analgesic (buprenorphine, 0.2 mg/kg, i.p.) at least 30 minutes prior to impacts. Mice were anesthetized with isoflurane anesthesia before being placed in the prone position perpendicular to the axis of weight drop, with the hind legs extending away from the body. A 120 mg slug was released from a height of 110 cm and traveled along a low-friction monorail track. Impact weight and height were set to cause mild brain injury as determined by a transient reduction in NSS score and rare mortality. Traumatic head motion was initiated by a final momentum transfer from the slug to a captive impact rod on the animal's head. Impacts were delivered in the center of the mouse skull between the lambda and bregma suture lines. Concussive mTBI was determined by transient reduction in neurobehavioral performance (NSS) and rare mortality. Repetitive mTBI involved one mTBI per day for five consecutive days. Sham controls were exposed to the same procedures, including isoflurane anesthesia, with no TBI.

Behavioral assessments

Behavioral assessments were conducted on adult male mice (n = 27) beginning ~2.5 weeks after the fifth and final impact. Assessments were conducted in even ambient lighting in the animal's light cycle. Behavioral apparatuses were thoroughly cleaned with 70% ethanol and dried between trials.

Y-maze spontaneous alternation

Exploratory behavior and spatial acuity were assessed with a mouse-specific Y-maze apparatus (Noldus). Mice were placed in the center and allowed to freely explore for 5 minutes. Animal activity was recorded from above and videos were blindly scored by manually recording the arm entry sequence. Entry into an arm was defined as having the two front paws cross the threshold from the triangular center into an arm of the Y-maze. Actual alternation was defined as

entry into all three arms consecutively without repetition (e.g., ABC, BCA, CAB). The number of maximum spontaneous alternations equals the total number of arms entered minus two, and therefore percent alternation was calculated as (actual alternations/maximum alternations) $\times$ 100. In the Y-Maze, chance level of alteration is calculated at 22.2%.

Elevated plus maze

Anxiety-like behavior was assessed in mice placed at the center of a mouse-specific Elevated Plus Maze apparatus (Noldus). Animals began the experiment placed in the same location facing an open arm. The trajectory of each animal was recorded for 10 minutes, and behavior was scored using the video tracking software EthoVision XT.

Barnes maze

Spatial learning and memory retention were assessed in mice using a mouse-specific Barnes Maze apparatus (Noldus) and an established Barnes Maze protocol [237]. In brief, animals are tested on their ability to locate a previously learned escape hole in the presence of an aversive noise stimulus (65-decibel metronome) placed at the center of the maze. For the first day of habituation, the holding container was transparent to allow mice to observe their surroundings as they are guided to the target escape hole with an escape box underneath. Once they successfully escape, the metronome is turned off. On the following 2 consecutive training days, mice are released from the holding container to explore as they are operantly conditioned to associate successfully entering into the escape box with the termination of the aversive metronome noise. There were 3 training trials on day 2 and 2 training trials on day 3. After a 72-hour delay, the final probe trial consists of the animal exploring the maze for up to 2 minutes to assess their ability to find the escape hole. The time in seconds to locate the escape hole was measured (i.e., escape latency). All trials were recorded by camera and analyzed with EthoVision XT. The apparatus and objects in the testing room remained unmoved through the full duration of the assessment.

Mouse tissue sampling

Mice were sacrificed 30 days after the fifth and final impact and brains were removed and dissected in PBS. Brain regions were collected in Eppendorf tubes and snap-frozen for downstream molecular experiments.

Western blot analysis

Mouse hippocampal tissues were manually homogenized, and protein lysates were extracted using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) including a protease (Calbiochem) and phosphatase inhibitor cocktail (Roche). Protein samples were run under reducing conditions. 20 μ g of protein lysate was mixed with 4x Laemmli buffer (Bio-Rad) containing 5% 2-mercaptoethanol (Sigma) and fractionated by SDS-PAGE using the Mini-PROTEAN Tetra System (Bio-Rad) and Mini-PROTEAN TGX Precast gels (4-20%, 15-well comb, 15 μ L/well). Each gel was loaded with 7 μ L of Chameleon Duo pre-stained protein ladder (LI-COR) for reference. Following separation, samples were transferred to a 0.45 μ m polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked for 1 hr at RT with Intercept TBS blocking buffer (LI-COR) and incubated overnight at 4°C with primary antibodies. Membranes were then washed 3 times for 10 mins with TBST (0.05% Tween-20 in TBS) and incubated with secondary antibodies in blocking buffer for 1 hr at RT in the dark. Membranes were washed 3 times for 10 mins with TBST and visualized using the

Odyssey DLx Imaging System (LI-COR). Fluorescence analysis was performed in Image J (NIH). Blot values were normalized to GAPDH internal control and mean relative changes in protein levels were normalized to the control group. For details about the antibodies used, refer to Supplementary Table 1.

Evans blue dye perfusion and tissue sampling

4% Evans blue (EB) solution in 0.9% sterile saline was administered by bolus injection through the tail vein (2 mL/kg, i.v.) one week after the first impact. Thirty minutes after EB administration, mice were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold, heparinized (10 units/mL) physiological saline for 5 minutes followed by 4% paraformaldehyde (PFA) in PBS for 10 minutes (2 mL/min). Mouse brains were removed and post-fixed in 4% PFA in PBS for 24 hours at 4°C, then cryoprotected by saturating with 30% sucrose in PBS. Brains were embedded in Tissue Plus O.C.T. compound (Scigen) and frozen at -80°C. Brains were later sliced on a CryoStar NX70 cryostat into 20 µm coronal sections and mounted onto charged slides for analysis. Coverslips were adhered using Entellan (Millipore). Sections were imaged at 20x magnification using an Axio Scan.Z1 slide scanner (Zeiss). Six representative coronal sections (located +1.145, +0.445, -0.18, -1.155, -1.655, and -2.255 mm anterior and posterior from Bregma, respectively) were selected across all animals with the 2008 Allen Mouse Brain Atlas, converted into 8-bit grayscale .TIFF files on ZEN 3.1 (Blue edition), and variables were quantified by a blinded volunteer in ImageJ. EB intensity values (raw integrated density/µm²) were normalized to background corpus callosum EB intensity values.

Statistical analysis

Statistics and graphs were performed using GraphPad Prism 8 software. For multi-day experiments, ordinary two-way ANOVA was used followed by post-hoc tests to compare sham and rmTBI groups when a column effect was detected. For comparisons between two groups, unpaired, two-tailed t-tests with Welch's correction were used. For correlations, two-tailed linear regressions were performed to determine Person's r and p-value. The ROUT method of outlier removal was applied to all data sets using Prism's default Q = 1% detection parameter. One outlier was detected (sham animal) and removed from the Barnes Maze data set. Significance thresholds were set at *p < 0.05, **p < 0.01, ***p < 0.001.

3.3. Results

rmTBI results in blood-brain barrier disruption

To investigate the physiological consequences of rmTBI in the brain, we established a closed-head weight-drop mouse model of rmTBI that induces rotation of the head and body upon impact. This model was chosen as it simulates the repetitive head injuries commonly experienced by contact sport athletes. Seven-week-old male mice (n = 30) were handled and acclimatized for one week prior to treatment. The rmTBI group (n = 19) was subjected to one mTBI per day for five consecutive days (Fig. 1A). The control group (n = 11) was subjected to the same procedures, including analgesic and isoflurane anesthesia, with no TBI. 4 rmTBI mice died due to impacts and were removed from analysis. After the final impact, mice were allowed to recover for 3 days. To investigate the effects of rmTBI on BBB permeability, animals were injected through the tail vein with Evans blue (EB) dye, a fluorescent azo dye that binds albumin with high affinity [238]. Since serum albumin does not normally cross the BBB, EB is used to evaluate the extent of BBB and

albumin leakage in neural tissue [238]. In control mice, neural tissue remains unstained, indicating EB is confined to the capillary blood vessels (Fig. 1B). In contrast, pronounced EB fluorescence was observed in the neural tissue of rmTBI brains, especially in cortical and hippocampal regions, indicating BBBD (Fig. 1B). Ventricular enlargement is a common occurrence in severe TBI and is related to coma duration and intellectual and memory deficits in humans [239]. To test for ventricular dilation, we measured lateral ventricle area in coronal sections. No significant increase in LV area was detected in rmTBI brains compared to control (Fig. 1C). EB dye fluorescence levels in neural tissue in the whole brain (Fig. 1D) and in the granule cell (GC) layer of the hippocampal dentate gyrus (Fig. 1E) were significantly higher in rmTBI mice compared to controls, indicating diffuse brain damage that extended deep into the temporal lobe. However, there was no significant difference between groups in EB levels in the hilus of the hippocampal dentate gyrus (Fig. 1F). There were no significant differences in EB levels between the left and right hemispheres of control (Fig. 1G) or rmTBI (Fig. 1H) groups. Together, these results show that five days of rmTBI causes diffuse blood-brain barrier disruption in the brain and concomitant albumin leakage.

rmTBI causes acute neurobehavioral impairments

To investigate the mechanisms underlying rmTBI-associated neuropathology, seven-week-old male mice ($n = 30$) were handled and acclimatized for one week prior to treatment. The rmTBI group ($n = 20$) was subjected to one mTBI per day for five consecutive days. The control group ($n = 10$) was subjected to the same procedures, including analgesic and isoflurane anesthesia, with no TBI. Three rmTBI mice died due to impacts and were removed from analysis. Acute neurobehavioral assessment was conducted using the neurological severity score (NSS) paradigm as described in the methods. NSS scoring was performed daily before and after-impact, as well as 24 hours after the final impact. Enduring neurobehavioral changes were assessed approximately 2.5 weeks after the final impact using elevated plus maze (EPM), Y-Maze, and Barnes maze (BM) (Fig. 2A). rmTBI mice experienced significantly more weight loss across all days compared to the sham group, suggesting physical complications due to injury (Fig. 2B). After anesthesia (with or without impact), animals were placed in a supine position. Recovery time was measured as the time it took for the animals to right themselves. rmTBI mice had significantly longer recover times compared to control animals across impact days (Fig. 2C). The average difference from baseline in post-impact NSS scores in the rmTBI group and were significantly greater compared to control (Fig. 2D). The average difference from baseline in pre-impact composite NSS scores were significantly greater on impact day 4 but were not significantly different at the end of the treatment period (POST = 24 hours after the final impact), indicating the observed neurobehavioral impairments were transient (Fig. 2E). Despite tight control of the experimental injury parameters, the spectrum of acute neurobehavioral responses to rmTBI showed wide variation (Fig. 2C-E).

rmTBI mice exhibit increased $TGF\beta$ signaling and senescence markers

To investigate whether rmTBI increases $TGF\beta$ signaling and cellular senescence in the hippocampus as a result of increased BBBD, protein expression was analyzed from left hippocampal tissue. rmTBI mice had significantly higher levels of $TGF\beta$ signaling compared to control mice, as measured by levels of phosphorylated SMAD2 (pSMAD2) and the ratio of pSMAD2 to unphosphorylated SMAD2 (Fig. 3A). To investigate pathways involved in senescence, we assessed activation of the p38 mitogen-activated protein kinase (p38MAPK) pathway, as indicated by its phosphorylated state (p-p38MAPK). Activation of p38MAPK plays a

causal role in cellular senescence following persistent low-level stresses [197] and is essential for SASP expression [198]. Thus, p-p38MAPK has been used as one indicator of senescence in the brain [144,199,200]. In rmTBI mice, the levels of p38MAPK, p-p38MAPK, and the ratio of p-p38MAPK to p38MAPK were significantly higher than controls (Fig. 3B). One of the most salient senescence markers p16^{INK4A}, a cyclin-dependent kinase inhibitor that arrests the cell cycle [240,241]. Importantly, rmTBI mice exhibited significantly increased p16^{INK4A} expression compared to controls (Fig. 3C). Regression analysis revealed there were significant positive correlations between the pSMAD2:SMAD2 ratio and p16^{INK4A} expression ($r = 0.60$, $p < 0.001$) (Fig. 3D) and the p-p38MAPK:p38MAPK ratio and p16^{INK4A} expression ($r = 0.43$, $p = 0.024$) (Fig. 3E) across all groups. Together, these data support a relationship between rmTBI-induced TGF β signaling and elevated senescence pathways in the brain.

rmTBI mice demonstrate deficits in spatial learning and memory retention

To determine the effects of rmTBI on spatial learning and memory retention, mice were evaluated using a Barnes maze protocol that involved 3 days of acquisition training, culminating in a single probe trial on day 5 that tests an animal's ability to locate a previously learned escape hole (Fig. 4A). There were no significant differences in escape latency between sham ($n = 9$) and rmTBI ($n = 17$) groups during the day 2 training trials (Fig. 4B). On day 3, the rmTBI group had a significantly longer average escape latency during trial 5 compared to controls, demonstrating a spatial learning impairment (Fig. 4C). On day 5, the rmTBI group had a significantly longer average escape latency on the probe trial compared to the control group (Fig. 4D), demonstrating deficits in memory retention. Regression analysis revealed weak, nonsignificant positive correlations between escape latency and the protein expression levels of p16^{INK4A} (Fig. 4E), the ratio of pSMAD2:SMAD2 (Fig. 4F), and the ratio of p-p38MAPK:p38MAPK (Fig. 4G). Together, these data suggest that rmTBI results in enduring deficits in spatial learning and memory retention. However, these deficits did not correlate with the levels of TGF β signaling or senescence markers in this data set.

rmTBI mice demonstrate normal exploratory behavior and spatial acuity

To investigate the effects of rmTBI on exploratory behavior and spatial acuity, the spontaneous alternation of mice was assessed using a Y-maze (Fig. 5A). This measurement is based on the propensity of rodents to explore new environments. In this test, healthy mice prefer to visit a new arm of the maze rather than a familiar arm and will thus alternate entering different arms. Therefore, repetitive entries can be interpreted as a behavioral deficit (i.e., lack of curiosity) or loss of spatial acuity. Both sham and rmTBI groups demonstrated normal spatial acuity and exploratory behavior, exhibited by percent alternation levels that were above chance, which is 22.2% on the Y-Maze. No significant difference was observed in percent alternation between sham ($n = 10$) and rmTBI ($n = 17$) groups (Fig. 5B). Regression analysis revealed weak, nonsignificant correlations between escape latency and the protein expression levels of p16^{INK4A} (Fig. 5C) the ratio of pSMAD2:SMAD2 (Fig. 5D), and the ratio of p-p38MAPK:p38MAPK (Fig. 5E).

Senescence markers are associated with reduced anxiety-like behavior

To evaluate the effects of rmTBI on anxiety-like behavior, mice were subjected to the elevated plus maze (EPM), which consists of two open arms, two enclosed arms, and a partially-enclosed center (Fig. 6A). This assay is based on the dual behavioral preferences of rodents to explore new environments while preferring dark, enclosed spaces for safety. Rodents generally prefer the enclosed arms, but rodents with higher anxiety tend to spend an even smaller percentage

of time in the open arms (i.e., “anxiogenic” zones) compared to normal mice. The enclosed arms and the center of the maze are considered non-anxiogenic. No significant difference was observed between sham (n = 10) and rmTBI (n = 17) groups in the percent time spent in the anxiogenic zones (Fig. 6B), enclosed arms (Fig. 6C), or center (Fig. 6D). However, the rmTBI group trended towards spending a greater percentage of time in the anxiogenic zones compared to controls ($p = 0.055$) (Fig. 6B). Interestingly, regression analysis revealed positive correlations between percent time in anxiogenic zones and the protein expression levels of p16^{INK4A} (Fig. 6E), the ratio of pSMAD2:SMAD2 (Fig. 6F), and the ratio of p-p38MAPK:p38MAPK (Fig. 6G). In particular, the expression levels of both senescence markers were significantly correlated with time spent in anxiogenic zones (Fig. 6E,G). Together, these results suggest that rmTBI-induced senescence is associated with reduced anxiety-like behavior.

3.4. Discussion

Our previous study found that an *in vivo* model of BBBB is associated with albumin-induced TGF β hyperactivation and cellular senescence [232]. Therefore, we sought to determine whether rmTBI-induced BBBB would also trigger increased cellular senescence in the brain. In this study, we developed a novel closed-head injury model of rmTBI in which a single injury results in brief loss of consciousness and acute transient neurological symptoms, similar to the concussion-like symptoms experienced by patients with mild brain injury in the contact sports setting. Deaths were unpredictable and occurred in 15-20% (7/40 impacted mice). The observed immediate mortality upon repetitive impacts is consistent with reports of sudden death associated with second-impact syndrome in rmTBI patients [242,243]. Repeated administration of similar impacts over the course of 5 days resulted in diffuse BBBB as demonstrated by increased EB dye leakage into neural tissue across the whole brain of rmTBI mice compared to controls. Notably, BBBB was present in the GC layer of the hippocampus of rmTBI mice. Hippocampal protein expression analysis revealed increased activation of TGF β signaling and senescence pathways in the brains of rmTBI mice compared to controls, suggesting that rmTBI-induced BBBB is associated with TGF β hyperactivation and cellular senescence.

To examine if rmTBI-induced cellular senescence is associated with enduring neurobehavioral impairments, we performed BM, Y-maze, and EPM assays at least 2 weeks after animals had recovered from acute symptoms. rmTBI mice demonstrated pronounced deficits in spatial learning and memory retention compared to controls on the BM test. Since rmTBI mice exhibited hippocampal BBBB, these results are consistent with previous studies that have linked hippocampal BBBB with cognitive deficits in the context of aging [70,98]. Yet, rmTBI mice exhibited normal exploratory behavior and spatial acuity in the Y-maze, indicating that their spatial working memory was preserved. The relative expression of hippocampal senescence markers was not correlated with BM or Y-maze performance in this data set.

Notably, the rmTBI group exhibited a trend towards reduced anxiety-like behavior on the EPM assay, and higher senescence marker expression was associated with reduced anxiety-like behavior in this data set. These results are consistent with previous studies that have observed decreased anxiety-like behavior on the EPM and elevated zero maze (EZM) following TBI [244,245]. This phenotype has alternatively been described as “behavioral disinhibition” and is associated with increased risk-taking and impulsivity in TBI patients [246,247]. However, other investigators have reported increased anxiety-like behaviors during these tests following TBI in rodents [248,249]. Comprehensive literature reviews have tried to reconcile these discrepancies in behavioral results, but have not yet converged on experimentally-validated conclusions [250,251].

Future studies should determine whether anxiety phenotypes are dependent on the type and severity of injury or the time after injury at which anxiety testing is performed. For example, focal injuries such as those achieved by the controlled cortical impact (CCI) model of TBI may induce different behavioral results compared to the diffuse injuries caused by closed-head TBI models.

Notably, behavioral disinhibition indicates loss of executive function, which is largely attributed to the frontal cortex, hippocampus, and striatum. Given that senescence markers were significantly correlated with reduced anxiety-like behavior in our study, it's possible that BBBD-induced activation of TGF β and senescence pathways disrupts normal signaling in these brain regions. Understanding how rmTBI alters executive function may enable development of treatments for neurobehavioral deficits following injury.

3.5. Figures

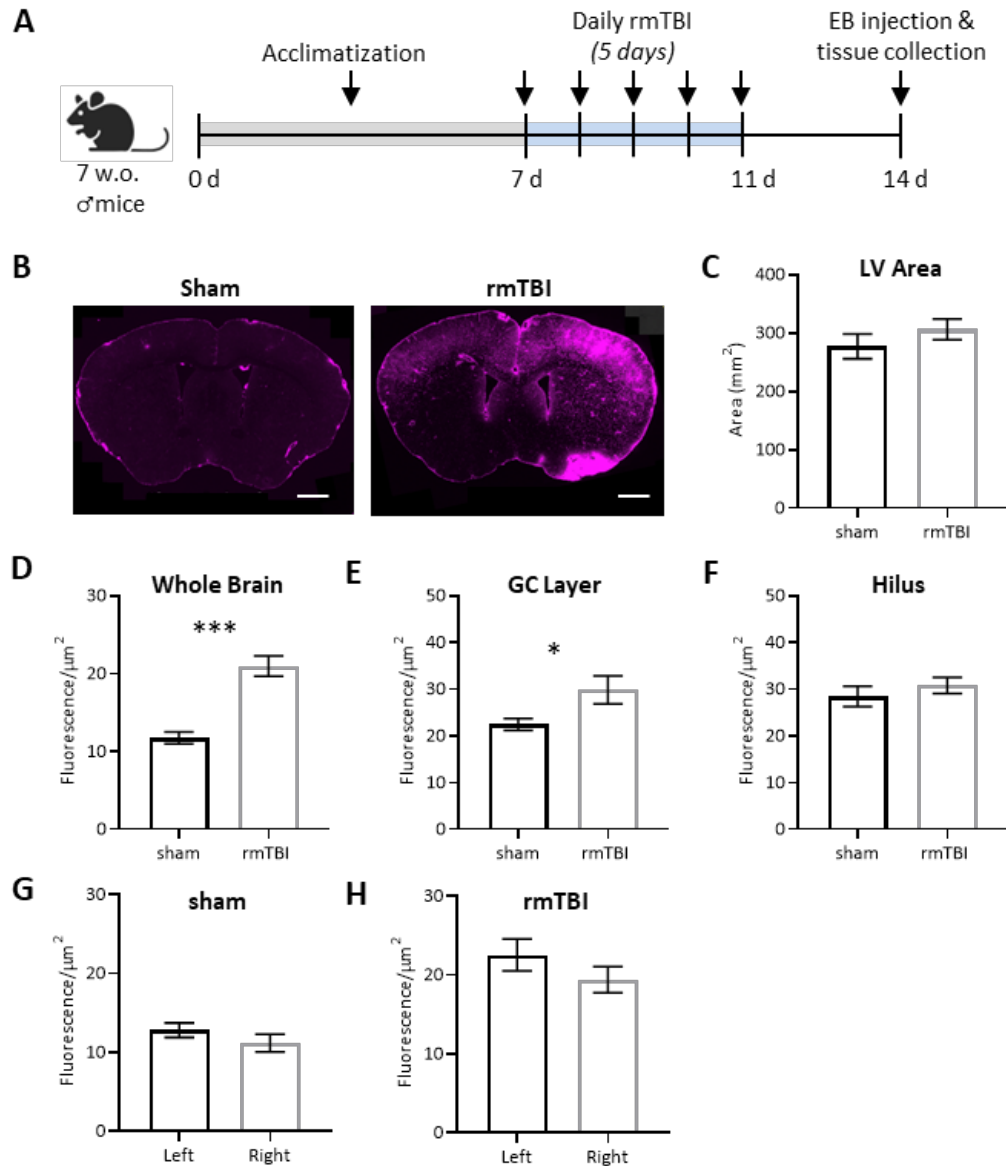


Figure 1. rmTBI induces diffuse blood-brain barrier disruption. (A) After one week of acclimatization, 7-week-old male mice ($n = 30$) were subjected to 5 consecutive days of rmTBI impacts. Mice were sacrificed and brains were extracted 3 days after the final impact. (B) Representative coronal sections of sham and rmTBI mouse brains following Evans blue (EB) dye injection. (C) There was no significant difference in average lateral ventricle area between rmTBI ($n = 15$) and control ($n = 11$) mice. 4 rmTBI mice died due to impacts and were removed from analysis. Average integrated density measurements of EB in the (D) whole brain and (E) granule cell (GC) layer of the hippocampal dentate gyrus were significantly higher in the rmTBI group compared to controls, indicating increased diffuse vessel damage and blood-brain barrier leakage. (F) There was no significant difference between groups in the hilus of the hippocampal dentate gyrus. There were no significant differences in EB leakage between left and right cerebral hemispheres in (G) sham or (H) rmTBI mice. Error bars depict mean $\pm$ SEM of each experimental

group. Scale bar = 1 mm. Asterisks denote a significant difference from control *p <0.05, **p<0.01, ***p<0.001.

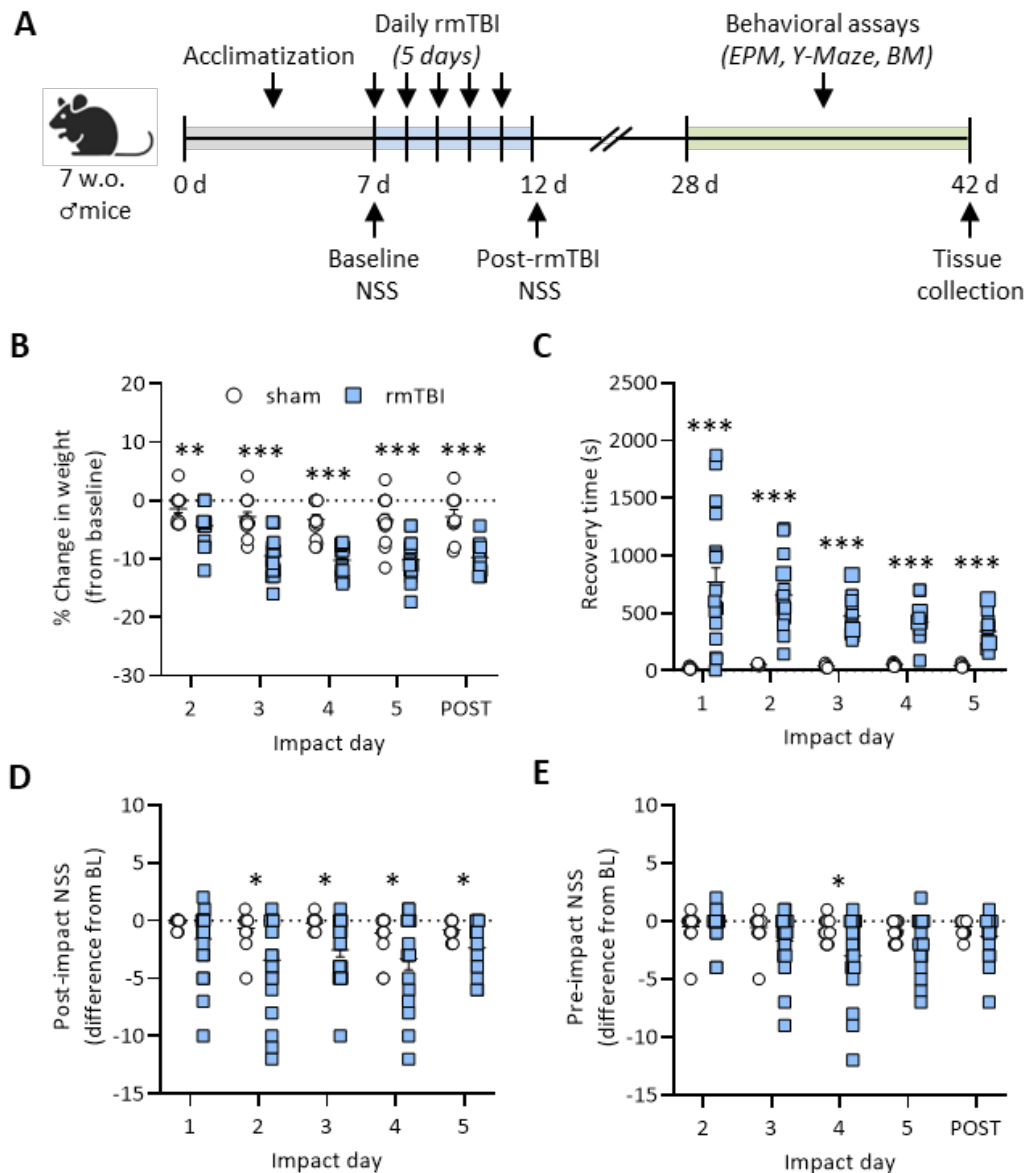


Figure 2. Closed-head model of rmTBI causes transient neurobehavioral impairment. (A) After one week of acclimatization, 7-week-old male mice (n = 30) were subjected to 5 consecutive days of rmTBI impacts. Pre- and post-impact neurological severity scoring (NSS) was performed daily to assess acute neurobehavioral deficits. Comprehensive behavioral assessments were performed approximately 2.5 weeks after rmTBI to examine enduring neurological effects. Mice were sacrificed and tissue was harvested 31 days after the final impact. (B) The average percent change in weight from baseline was significantly greater in rmTBI mice (n = 17) compared to control mice (n = 10), suggesting physical complications due to injuries. 3 rmTBI mice died due to impacts and were removed from analysis. (C) rmTBI mice had a significantly longer recovery time after anesthesia, suggesting loss of consciousness. Average recovery times for rmTBI mice decreased across treatment days. (D) The average difference from baseline in post-impact NSS composite scores in the rmTBI group and were significantly greater compared to control. (E) The average difference from baseline in pre-impact composite NSS scores were significantly greater

on impact day 4 but were not significantly different at the end of the treatment period (POST = 24 hours after the final impact), indicating neurobehavioral impairments were transient. Error bars depict mean $\pm$ SEM of each experimental group. Asterisks denote a significant difference from control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

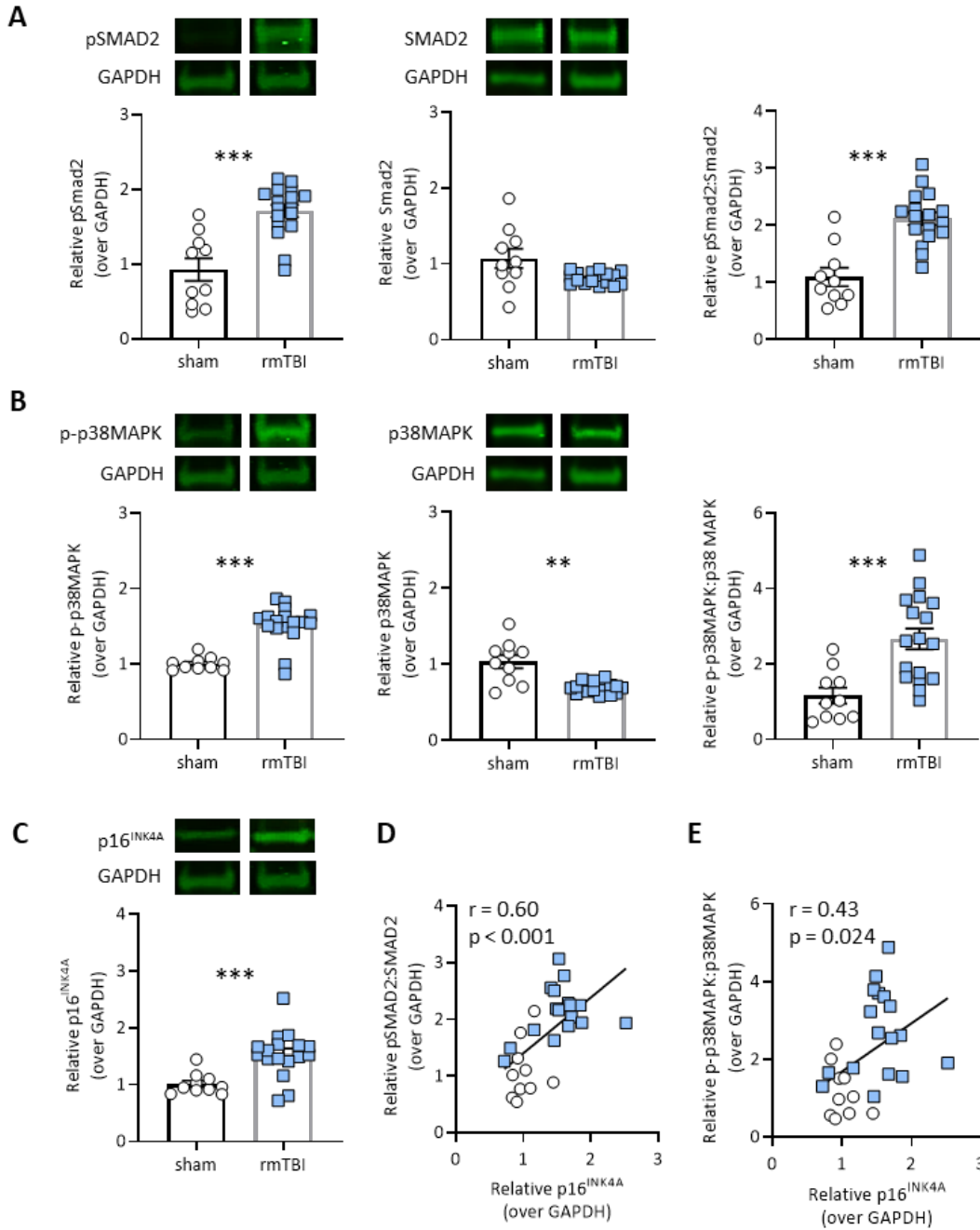


Figure 3. rmTBI increases hippocampal TGF β signaling and senescence marker expression. Representative Western blot images and bar graphs show protein expression levels normalized to GAPDH of (A) pSMAD2, SMAD2, and the ratio of pSMAD2:SMAD2, (B) p-p38MAPK, p38MAPK, and the ratio of p-p38MAPK:p38MAPK, and (C) p16^{INK4A} in sham (n = 10) and rmTBI (n = 17) mice. Regression analysis revealed significant positive correlations between the protein expression levels of (E) p16^{INK4A} and the ratio of pSMAD2:SMAD2, and (F) p16^{INK4A} and the ratio of p-p38MAPK:p38MAPK. Bar graphs depict mean $\pm$ SEM of each experimental group. Asterisks denote a significant difference from control *p < 0.05, **p < 0.01, ***p < 0.001.

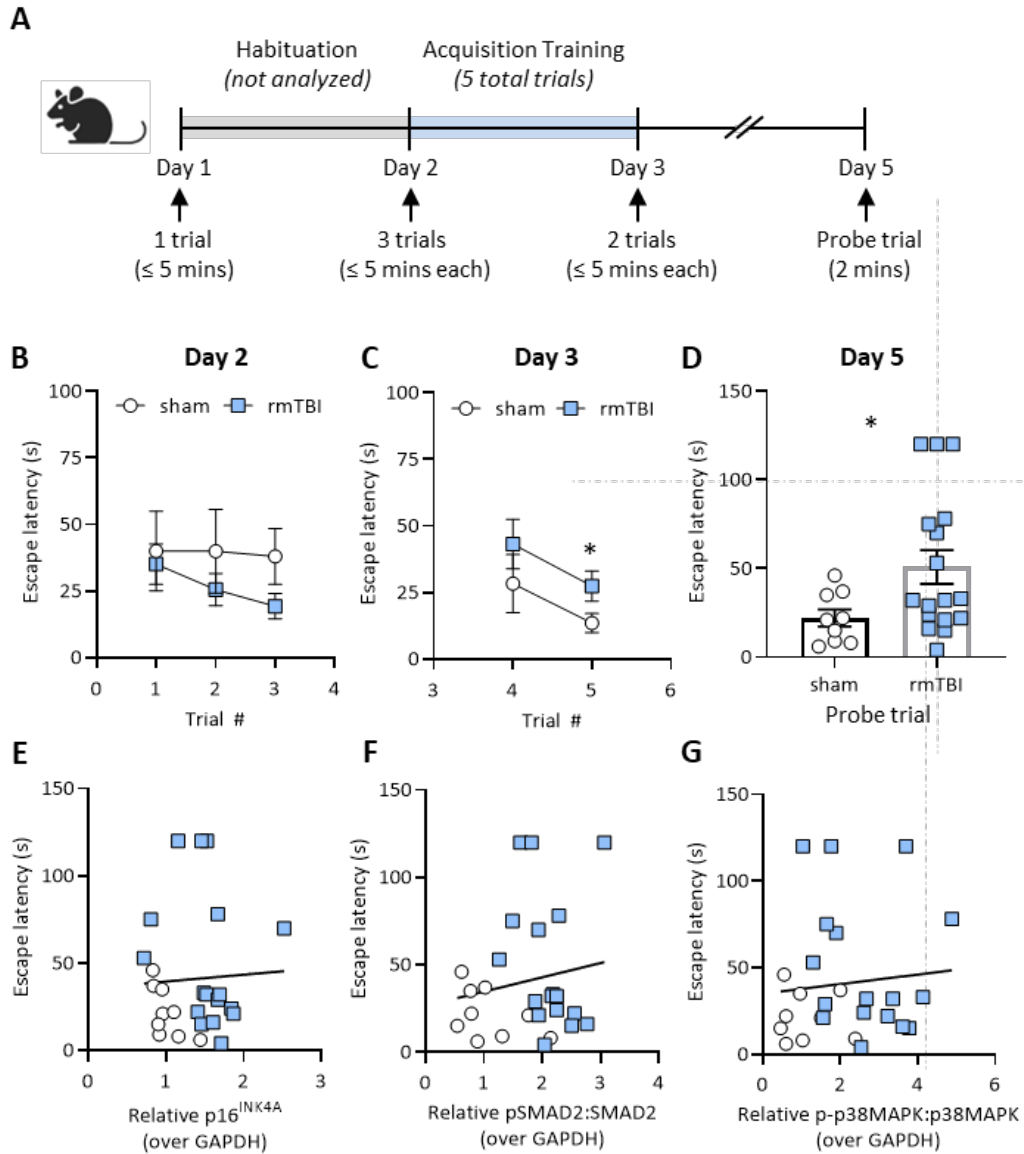


Figure 4. rmTBI mice demonstrate deficits in spatial learning and memory retention. (A) Barnes Maze protocol involved 3 days of training and assessment, culminating in a single probe trial on day 5 that tests an animal's ability to locate a previously learned escape hole. (B) There were no significant differences in escape latency between sham ($n = 9$) and rmTBI ($n = 17$) groups on day 2. (C) On day 3, the rmTBI group had a significantly longer average escape latency compared to controls during trial 5, demonstrating a learning impairment. (D) On day 5, the rmTBI group had a significantly longer average escape latency on the probe trial compared to the control group. Regression analysis revealed weak, nonsignificant positive correlations between probe trial escape latency and the protein expression levels of (E) p16^{INK4A}, (F) the ratio of pSMAD2:SMAD2, and (G) the ratio of p-p38MAPK:p38MAPK. Graphs depict mean $\pm$ SEM of each experimental group. Asterisks denote a significant difference from control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

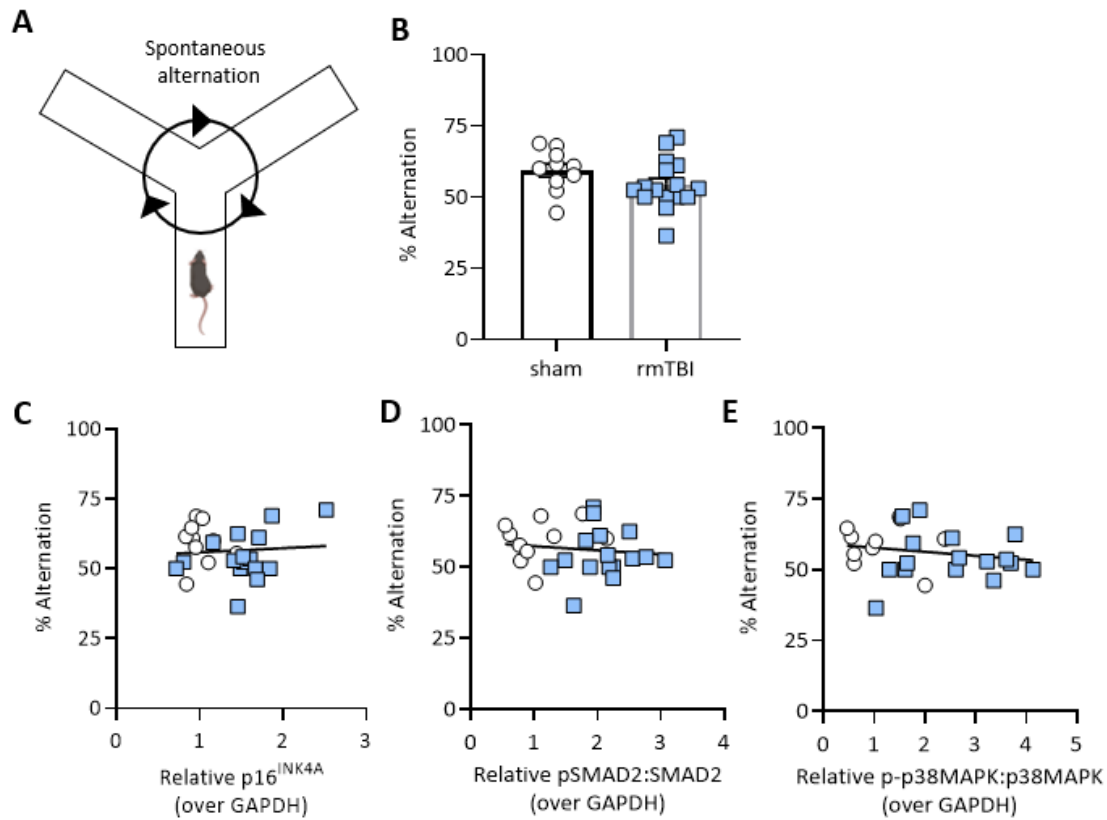


Figure 5. rmTBI mice demonstrate no deficits in short-term spatial learning. (A) Y Maze schematic demonstrating the spontaneous alternation pattern. (B) No significant difference was observed in percent alternation between sham and rmTBI groups. Regression analysis revealed weak, nonsignificant correlations between escape latency and the protein expression levels of (C) p16^{INK4A}, (D) the ratio of pSMAD2:SMAD2, and (E) the ratio of p-p38MAPK:p38MAPK. Graphs depict mean $\pm$ SEM of each experimental group.

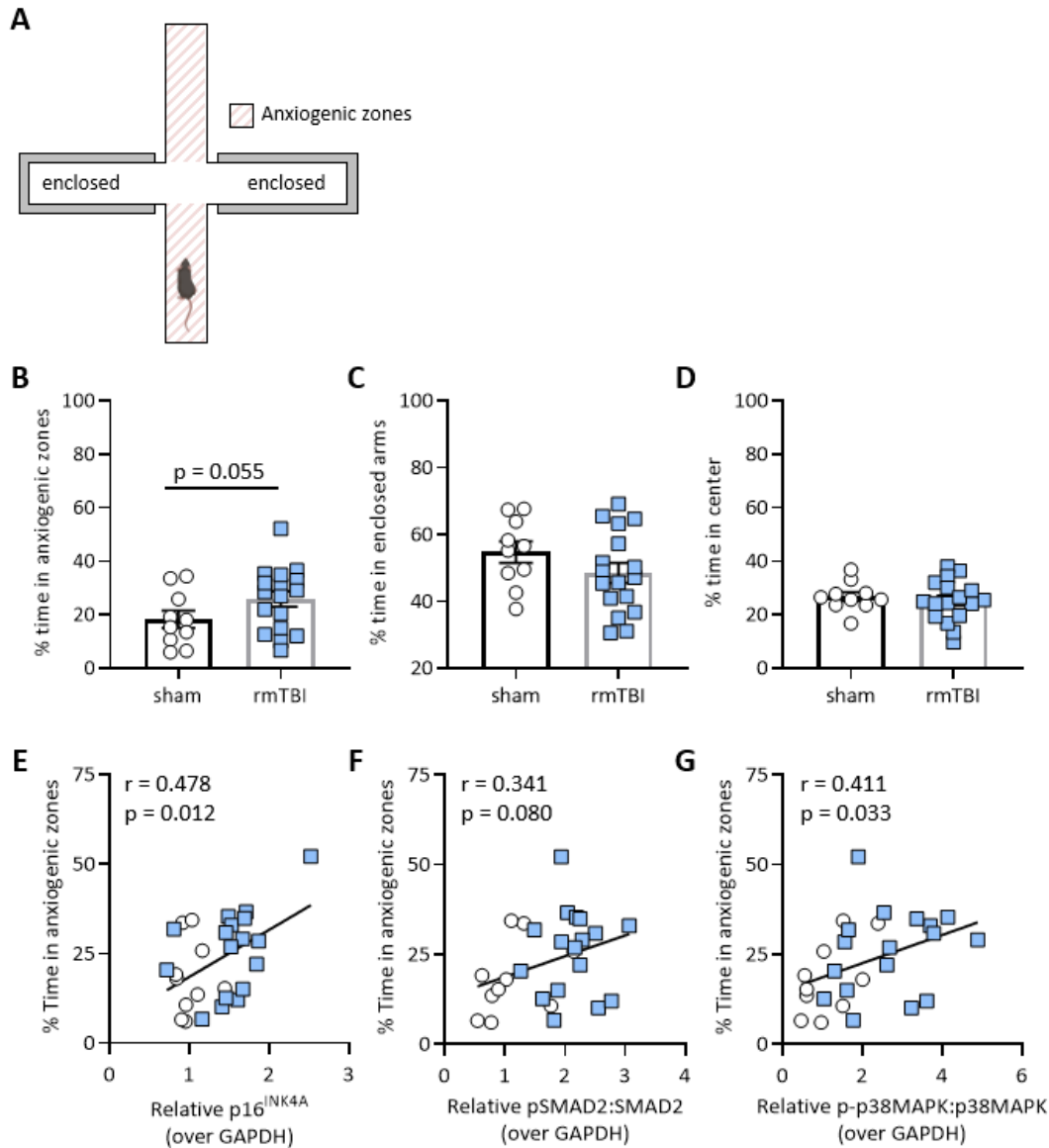


Figure 6. Senescence markers are associated with reduced anxiety-like behavior. (A) Elevated plus maze (EPM) schematic showing the “anxiogenic zones” on the exposed arms of the maze in contrast to the enclosed arms and the center, which are considered non-anxiogenic. (B) No significant difference was observed in percent time spent in the anxiogenic zones, (C) enclosed arms, or (D) center between sham (n = 10) and rmTBI (n = 17) groups. Regression analysis revealed positive correlations between percent time in anxiogenic zones and the protein expression levels of (E) p16^{INK4A}, (F) the ratio of pSMAD2:SMAD2, and (G) the ratio of p-p38MAPK:p38MAPK. Graphs depict mean $\pm$ SEM of each experimental group.

Supplementary Table 1. Antibodies

Target	Isotype	Supplier	Catalog #	Dilution
p16 ^{INK4a}	Rabbit IgG	Assay Biotech	C0285	1:500
SMAD2	Rabbit IgG	Cell Signaling	5339S	1:1000
pSMAD2	Rabbit IgG	Millipore Sigma	AB3849-I	1:1000
p38 MAPK	Rabbit IgG	Cell Signaling	9212S	1:1000
p-p38 MAPK	Rabbit IgG	Cell Signaling	4511S	1:1000
GAPDH	Rabbit IgG	Cell Signaling	2118S	1:1000
IRDye 800CW	Goat anti-rabbit IgG	LI-COR	926-32211	1:10,000

Chapter 4. Discussion

Adapted from: Preininger, M.K., Kaufer, D. (2022) Blood-brain barrier dysfunction and astrocyte senescence as reciprocal drivers of neuropathology in aging. Manuscript under review.

4.1. Perspectives on blood-brain barrier dysfunction, TGF β signaling, and senescence in neuropathology

Studies show that TGF β activation mediates senescence in various cell types *in vitro*, including fibroblasts [175–177], keratinocytes [178], mesenchymal stem cells (MSCs) [179], glioblastoma cells [180], cardiomyocytes [181], and lung epithelial cells [182]. Also, TGF β 1 as a SASP component was found to be an important mediator of paracrine senescence *in vivo* [183]. Furthermore, pharmacological attenuation of age-associated TGF β activation has rejuvenating effects in the aged brain [70,252], including suppression of cellular senescence [253]. In astrocytes, previous reports have identified triggers of astrocyte senescence such as oxidative stress [142], A β oligomers [144,254], HIV infection, and drug abuse [143]. Notably, TGF β signaling is also implicated in these senescence mechanisms. In the context of oxidative stress, TGF β activation increases the production of reactive oxygen species (ROS) by impairing mitochondrial function and inducing NADPH oxidases [211,212]. Indeed, TGF β 1 was found to induce senescence of bone marrow MSCs via increased mitochondrial ROS [179]. TGF β activation also suppresses the synthesis of antioxidant enzymes such as glutathione, causing redox imbalance and promoting further oxidative stress [210]. In the case of A β pathology, astrocytic TGF β overexpression increases A β generation in transgenic mice [213–215], and TGF β 1 expression is elevated in cortical astrocytes surrounding A β plaques in AD patients [216,217]. HIV infection and drug abuse were found to mediate astrocyte senescence in a β -catenin-dependent manner [143]. Notably, TGF β 1 operates through the canonical WNT/ β -catenin pathway, and these two pathways stimulate each other via SMAD effector proteins [255]. The precise molecular mechanisms by which TGF β signaling mediates senescence in astrocytes are unknown, but some evidence suggests that epigenetic alterations may be involved. A recent study found that canonical TGF- β /SMAD signaling promotes SIPS in cardiomyocytes via miR-29-induced loss of H4K20me3, and that disrupting TGF β signaling improves cardiac function in aged mice [181].

Together, these findings indicate that TGF β activation is a salient factor in age-related pathological changes and can directly mediate the senescence response under certain conditions. Indeed, the results presented here show that BBBD-induced TGF β activation via albumin extravasation is a potential physiological trigger of astrocyte senescence in the aging brain. Prolonged exposure to albumin via experimental treatment or via traumatic brain injury was sufficient to induce senescence marker expression. In a mouse model of BBBD, astrocyte senescence was preventable by pharmacological inhibition of TGF β R ALK5 or astrocyte-specific knockdown of TGF β R2.

In addition to activating canonical TGF β signaling, albumin uptake activates the p38MAPK pathway in astrocytes [90], which is known to play a central role in the regulation of astrocyte senescence and SASP expression [144,199]. Thus, it follows that simultaneous activation of canonical TGF β , p38MAPK, and potentially other unknown pathways by albumin can mediate astrocyte senescence.

Reciprocally, the molecular and functional effects of senescent astrocytes on BBB integrity have not been established. Certainly, astrocytes produce SASP factors such as MMPs and IL-6,

which are known to increase endothelial barrier permeability. MMPs induce BBBD via disruption of TJ proteins [256,257], and IL-6 upregulates cell adhesion molecules (CAMs) on ECs, which facilitates leukocyte transmigration across the endothelium. Interestingly, one study found that accumulation of senescent ECs and pericytes is associated with compromised BBB integrity due to reduced TJs; however, the blood vessel coverage by astrocytic end-feet was not altered [258]. This suggests that EC and pericyte senescence is not the proximal cause of reduced astrocyte end-foot coverage observed in age-associated BBBD. These findings raise another possibility—that each cellular component of the NVU is differentially susceptible to physiological triggers of senescence. Indeed, TGF β signaling is complex and context-dependent [259,260]; therefore, TGF β hyperactivation may have variable effects among different cell types and conditions. Future studies should seek to elucidate the context in which various triggers promote senescence during aging and the extent of their respective contributions.

4.2. Concluding remarks

Astrocytes are a diverse population of cells that perform essential functions in the brain related to neuronal support, immune modulation, and blood-brain barrier regulation and maintenance. Collectively, these functions ensure that the brain environment remains optimal for neuronal function. In aging, astrocytes adopt a neurotoxic phenotype following age-related BBBD that is mediated by TGF β hyperactivation. Furthermore, aging is associated with an accumulation of senescent astrocytes, which contribute to neuroinflammation and neurodegeneration. Given that TGF β is a salient factor in aging pathologies and is known to mediate senescence in various cell types, BBBD-induced TGF β activation via blood protein extravasation should be further explored as a potential trigger of astrocyte senescence in the aging brain. Identifying physiological triggers of astrocyte senescence is important for understanding the complex series of events that facilitate the development of neurodegenerative disease.

Astrocytes play an important role in BBB regulation and maintenance through their involvement in the NVU. Since the NVU is highly interdependent, the impacts of perivascular astrocyte senescence on each component of the NVU merit investigation. Genetic tools that enable selective ablation of senescent astrocytes [145] should be leveraged to investigate the role of senescent astrocytes in the context of BBBD. Another outstanding question in the context of aging is how the global shift in transcytosis mechanisms manifests in each NVU cell type. Specifically, how do perivascular astrocytes experience this shift, and does cellular senescence play a role in facilitating this shift? In other words, do senescent cells experience more nonspecific transcytosis than their youthful counterparts?

Given the importance of astrocytes in preserving the BBB, it's likely that initial BBBD-induced astrocyte senescence establishes a positive feedback loop that results in progressive neurological decline. In this scenario, the accumulation of functionally impaired senescent astrocytes causes more BBBD and thus more BBBD-induced astrocyte senescence—perpetuating a vicious cycle of BBBD and associated functional decline. Further studies should investigate how this process is regulated and explore potential strategies to prevent its onset.

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