

TARGETING THE EXTENDED RENIN-ANGIOTENSIN SYSTEM TO TREAT
TRAUMATIC BRAIN INJURY

by

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DEDICATION

To my father: for serving as Polaris when I am without a compass.

To my mother: for keeping me close to home no matter how far I stray.

To my brother: for sharing his talent and giving me someone to admire.

I owe everything to them.

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ABSTRACT

Targeting The Extended Renin-Angiotensin System To Treat Traumatic Brain Injury:

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Molecular Therapeutics

Angiotensin II (Ang II), the vasoactive peptide of the classic renin-angiotensin system (RAS), serves many roles beyond blood pressure modulation. Activation of angiotensin II type I receptor (AT1R) by Ang II within the central nervous system can promote glial proliferation, local inflammation, and a decrease of cerebral blood flow. Angiotensin-(1-7) (Ang-(1-7)) is cleaved from Ang II by the actions of angiotensin converting enzyme 2 (ACE2) and is a ligand of the extended RAS. Ang-(1-7) has low affinity for AT1R instead acting as the ligand to the Mas receptor (MasR), which is widely expressed in the brain. The ACE2/Ang-(1-7)/MasR axis acts to counter Ang II signaling at many levels and has anti-inflammatory, vasodilatory, and neuroprotective effects. We are investigating novel therapeutics to improve recovery from traumatic brain injury (TBI), using a mouse model of controlled cortical impact (CCI). Our laboratory has previously shown that inhibition of AT1R signaling improves recovery from CCI injury in mice. Here we sought to determine if activation of Ang-(1-7)/MasR signaling would also be beneficial in this TBI model.

Male adult C57BL/6 mice were injured by CCI. Ang-(1-7) or vehicle was administered by daily subcutaneous (SQ) injection or continuous SQ infusion, starting at 1 or 6 hours post injury until animals were sacrificed at 3 or 29 days post injury (dpi). Ang-(1-7) treatment attenuated motor deficits determined by the rotarod test at 3 dpi and improved cognitive recovery in the Morris water maze at 29 dpi. Brain histology and/or MRI indicated that Ang-(1-7) treated mice had a smaller lesion volume at 3, 10, 24, and 29 dpi. Pretreatment with the MasR antagonist A779 blocked Ang-(1-7)-reduced lesion volume at 3 dpi suggesting that this beneficial effect of Ang-(1-7) treatment was mediated through the MasR. Immunohistochemistry revealed that Ang-(1-7) treatment reduced microgliosis and astrogliosis and decreased neuronal and capillary loss at different time points following injury. In summary, Ang-(1-7) treatment, administered SQ after injury had anti-inflammatory, neuro- and cerebrovascular protective actions leading to improved functional and pathological recovery in a mouse model of TBI. These data show for the first time that Ang-(1-7) has potential therapeutic use for TBI.

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LIST OF ABBREVIATIONS

- ACE - Angiotensin Converting Enzyme
- ACE2 - Angiotensin Converting Enzyme 2
- ADH - Antidiuretic Hormone
- AGT - Angiotensinogen
- Alam - Alamandine
- AMPK – AMP activated protein kinase
- Ang A – Angiotensin A
- Ang I – Angiotensin A
- Ang II – Angiotensin II
- Ang III –Angiotensin III
- Ang IV – Angiotensin IV
- Ang-(1-7) – Angiotensin-(1-7)
- ANOVA –Analysis Of Variance
- APA –Aminopeptidase A
- APN –Aminopeptidase N
- ARB –Angiotensin Receptor Block
- AT1R – Angiotensin II Receptor Type 1
- AT2R – Angiotensin II Receptor Type 2
- AT4R – Angiotensin IV Receptor
- ATP – Adenosine Triphosphate
- BBB – Blood-Brain Barrier
- BDNF - Brain-derived neurotrophic factor
- BrdU - 5-bromo-2'-deoxyuridine
- CBF – Cerebral Blood Flow
- CCI – Controlled Cortical Impact
- CD11b – Cluster of Differentiation 11b
- CD31 –Cluster of Differentiation 31
- CNS –Central Nervous System
- COX-2 - cyclooxygenase-2
- CXCR4 - C-X-C chemokine receptor type 4
- D -Deletion
- DAMP - Damage-associated molecular patterns
- DIZE - diminazene aceturate
- DMNV - dorsal motor nucleus of the vagus
- Dpi – days post-injury
- eNOS - Endothelial Nitric Oxide Synthase
- FCCS - Fluorescence Cross Correlation Spectroscopy
- FDA – Food and Drug Administration
- FPI –Fluid Percussion Injury
- FRET - Fluorescence Resonance Energy Transfer Efficiency

- GFAP - Glial fibrillary acidic protein
- GFR - Glomerular filtration rate
- GLUT4 - Glucose transporter type 4
- GPCR - G protein-coupled receptors
- H - hour
- HEK - Human Embryonic Kidney
- HMGB1 - High Mobility Group Box 1 protein
- hpi –hours post-injury
- I - Insertion
- Iba1 - Ionized calcium binding adaptor molecule 1
- ICAM-1 - Intercellular Adhesion Molecule 1
- ICP – Intracranial Pressure
- ICV - Intracerebroventricular
- IL-1 α - Interleukin 1 alpha
- IL-1 β – Interleukin 1 beta
- IL-2 – Interleukin 2
- IL-6 – Interleukin 6
- iNOS – Inducible Nitric Oxide Synthase
- IP - Intraperitoneal Injection
- IRAP - Insulin-Regulated Aminopeptidase
- JAK –Janus Kinase
- KO – Knockout
- LPBN - Lateral Parabrachial Nucleus
- LPS - Lipopolysaccharide
- MAPK - Mitogen-Activated Protein Kinases
- MasR – Mas receptor
- MCAO –Middle Cerebral Artery Occlusion
- MLCK –Myosin Light-Chain Kinase
- MMP-9 - Matrix Metalloproteinase 9
- MnPO – Median Pre-Optic Nucleus
- MrgD - MAS-related G-protein Coupled Receptor
- MRI – Magnetic Resonance Imaging
- mRNA – Messenger Ribonucleic Acid
- MWM – Morris Water Maze
- NADPH - Nicotinamide Adenine Dinucleotide Phosphate
- NF κ B - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
- NO – Nitric Oxide
- Nox2 - NADPH Oxidase 2
- PBBI - Penetrating Ballistic-Like Brain Injury
- PBS – Phosphate Buffered Solution
- PDW - Proton Density Weighted
- PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase
- PPAR γ - Peroxisome Proliferator-Activated Receptor gamma

- PRO - Prorenin
- PRR – Prorenin receptor
- PTV - Posttraumatic Cerebral Vasospasm
- PVN - Paraventricular Nucleus
- RAGE - Receptor for Advanced Glycation End Product
- RAS –Renin-Angiotensin System
- ROS –Reactive Oxygen Species
- RPa - Raphe Pallidus Nucleus
- RVLM - Rostral Ventrolateral Medulla
- s - seconds
- S1BF - Primary Somatosensory Cortex Barrel Field
- SAH - Subarachnoid Hemorrhage
- SEM – Standard Error of the Mean
- SFO - Subfornical Organ
- SN – Solitary Nucleus
- SON - Supraoptic Nucleus
- SQ – Subcutaneous
- STAT - Signal Transducer and Activator of Transcription
- TBI – Traumatic Brain Injury
- TGFβ – Transforming Growth Factor Beta
- TICA - Traumatic Intracranial Aneurysms
- TIMP-1 - Tissue Inhibitor of Metalloproteinases 1
- TLR2 – Toll-like Receptor 2
- TLR4 – Toll-like Receptor 4
- TNFα – Tissue Necrosis Factor Alpha
- TUNEL - Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
- V2R – Vasopressin Receptor 2
- VA – anterior thalamic nucleus
- VCAM-1 - Vascular Cell Adhesion Molecule 1
- VEGF-A - Vascular Endothelial Growth Factor A
- VEGF-B - Vascular Endothelial Growth Factor B
- VOLT - Vascular Organ of the Lamina Terminalis
- ZO-1 - Zonula Occludens-1

CHAPTER 1: Introduction

Traumatic brain injury (TBI) has been called the “signature injury” of the recent conflicts in Iraq and Afghanistan. Since the start of the Global War on Terrorism, over 380,000 cases of TBI have been diagnosed in U.S. military active duty service members, National Guard members, and reservist (32). Moreover, TBI is a leading cause of death and disability worldwide. Global assessments estimate that over 27 million cases of TBI occur annually, resulting in over 8 million years lived with disability (155). Of these cases, the most common mechanisms of injury are motor vehicle accidents and falls (155).

TBI results from an acute transfer of kinetic energy through the skull and into the brain. This occurs either when an object strikes the skull, or when the skull strikes an object (128; 415). Severity varies based on many factors including impact location, force of impact, and whether the skull is intact. TBIs are classically stratified on a severity scale that includes mild, moderate, severe, and penetrating strata (342). Recently, injuries from blast (such as seen in military personnel) were recognized to have unique pathophysiological features and thus are classified separately (183; 213; 227; 315). The pathophysiology of TBI is commonly divided into two separate injury types: primary and secondary. Primary injury is in reference to the parenchymal destruction that occurs as an immediate consequence of acute kinetic energy transfer to the brain (i.e. membrane rupture, hemorrhage, axotomy, etc). Secondary injury is in reference to the deleterious molecular, cellular, and tissue responses that occur hours to days following the primary injury (274).

Primary injury can be prophylactically avoided through the employment of safety features such as seatbelts, airbags, and helmets. In the U.S., for example, safety features such as motorcycle helmets are estimated to reduce collision-based fatality by up to 37 percent and reduce the frequency of TBI by up to 67 percent (76; 383). Though protective measures provide a way to mitigate primary brain injury, treatment options are limited once a TBI has been sustained. In fact, over 30 clinical trials have failed to show efficacy in TBI, leaving patients without any promising therapeutic options (79). Current medical and surgical management of acute TBI focuses on prophylactic measures to decrease intracranial pressure (ICP) and seizure frequency, with other treatments addressing symptoms rather than injury; and there are no available treatments that mitigate the secondary injury of TBI (49). The lack of treatment options in TBI underlies the need to develop and test novel neuroprotective agents that modulate secondary injury. Endogenous hormonal axes, such as the renin-angiotensin system (RAS), are implicated in many of the pathophysiological processes that constitute the secondary injury of TBI (401; 402). Thus, the RAS serves as a logical system to target in hopes of developing a therapeutic targeted at reducing the burden of TBI.

The RAS is a highly characterized hormonal axis whose most studied role in vertebrates is the modulation of blood pressure (360). In the simplest canonical paradigm, a reduction of blood pressure results in increased secretion of the enzyme renin from the kidneys into the circulation. Once within the circulation, renin actively cleaves the liver-produced peptide known as angiotensinogen (AGT) into a 10-residue peptide known as angiotensin I (Ang I) (360). Angiotensin converting enzyme (ACE), produced by lung endothelium, further cleaves Ang I, resulting in a highly active octapeptide product

known as angiotensin II (Ang II) (Figure 1a). Ang II is the main effector peptide of the RAS and the primary ligand for the G-protein coupled receptor (GPCR) Angiotensin II Type 1 receptor (AT1R) (Figure 1b). Signaling of AT1R in the vasculature, brain, pituitary gland, and adrenal glands by Ang II activates various physiological mechanisms that increase blood pressure (i.e. vasoconstriction, sympathetic stimulation and thirst, water retention, and salt uptake, respectively), which in turn combats the initial hypotensive stimuli (360).

Blood pressure can be modulated through physiological means other than RAS signaling and conversely, RAS signaling can modulate physiological characteristics beyond blood pressure (48; 58). Furthermore, it is now very clear that the regulation of blood pressure that occurs via RAS modulation cannot be attributed solely to its classical signaling pathway (210). Over the last few decades, an entire “extended RAS” system has been described that includes unique cleavage products of AGT, multiple enzymes that catalyze these cleavage events, and new receptors for the multiple cleavage products (210). Almost all components of both the RAS and the extended RAS play a role in blood pressure modulation, with the extended RAS possessing more of a “fine-tuning” character (210). Some components of the extended RAS, such as the Ang II cleavage product known as Angiotensin-(1-7) (Ang-(1-7)), act to oppose many of the effects of Ang II on blood pressure. However, just as with the classical RAS, duties of the extended RAS extend beyond hemodynamic regulation (120; 275; 430; 431).

Components of both the classic RAS and the extended RAS are found in many organ systems throughout the mammalian body. It is now understood that in addition to the classical renal-pulmonary-hepatic RAS that regulates blood pressure, there are tissue

specific RASs in several different organ systems that contain all the components needed to support a local RAS independent of the classical system (43; 281). Exactly what function these localized RAS systems serve remains a subject of controversy. Aside from the function of organ-specific RASs, there is no debate that the *individual* components found in these spaces serve active functions (43; 281).

In addition to its role in regulating blood pressure, RAS activity influences inflammation, oxidative stress, autoimmunity, cardiovascular disease, neuroglia dysfunction, and cognitive decline (46; 390; 431). Certain pathologies, such as stroke and TBI, present with a mixture of these disease processes (36). As the underlying pathophysiology of TBI is composed of various RAS-influenced processes, we hypothesized that we could manipulate the extended RAS to benefit outcomes after TBI. Specifically, we hypothesized that administration of Ang-(1-7) would benefit recovery in a controlled cortical impact model of TBI in mice. We aimed to assess the effect of Ang-(1-7) on various morphological, histological, and behavioral consequences classically associated with the CCI model of rodent TBI.

This dissertation will serve to summarize both potential and established relationships of the RAS with TBI, to highlight our contribution to this body of knowledge, and to discuss the implications of our findings. We start by reviewing classic RAS signaling, and then follow by addressing TBI pathophysiology in a cell-specific manner. We proceed by reviewing potential or established roles of the RAS and extended RAS following TBI, and do so in a RAS component-specific manner. We then introduce our recent findings regarding the benefits of extended RAS modulation for TBI in mice.

Finally, we close by discussing the implications of our findings and propose various future studies that merit investigation.

1A - TRAUMATIC BRAIN INJURY (TBI)

1A.1 - Gross pathological features

Depending on the severity of injury, gross pathological features will vary drastically. In the mildest case of TBI, brain swelling is not always observed, neurological deficits may or may not be present, and sophisticated imaging modalities are required to identify the region of impact (180). In moderate and severe TBI, neurological deficits are almost always present. Cell membrane damage and hemorrhage result in failure of cellular fluid homeostasis. Such failure allows for intracellular fluid shifts that cause cytotoxic brain edema around the site of injury and an increase of ICP that can eventually lead to fatal brain herniation (88; 391). Mechanical membrane damage also allows for leakage of intracellular contents; creating a hyperexcitable neuronal environment that leaves patients seizure prone (418; 429). Furthermore, heightened ICP can collapse vessels and impair both cerebral perfusion and oxygenation (97; 147). In the case of penetrating injury, increases of ICP can force both damaged and undamaged brain tissue to herniate from the cranial defect, ultimately strangulating the healthy tissue and exacerbating the extent of primary injury (174).

1A.2 - Neurons

TBI often results in neuronal compromise and/or death. However, location and severity of injury contribute largely to outcome (182; 311). Immediately following TBI, biomechanical disruption of neuronal membranes causes uncontrolled release of intracellular ions and neurotransmitters. In attempt to restore membrane potential, sodium

– potassium pumps must increase ionic flux; and doing so requires adenosine triphosphate (ATP). The increase in ATP expenditure dramatically increases their need for glucose, which can further strain already distressed neurons (17; 139; 338; 436). Depending on the severity of TBI, metabolic demands of injured neurons are not always met. This results in persistent leakage of ions and various neurotransmitters, including glutamate (44; 146; 171). The increase of extracellular glutamate, exposure to extravasated blood products, lack of oxygen, and stress associated with edema, all contribute to a neurotoxic environment and can render neurons hyperexcitable (192; 280; 418). As such, post-traumatic seizures are often observed after TBI (398).

For many neurons the biomechanically induced damage is beyond repair. These neurons take in sodium, swell, and undergo necrotic death (81; 371). Some neurons remain viable and return fully to basic function. Others neurons avoid a necrotic fate and remain viable yet compromised. Subject to an array of noxious stimuli in the post-TBI milieu, compromised neurons are directed towards apoptosis. Here, the neurons shrink and are slowly disassembled until apoptotic death is complete (59; 93; 224). In post-mortem brain samples, neuronal presence can be assessed with histological and immunohistochemical tools including Fluoro-Jade B staining (tags degenerating neurons), as well as NeuN staining (generic nuclear protein marker found in neurons) (70; 251; 331). Indeed, decreases of NeuN immunoreactivity and increases of Fluoro-Jade B staining have been observed after TBI in mammals (116; 327). However, biomechanical insult is only one of many factors that contribute to neuronal compromise following TBI. In fact, much of the neuronal death that ensues hours to days after TBI results from actions of neighboring non-neuronal cells. Such is the case when neurons are

exposed to inflammatory mediators from glia, gliotic scarring from astrocytes, and hypoxemia following cerebrovascular damage (170; 433; 438).

1A.3 - Microglia and Inflammation

TBI is characterized by acute cell injury and inflammation followed by low-level chronic inflammation (217; 349). Upon mechanical insult, ruptured cells release small molecules known as damage associated molecular patterns (DAMPs) (341). DAMPs stimulate receptors commonly found on inflammatory cells, including toll like receptor 4 (TLR4), toll like receptor 2 (TLR2), and receptor for advanced glycation end products (RAGE). Of particular importance in the CNS is the action of DAMPs on microglia. Activation of these receptors on microglia triggers a robust inflammatory response (355; 440).

Activated microglia produce an array of pro-inflammatory mediators including interleukin 1 beta (IL-1 β), tissue necrotic factor alpha (TNF α), and many reactive oxygen species (ROS) (39; 82; 263; 306; 375). Similar to microglia, activated astrocytes secrete pro-inflammatory mediators such as TNF α , α -chemokines, cyclooxygenase 2 (COX-2), and matrix metalloproteinase 9 (MMP-9) (125; 284; 294). Most of these inflammatory mediators have well-established neurotoxic potential (209; 363). Activated microglia also release glutamate, which can potentiate hyperexcitability in neurons (20; 21). This wave of inflammation can disrupt blood-brain barrier (BBB) function and allow immune cells such as neutrophils, meningeal macrophages, and lymphocytes to converge on injured CNS tissue, augmenting the inflammatory process (28; 374). Furthermore, any physical breach of the immune privileged CNS — whether by hemorrhage or by penetrating brain injury — can expose the cells to non-native pathogens; most of which serve as extremely

potent inflammogens that further exacerbate injury (114). Though inflammation is a necessary physiological response for survival, it responsible for much of the secondary injury that follows TBI (392).

1A.4 - Astrocytes

Astrocytes provide nutrients to nervous tissue, maintain balance of extracellular ion concentrations, provide chemical and structural support to neurons, and interact with the cerebrovasculature to form the BBB (352). In response to traumatic insult, astrocytes form an astrogliotic scar that serves to wall off healthy tissue from dead or damaged tissue, reducing further exposure of neurons to noxious stimuli (285). However, the formation of an astrogliotic scar has both benefits and consequences regarding pathological progression of TBI. In particular, physical and functional recoveries are often hindered in neuroanatomical regions where gliotic scarring has occurred. This is in part due to the physical barrier formed by the scar tissue itself, and in part due to the secretion of various proteoglycans — molecules known to inhibit neurite outgrowth (100; 237; 285; 419).

1A.5 - Cerebrovasculature

Deregulation of cerebral blood flow is a key feature of TBI (35; 60). While milder TBI can cause disturbances to cerebral microcirculation, severe TBI can shear both small and large vessels, promoting an ischemic injury by compromising the cerebrovasculature (312; 396). Such complications are readily observed in animal models. In rat models for instance, diffuse TBI can decrease cerebral blood flow (CBF) by up to 50 percent (165; 433; 438). Unfortunately, because brain glucose usage spikes dramatically following

TBI, there exists a mismatch between CBF and glucose demand. This relationship potentiates damage in already compromised nervous tissue (56).

Posttraumatic cerebral vasospasm (PTV), the transient constriction of cerebrovascular arteries and/or arterioles, occurs in at least 30 percent of patients within 10 days of sustaining a severe TBI. For blast injuries, the observed rates are often higher (16; 213). A 57-patient study by Armonda et al. reported that nearly half of the patients who sustained a severe blast-TBI developed PTV that peaked between 14 and 16 days after injury, and lasted up to one month following injury (16). Though subarachnoid hemorrhage is a regular cause of cerebral vasospasm, PTV can occur without it (266). Radiographic studies report rates of PTV between 10 and 30% in TBI patients without subarachnoid hemorrhage (266). Similar findings are observed with regard to severe blast-TBI (16). PTV likely occurs via multiple mechanisms; these include endothelial and smooth muscle reactivity to inflammatory mediators, calcium dysregulation, expression of endothelin, and upregulation of vascular contractile proteins within the vascular walls (33; 136; 151; 289). Whether causative or not, there is an established association of poorer outcomes for TBI patients with PTV when compared to those with non-reactive vasculature (7; 16).

Another consequence of TBI is the formation of traumatic intracranial aneurysms (TICA) (193). TICAs are prone to rupture, thus increasing the chance of subarachnoid hemorrhage (SAH) (75). The biomechanics of severe and/or penetrating brain injury increases the risk for developing a TICA (256). For penetrating brain injury, disruption of the vascular wall from contact with a penetrating missile or bone fragment may weaken vascular integrity (244). Similarly, severe closed head injury can exert damaging strain

on vessels (244). In either case, once weakened, cerebral vessels are prone to aneurysm formation (193). Following TBI, the presence of a TICA can increase the risk of mortality by up to 50 percent (193).

1A.6 - Oligodendrocytes

Oligodendrocytes are the primary cells responsible for myelinating axons within the CNS and their injury or death underlies the demyelination that can occur subsequent to TBI. Furthermore, oligodendrocytes are susceptible to a variety of noxious stimuli that occur following TBI (18; 47; 257; 291). Reports of glutamate-induced oligodendrocytic death are abundant, and suggest one possible reason why demyelination occurs following TBI. Additionally, cytokines such TNF α , IL-1 β , interleukin 2 (IL-2), and interferon gamma (IFN γ) all exert direct toxicity on oligodendrocytes. As each of these cytokines dramatically increases following TBI, pro-inflammatory mediated oligodendrocyte damage likely underlies demyelination after trauma (13; 63; 67; 204; 321; 343).

1A.7 - Blood-brain barrier

The BBB is a selective, semipermeable barrier made up of cerebrovascular endothelial cells, astrocytic foot processes, and pericytes that collectively separate circulating blood from the CNS and functions to protect the brain from blood-borne pathogens (41). Specifically, expression of tight junctions proteins between adjacent cerebrovascular endothelial cells restrict free passage of solutes, while various molecules found on astrocytes and pericytes serve to maintain BBB homeostasis (41). Following TBI, mechanical force often disrupts or destroys cerebrovascular vessels causing cerebral hemorrhage. In this early phase of injury, the BBB is grossly compromised (57). As BBB

permeability is directly associated with injury severity, time required to restore BBB integrity varies (22; 135; 295). One rodent study demonstrated biphasic peaks of BBB compromise at 6 and 72 hours after injury in a controlled cortical impact (CCI) model of TBI. A similar study showed that the BBB is still compromised up to 7 days after injury (22; 135; 295). However, BBB integrity is influenced not only by the cells that constitute its building blocks, but also by microglia and neurons. Glutamate, released upon neuronal death following TBI, promotes endothelial permeability. TNF α and IL-1 β , the prototypic pro-inflammatory cytokines secreted by microglia following TBI, also increase endothelial cell permeability (61; 148). Furthermore, tissue transforming growth factors (TGF β), vascular endothelial growth factors (VEGF), ROS, and matrix metalloproteinases — all of which are deregulated following TBI — alter endothelial permeability (57).

1A.8 - Rodent models

Rodent neuroanatomy is distinct from that of humans, particularly as the lissencephalic structure of the rodent brain is very different from the gyrencephalic brain of higher mammals, including humans. Nonetheless, the ease of rodent experimentation and lower price point keeps them at the forefront of TBI research. Mouse and rat brains do not contain each cell type found in adult humans brains, but the general role of each shared cell type is conserved between species; thus, they serve as invaluable models (32). However, when TBI studies are focused on physical brain dynamics, rather than molecular or cellular studies, the need to resemble human neuroanatomy is of higher importance. In these cases, lab ferrets (*Mustela putorius furo*) are frequently utilized since they possess gyrencephalic brains that allow for better modeling of force vectors

experienced by the human brain (335). Frequently used rodent models of TBI include CCI injury, fluid percussion injury (FPI), weight drop-impact/acceleration injury, blast injury, and penetrating ballistic-like brain injury (PBBi). Mice and rats are among the most commonly used animals for these TBI models (429). Areas of the mouse or rat brain that underlie memory and motor coordination are frequently targeted for injury, and simple behavioral assays exist to measure these CNS functions. Tests such as the Morris Water Maze (MWM, a test of spatiotemporal learning and memory) and the rotarod (a test of motor coordination) are commonly employed to assess neurological damage in mice or rats (72; 248).

1A.9 - Functional consequences

Depending on the location and severity of TBI, neuropsychological alterations often follow. Beyond the gross deficits of motor, sensory, and autonomic function associated with moderate to severe TBI, deficits can arise as nuanced alterations in cognition after TBI of any severity (1; 95; 145; 404). However, certain brain regions are more prone to traumatic insult than others, and the neuropsychological sequelae that results from their injury is well documented (102; 235). Traumatic insult to the dorsolateral prefrontal cortex, for instance, can disrupt working memory, attention, abstraction, insight, and problem solving; while trauma to the orbitofrontal cortex can disrupt emotional and social responding (42; 94; 103; 235). Injury to the temporal polar cortex can disrupt memory retrieval and sensory-limbic integration, while damage to the amygdala can alter fear conditioning (235; 242; 243). Furthermore, traumatic injury to the cerebellum or brain stem, when not lethal, can alter coordination and arousal, respectively (235;

394). Thus, treatment for the brain-injured patient must not only address the molecular, cellular, and physiological consequences of TBI, but also the unique neuropsychological sequelae that follow.

1B – CLASSICAL RAS

The RAS is the primary hormonal regulator of blood pressure in mammals. Through finely balanced feedback loops, cross talk between proteins of the lungs, kidneys, and liver can modulate salt uptake, vascular resistance, and fluid retention (196). However, the effects of the RAS span beyond blood pressure modulation. Proteins of the RAS can alter cellular proliferation, inflammation, autophagy, cellular death, behavior, and various other molecular, cellular, and physiologic phenomena (5; 117; 122; 152; 281; 296). As such, physiologic stimuli (such as hypotension) that alter RAS signaling may have various off-target consequences that do not feedback to combat the initial physiologic stimuli. Thus, though it is critical to review the association of the RAS to TBI, it is also important that we discuss the primary mechanism through which RAS signaling is triggered: as a response to hypotension. In order to do this, we must first review basic kidney physiology.

1B.1 Basics function of the kidney

The kidney's primary function is to regulate blood homeostasis. Through processes of reabsorption, secretion, excretion, and filtration, the functional unit of the kidney (the nephron) is able to regulate blood volume, electrolyte balance, acid-base homeostasis, and the removal of toxins (298). The RAS primarily acts to alter how nephrons regulate electrolyte balance and overall blood volume. Thus, rather than

performing an all-exhaustive overview of renal function, we will focus on renal function as it pertains to electrolyte balance and fluid homeostasis (298).

Millions of microscopic blood filtration systems, known as nephrons, exist within the kidney (29). Each nephron is made up of a renal corpuscle and a renal tubule (298). The renal corpuscle consist of two components: 1) the glomerulus — a network of capillaries supplied with blood from a single afferent arteriole that drain into a single efferent arteriole; and 2) the Bowman's capsule - a specialized semi-permeable structure made up of simple squamous endothelium and podocytes that surrounds the glomerulus (336). Water, ions, glucose, amino acids, and various small molecules permeate from the glomerular capillaries into the Bowman's capsule and collectively constitute the glomerular filtrate (336). From here, the glomerular filtrate enters the renal tubule.

The renal tubule is made up of various types of endothelium and a tubular basement membrane. Unique gene expression in specific regions of the renal tubule gives rise to specific patterns of solute permeability (445). Furthermore, the renal tubule is closely paralleled by a network of peritubular capillaries that flow in the opposite direction of the glomerular filtrate (196). This counter-current allows for quick return of ions reabsorbed from the renal tubule into systemic circulation (276).

The first segment of the renal tubule that the glomerular filtrate must pass through is the proximal convoluted tubule in the renal cortex (445). The proximal convoluted tubule is primarily made of simple cuboidal endothelium with brush borders that increase surface area for maximal reabsorption of glucose, amino acids, urea, sodium, chloride, maganesium, phosphorus, calcium, and water into the peritubular capillaries. From the proximal convoluted tubule, filtrate enters the loop of Henle (445).

The loop of Henle consists of a descending limb and an ascending limb that vary in function. The descending limb is the first to receive filtrate from the proximal convoluted tubule. The descending limb of the loop of the Henle dives from the renal cortex into the renal medulla, and is permeable only to water (249; 278). As tonicity increase from the cortex to the medulla, water exits the filtrate into the medullary interstitium, leaving behind hypertonic filtrate. From the descending loop, hypertonic filtrate moves into the water-impermeable ascending loop, which traverses the renal medulla yet again, this time to return to the renal cortex (249; 278). Furthermore, active ionic transport is supported by expression of various transporters in the walls of the ascending limb. As the filtrate moves through the ascending limb from the renal medulla towards the renal cortex, ions from the hypertonic filtrate, including sodium, chloride, and potassium are reabsorbed into the medullary intersitium (249; 278). The active transport of ions into the medullary interstitium is what maintains the medullary hypertonicity required for the previously-mentioned water reabsorption that occurs in the descending limb of the loop of Henle (319).

The end of the ascending limb of the loop of Henle marks the beginning of the distal convoluted tubule (236; 368). Reabsorption of sodium chloride continues via sodium-chloride symporters expressed on the mitochondria-rich simple cuboidal endothelium that make up the vessel wall of distal convoluted tubule. Subsequently, the filtrate passes through a final branch of the tubule, known as the connecting tubule, which empties into the collecting duct (236; 368). At this point, much of the reusable ions have been reclaimed. Thus, upon entrance into the collecting duct, the filtrate is a hypotonic

aqueous solution that contains residual ions, as well as biological waste products (such as urea), from the blood (282).

From the renal cortex, the collecting duct dives back into the hypertonic renal medulla. Here, a final round of reabsorption occurs to reclaim any last reusable resources, while leaving toxins behind for excretion. This occurs via principal cells found within vessel wall of collecting duct (282). Through sodium and potassium channels on principal cells, sodium is removed from the filtrate while potassium is added (282). Aquaporin 2, a transporter molecule also expressed on principal cells, allows for the reabsorption of water from the collecting duct into the interstitium (80). Finally, after the filtrate has been titrated in the collecting duct, it enters the renal pelvis where it will eventually exit through the ureter and into the bladder as urine (298).

1B.2 Classical RAS modulation of blood pressure

At the junction of the ascending loop of Henle and the distal convoluted tubule, a specialized collection of cells exist that make up the macula densa (290). These cells are extremely sensitive to sodium chloride concentration within the distal convoluted tubule. When there is a reduction of blood pressure, hydrostatic pressure of the peritubular capillaries decreases. Thus, more sodium chloride can be reabsorbed from the renal tubule into the peritubular capillaries (290). Furthermore, a reduced blood pressure may decrease glomerular filtration rate (172). Collectively, these two effects of hypotension result in lower sodium chloride concentrations within the renal tubule. When cells of the macula densa sense a decrease of sodium chloride concentration within the filtrate, they trigger the dilation of the afferent arteriole and secrete prostaglandins (141).

Dilation of the afferent arteriole increases blood flow through the glomerulus; thus, increasing glomerular filtration rate (GFR) and subsequently increasing sodium chloride concentration within the filtrate (172). Prostaglandins secreted from the macula densa act in a paracrine fashion on specialized juxtaglomerular cells that line the afferent arteriole (141). Prostaglandins stimulate the juxtaglomerular cells to secrete renin into the blood stream (290). Additionally, when a decrease of blood pressure is sensed by arterial baroreceptors, juxtaglomerular cells are signaled to secrete renin (378). Renin secretion by juxtaglomerular cells can also occur from direct sympathetic nervous stimulation (254).

The liver constitutively produces and secretes a functionally inactive globular protein, known as AGT, into circulation (225). Upon contact with renin, AGT is cleaved into a 10-residue peptide known as Ang I (365). Ang I stays in circulation while remaining inactive relative to its cleavage products (30; 258). ACE is found on the surface of the pulmonary endothelium. When circulating Ang I comes in contact with ACE, ACE cleaves two terminal residues from Ang I, resulting in an octapeptide product known as Ang II (30; 258). Ang II primarily acts through the AT1R: a G-protein coupled receptor (GPCR) that is expressed in the vascular smooth muscle, the pituitary gland, the adrenal glands, and various cardiovascular regulatory regions within the brain (86; 142; 154; 166).

When Ang II acts on AT1R within the vascular smooth muscle, it promotes vasoconstriction that increases mean arterial pressure (167). This helps combat the initial hypotensive stimuli that resulted in the secretion of renin and subsequent production of Ang II. Furthermore, Ang II's action on AT1R in the efferent arteriole of the glomerulus

reduces its luminal diameter (74). Thus, Ang II increases pressure within the glomerulus to help restore a functional GFR (137).

Ang II also perturbs blood volume and blood electrolyte balance. When Ang II acts on AT1R within the adrenal cortex, it promotes adrenal release of aldosterone (123). Aldosterone moves through the circulation, and eventually reaches the distal convoluted tubule and collecting duct of the nephrons (253). Here, aldosterone acts by altering sodium and potassium transport, ultimately resulting in an increase of sodium uptake (253). Consequently, blood volume and blood pressure rise as an indirect result from the fluid retention that follows sodium reabsorption.

When circulating Ang II reaches AT1R receptors found in the posterior pituitary gland, signaling by AT1R promotes the secretion of antidiuretic hormone (ADH) into the circulation. ADH acts at the vasopressin V2 receptor (V2R) on cells within the collecting ducts (73; 222; 261). Activation of V2R by ADH promotes translocation of intracellular aquaporin 2 to the apical plasma membrane, thus increasing the collecting duct's permeability to water (188; 405). Activation of V2R by ADH also results in an overall increase in aquaporin 2 expression. Consequently, water leaves the collecting duct and eventually enters the systemic circulation, restoring blood volume and increasing blood pressure (189).

Finally, within the brain, the RAS exerts a regulatory role on blood pressure through mechanisms unique to those previously mentioned. Ang II stimulates AT1R in neurons of the circumventricular organs, the median preoptic nucleus, and regions surrounding the anteroventral third ventricle (397). Doing so promotes thirst and sodium appetite, thus increasing intravascular fluid volume and restoring blood pressure (397).

Furthermore, action of Ang II in cardioregulatory regions of the brain, such as the solitary nucleus (SN), the rostral and caudal ventrolateral medulla, and the paraventricular nucleus (PVN) within the hypothalamus, can promote sympatho-adrenomedullary outflow that directly stimulate juxtaglomerular cells to secrete renin (254).

1B.3 - Renin and TBI

Renin's production of Ang I by enzymatic cleavage of AGT is the rate-limiting step of the RAS (113). Renin and its zymogen, prorenin (PRO), co-exist and are both able to cleave AGT into Ang I; though renin is much more active (372). However, renin's ability to affect downstream targets begins before the enzyme has been rendered into its most catalytic state. The prorenin receptor (PRR) primarily serves to binds both PRO and renin and increases their enzymatic activity (260). Though PRR serves as a biochemical catalyst to increase PRO and renin activity, various signaling events occur following the binding of PRO to PRR (207).

In the human brain, PRR is most highly expressed in the pituitary gland and the frontal lobe (373). PRR is also co-localized with magnocellular neurons of the PVN and supraoptic nucleus (SON) that contain oxytocin and ADH. These suggest that PRR signaling may regulate blood pressure through water-electrolyte homeostasis. Furthermore, PRR mRNA and protein have been described in the PVN, subfornical organ (SFO), rostral ventral lateral medulla (RVLM), raphe pallidus nucleus (RPa), and SN; providing further support of PRRs role in cardiovascular regulation (206; 373).

Various studies have discovered intracellular renin within both neurons and astrocytes (87; 112; 130; 144; 195; 350). Since the majority of RAS-related receptors are membrane bound, the function of intracellular components remains unclear; though

outside of the cell renin readily alters neuronal function. Recent *in vitro* studies show that renin initiates MAPK signaling in neurons through the PRR, thereby reducing neuronal activity (339). As such, PRR blockers have been developed and are able to protect neuronal activity in the presence of excess renin (153; 260; 339). Interestingly, TBI in mice increases mRNA expression of renin but does not alter mRNA expression of PRR (379). However, PRR and renin's effects can be observed in various other cells within CNS.

PRR is also expressed in astrocytes and its actions are observed in microglia (206; 373). In microglia, PRO has direct pro-inflammatory effects that result in over-expression of TNF α and IL-1 β (344). These pro-inflammatory effects are similarly observed on vascular endothelium (328). In animal models of TBI, the release of various pro-inflammatory cytokines damages neurons in a paracrine fashion (226; 288). Small molecules that inhibit renin activity, such as aliskiren, can inhibit PRO-induced TNF α and IL-1 β production (444). Furthermore, aliskiren treatment in rats following cerebral hypoperfusion, a common feature of TBI that results in increased brain-renin activity, reduces injury-induced oxidative stress, glial activation, and spatial working memory deficits (60; 77; 87; 332). Collectively, these data support that inhibition of renin activity, whether by inhibiting the enzyme or the PRR through which it signals, can promote neuronal activity, decrease inflammation, and decrease memory deficits in various forms of nervous tissue insults. Thus, modulation of renin may be beneficial following TBI.

1B.4 - ACE and TBI

Following renin's cleavage of AGT, ACE must convert the inert decapeptide Ang I into the vasoactive octapeptide Ang II (400). ACE was originally discovered in lung and vascular endothelium, but has since been described in the majority of brain regions and cell types to include neurons, astrocytes, microglia, ependymal cell, and cerebrovascular endothelial cells (159; 366; 417). Ang II signaling can exacerbate TBI (400). Thus, overactivation of ACE may be detrimental to the pathophysiological progression of TBI. Rodent models of ischemic stroke have demonstrated the neuroprotective benefits of ACE inhibitors (277). Small molecules such as enalapril, an ACE inhibitor, benefits neurological activity, reduces cerebral infarct size, and decreases brain swelling following acute ischemic stroke in rats (277). However, Harford-Wright and Thorton showed that enalapril exacerbates motor deficits following TBI in rats, thus suggesting the benefits of ACE inhibition following ischemia vary from those in TBI (140).

Various polymorphisms are known to alter levels of ACE expression. One in particular is an Alu element insertion (I) - deletion (D) polymorphism in the ACE gene. Patients homozygous for the deletion have higher levels of circulating and tissue ACE (380). Furthermore, carriers of the deletion are at increased risk of cerebrovascular disease and cognitive dementia (12; 234). Ariza et al. found that carriers of this deletion performed worse on various neuropsychological functional tests following severe TBI (15). Thus, though in contradiction to Harford-Wright and Thorton's rodent study, increased ACE may indeed be harmful to TBI pathophysiological progression. Clearly,

more studies need to be performed to assess the value of ACE manipulation following TBI.

1B.5 - AT1R, AT2R, and TBI

1B.5.1 - Localization and Signaling

It is not known whether Ang II crosses the BBB (310). Regardless, its actions on the CNS following TBI remain relevant because of the BBB compromise that ensues after insult (57; 299; 310; 369). In rats however, even in the absence of BBB disruption, Ang II has been grossly identified in various hypothalamic nuclei, the circumventricular organs, and the SN; and specifically identified in magno- and parvo- cellular neuronal bodies of the PVN and supraoptic nucleus (SON). Ang II has also been identified in nerve fibers associated with the SFO, median preoptic nucleus (MnPO), SON, PVN, and posterior pituitary (110; 211).

Ang II serves as the primary ligand for both the Angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors (104). Though AT2R is a GPCR, most of its known effects are mediated through G-protein-independent signaling mechanisms (121; 149; 297). On the other hand, AT1R primarily signals through the Gq signaling pathways where it activates phospholipase C, increases intracellular calcium and inositol 1, 4, 5-triphosphate concentrations, and activates mitogen-activated protein kinases (MAPK), JAK/STAT signaling, and NFκB signaling (252; 326; 330). Increases of intracellular calcium results in the activation of myosin light chain kinase (MLCK) that then phosphorylates myosin and results in the smooth muscle contraction responsible for vasoconstriction (167). In general, AT1R signaling cascades promote various unrelated, often deleterious,

phenotypes depending on the cell type or organ system. In contrast, AT2R signaling counteracts many of these negative phenotypes (131).

AT1R is distributed in various brain regions, including but not limited to the: SFO, MnPO, vascular organ of the lamina terminalis (VOLT), PVN, lateral parabrachial nucleus (LPBN), RVLM, and the SN. Each of these regions takes part in the regulation of cardiovascular function and/or body fluid and electrolyte balance (69; 202; 214; 422; 424). Though AT2R is also expressed in many regions associated with cardiac function, such as the SN, area postrema, and the dorsal motor nucleus of the vagus (DMNV), it is also abundant in neuroanatomical regions involved with anxiety-like behavior, reward circuitry, sensory perception, movement, and learning (362). These include the hippocampus, cerebellum, amygdala, thalamus, septum, superior colliculus, subthalamic nucleus, acingulate cortex, superior colliculus, substantia nigra pars compacta, and the PVN (127; 131; 201; 202; 356-358). Neither AT1R or AT2R expression are limited to neurons within the CNS. Indeed, microglia, astrocytes, and cerebrovascular endothelial cells all express both receptors, and are all affected by the presence of Ang II (134; 159; 191; 203; 443). Furthermore, AT1R and AT2R are expressed on oligodendrocytes, and activation of AT1R leads to demyelination that ultimately perturbs neuronal function (101).

1B.5.2 - Overview of Angiotensin Receptor Blockers

Angiotensin receptor blockers (ARBs) are FDA approved drugs used extensively in the treatment of hypertension and other cardiovascular disorders. They are generally well tolerated with excellent safety profiles. Currently used ARBs include azilsartan, losartan, irbsartan, valsartan, eprosartan, telmisartan, olmesartan, candesartan and

fimasartan (169; 198). Newer ARBs under development include protasartan and forasartan (267; 272). Most ARBs are enterally administered in their active state, though candesartan, olmesartan, and losartan are administered as prodrugs that require hepatic metabolism for activation (169; 347).

ARBs primarily work by binding AT1R with high affinity and blocking the effects of Ang II. However, they have roughly 10,000 fold less selectivity for AT2R compared to AT1R; thus, they do not block activation of AT2Rs (51). As the benefits of AT2R signaling are often masked by the deleterious effects of AT1R signaling, selective blockade of AT1R by ARBs allows the beneficial effects of AT2R signaling to go unopposed (230). However, not all ARBs are created equal. One study showed that candesartan and azilsartan have the highest binding affinity for the AT1R, while others report a the highest affinity with telmisartan (241; 268).

Understanding Ang II signaling via AT1Rs in physiological and pathological conditions helps elucidate the mechanisms by which ARBs can be useful in the treatment of TBI. AT1Rs mediate the production of aldosterone by the adrenal gland and the vasoconstriction in blood vessels, ultimately leading to an increase in blood pressure (123) (167). Therefore by blocking AT1R, ARBs mediate their antihypertensive effects. However, besides mediating an increase in blood pressure, AT1R signaling can increase pro-inflammatory mediators, ROS, cell proliferation, and cerebral blood flow (316). Blockade of AT1Rs by ARBs should therefore reduce deleterious inflammatory processes and cellular proliferation, and improve circulation following TBI. Besides blocking AT1R, some ARBs regulate AT1R-independent signaling pathways. These include PPAR γ pathways, Akt pathways, and AMPK pathways (55; 109; 124; 255; 346;

402; 410; 432). Exactly how much these pathways contribute to the benefits of ARBs varies by disease process, and still requires further investigation.

Many molecules that typically do not cross the BBB do so in disease states where the BBB is compromised. Although it is generally accepted that ARBs cross the BBB poorly, it is expected that many of the ARBs will cross the BBB in conditions like stroke, meningitis, and TBI where the BBB is obviously compromised (169; 264). Telmisartan has however been shown to cross the BBB of intact brains and is able to modulate RAS effects within the CNS (169; 241; 265). For other ARBs, such as candesartan, conflicting reports exist regarding their BBB permeability (264; 337). However, ARBs may affect CNS tissue without significant BBB permeability via interaction through circumventricular organs, the cerebrovasculature, or other unknown mechanisms (169; 264).

1B.5.3 - Benefits of ARBs in CNS Pathology

1B.5.3.1 - Neurons and cognition

ARBs are generally considered to be neuroprotective through several different mechanisms, including direct effects on neurons (401). ARBs have been shown to reduce glutamate-mediated excitotoxicity in neurons *in vitro*, indicating one potential mechanism by which ARBs increase neuronal survival (409). Following neuronal stretch injury, Ang II production and AT1R expression are increased (4). In the same model of injury, AT1R blockade with losartan prevents neuronal death associated with increases of Ang II (4). Furthermore, in rodent models of ischemic insult, AT1R signaling exacerbates neuronal death by activating apoptotic pathways (223). Thus AT1R signaling can be

detrimental to neuronal viability. As expected, blockade of AT1R attenuates the cognitive deficits that typically ensue following ischemic stroke in rats (156; 223). The functional and cognitive benefits of ARBs have also been demonstrated in rodent models of TBI. In mice, motor function is acutely decreased following CCI, but returns to baseline levels within 7-10 days. On the other hand, spatiotemporal memory deficits last beyond 20 days. Work by Villapol et al. showed that treatment with ARBs following moderate TBI reduces both the motor function deficits and spatiotemporal memory deficits associated with CCI in mice (400).

1B.5.3.2 - Microglia and inflammation

Over activation of brain AT1R by Ang II is associated with a pro-inflammatory state leading to hypertension that has the potential to exacerbate cerebral ischemia, behavioral dysfunction, TBI, and various other disease processes (264; 317; 318; 407). Investigations of whether ARBs promote an anti-inflammatory effect following TBI confirm that blockade of AT1R is beneficial to TBI recovery. Indeed, mice treated with either telmisartan or candesartan show less microgliosis and neutrophilic infiltration within the perilesional area, and an overall reduction in lesion volumes, when compared to vehicle treated animals (400). Other studies have demonstrated that AMPK signaling is responsible for telmisartan's ability to reduce microglial activation (432). Indeed telmisartan has neuroprotective and anti-inflammatory action in a model of Parkinson's disease even in AT1R KO mice (119). It is well known that telmisartan and candesartan activate PPAR γ (27; 402). Unsurprisingly, when PPAR γ antagonist are co-administered with either of the ARBs telmisartan and candesartan following TBI, their ability to reduce lesion volume is hindered (400). Together, these suggest an AT1R-independent anti-

inflammatory mechanism of ARBs for microglia. Nonetheless, genetic deletion of AT1R reduces overall lesion size in mice after CCI, confirming the deleterious effects of AT1R activation after TBI and highlighting the potentially pleiotropic benefits of ARBs (400; 402).

As discussed earlier, exposure to DAMPs following injury activates various inflammatory cascades in microglia that can be detrimental to TBI. One of the key DAMPs critical in neuroinflammation is high mobility group protein B1 (HMGB1) (215; 269; 270; 279; 308). The effect of HMGB1 in neuroinflammation was first demonstrated in stroke and later in TBI (115; 250; 308). After release from injured cells, HMGB1 binds to its receptor, RAGE, to stimulate the HMGB1/RAGE pathway. HMGB1 also activates the TLR4 pathway. Inhibition of HMGB1 has been associated with beneficial effects both in stroke and TBI (178; 215; 270; 308). Evidence suggests that the various ARBs, including candesartan, irbesartan and telmisartan, inhibit the HMGB1 pathway in stroke (176). Thus, they may also have similar action after TBI

1B.5.3.3 - Cerebrovasculature

AT1R is highly expressed on both endothelial cells as well as smooth muscle cells within the vasculature (129; 300). Ang II signaling through the AT1R causes vasoconstriction (167). On the other hand, Ang II signaling through the AT2R promotes vasodilation. Due to the higher concentration of AT1R found within the vasculature, in the presence of Ang II, vasoconstriction typically prevails (68). Following insult, Ang II-induced vasoconstriction can restrict CBF and promote a state of ischemia that often results in toxicity to neurons if left unchecked (199). ARBs have generally been shown to benefit cerebral circulation by promoting flow during hypertension and stroke (232; 247).

Until recently, it was unknown whether these benefits would remain after TBI. Villapol et al. demonstrated that AT1R blockade with either telmisartan or candesartan completely attenuates the reduction of CBF associated with CCI in mice (402). Hence, ARBs may serve as beneficial therapeutics to retain physiologic CBF following TBI.

Loss of capillary density following TBI can result from mechanical destruction or noxious biochemical stimuli. Loss of vascular density compromises blood access to healthy brain parenchyma and can further exacerbate damage (220). Thus, preserving vascular density should serve as an important target for pharmacotherapies. Interestingly, ARBs such as candesartan influence the expression of angiogenic growth factors, ultimately promoting vasculature development. In rodent stroke models, candesartan promotes vascular protection, and increases vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor B (VEGF-B), and brain-derived neurotrophic factor (BDNF) (184; 353). Furthermore, in both normoxic and hypoxic conditions, candesartan promotes cell proliferation, migration, and tube formation in cultures of human brain endothelial cells (184; 353).

Beyond vasoconstriction, Ang II signaling through the AT1R activates an array of cellular and molecular cascades in cerebrovascular endothelial cells. Of particular importance with respect to TBI is Ang II's ability to promote a pro-inflammatory state through endothelial-dependent mechanism. Through stimulating COX-2 and NADPH oxidase, Ang II promotes endothelial dysfunction and ultimately increases ROS, which are particularly damaging to brain parenchyma (23; 24; 199; 259; 414). Ang II signaling in endothelial cells also promotes the expression of cytokines and chemoattractants, such as endothelin-1, vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion

molecule 1 (ICAM-1), and integrins, which recruit inflammatory cells to the vasculature (11; 31). Similar molecular expression patterns are observed in the cerebrovasculature of spontaneously hypertensive rats. In these rats, various ARBs are able to normalize endothelial/inducible nitric oxide synthase ratios, decrease TNF α , reduce ICAM-1 and VCAM-1 mRNA expression, and reverse cerebrovascular macrophage infiltration (14; 23; 434). As excessive CNS inflammation is deleterious after TBI, and because ARBs access the cerebrovasculature without crossing the BBB, targeting cerebrovascular AT1R to reduce CNS inflammation may hold potential for treating TBI.

1B.5.3.4 - Astrocytes

Gliotic scars are one long-term complication of TBI (173). Scar formation can serve as foci for abnormal neuronal firing resulting in post-traumatic epilepsy. Furthermore, gliotic scar formation can impair the regeneration of axons when repair is possible (173; 240). Scar formation in the brain is mainly mediated by astrocytes; however, pericytes and meningeal fibroblasts often contribute towards a fibrotic scar (2; 126; 348; 351). The ability of candesartan to reduce astrogliosis following TBI has been confirmed in a mouse CCI model (65; 177). However, the effects of candesartan on the astrogliotic scar are largely due to its antagonistic role of AT1R. Indeed, genetic deletion of AT1R robustly reduces the astrogliosis that ensues following CCI in mice (400). However, as PPAR γ agonists have been shown to reduce activation of astrocytes *in vitro*, it is also likely that ARBs, at least partially, reduce astrogliosis through PPAR γ activation (190; 364). Furthermore, the ARBs that reduce astrogliotic scarring (candesartan) in TBI are known to activate PPAR γ signaling (400).

The ARB losartan has not been assessed for its ability to attenuate astroglitic scarring following TBI. Unique to losartan is its confirmed ability to inhibit TGF β signaling in various cell types, including astrocytes. As TGF β signaling is a key activator of astrogliosis following TBI, modulation of TGF β through use of losartan may hold potential to reduce post-TBI astrogliosis (126; 239; 323). Thus, through both AT1R-dependent and independent pathways, ARBs show potential to directly decrease astroglitic scarring following TBI (19).

1B.5.3.5 - Blood-brain barrier

Much of the BBB disruption that ensues immediately after TBI is caused by mechanical force. However, lasting BBB permeability is likely due to prolonged inflammation as well as deregulated endothelial and astrocytic function. The role of Ang II on BBB permeability is still unclear. In opposition to many of the deleterious effects associated with AT1R signaling, various reports demonstrate that AT1R activation in astrocytes decreases BBB permeability, and that AT2R activation promotes BBB leakiness (108; 420). However, a 2010 study by Zhang et al. induced cerebral microvascular inflammation and increased BBB permeability in mice by chronically infusing Ang II for 14 days (439). Furthermore, Pelisch et al. showed that AT1R deletion attenuated Ang II-induced cognitive impairment by reducing BBB permeability (286). Thus, the role of AT1R blockade on the BBB following TBI is controversial and requires further study.

1B.5.4 - Selective AT2R agonists

As mentioned earlier, AT2R is expressed within neurons in cardioregulatory regions of the brain as well as regions known to affect cognitive function (201; 362). In

neurons, AT2R functions to modulate excitability and promote neurite outgrowth, as indicated by its increased expression during fetal development (201; 231; 357). However, signaling through the AT2R also affects behavior of adult mice. Upon deletion of AT2R, mice exhibit anxiety-like behavior, alterations in normal exploratory tendencies, and impaired spatial memory, suggesting that AT2R signaling facilitates cognition (233; 271). Due to the beneficial actions of AT2R signaling, selective AT2R ligands have been developed to stimulate AT2R without promoting the deleterious actions of AT1R. Small molecule agonists such as CGP42112A and Compound 21 (C21) specifically stimulate AT2R signaling. Multiple studies have shown that C21 attenuates damages and deficits associated with ischemic stroke in rats (159). The mechanisms through which AT2R signaling is neuroprotective after stroke are still under investigation. However, it has recently been demonstrated that treatment with the AT2R agonist C21 following stroke in rats increases expression of anti-inflammatory cytokines interleukin 10 (IL-10) and BDNF (9; 105; 334). Additionally, AT2R can activate PPAR γ (205). Hence, blockade of AT1R may increase Ang II signaling through AT2R and contribute to the increased PPAR γ signaling of ARBs (441). Astrocytic expression of AT2R is controversial. Though various studies report the lack of AT2R on astrocytes, recent reports suggest that it not only is present on astrocytes, but also is required for functional MasR signaling (read more in section 1C.1) (203; 362). The beneficial role of AT2R signaling has been demonstrated in a mouse model of TBI where AT2R agonism promotes neurogenesis and is neuroprotective (387; 388). Thus, AT2R holds promise as a potential therapy for TBI.

1C - EXTENDED RAS AND BRAIN INJURY

1C.1 - ACE2/Ang-(1-7)/MasR

1C.1.1 - Localization and Signaling

Though the effects of Ang II signaling through AT1R remain the most studied arm of the RAS, various cleavage products of Ang II exist that are biologically active. The enzyme Angiotensin Converting Enzyme 2 (ACE2) cleaves the terminal phenylalanine from Ang II to form the heptapeptide Angiotensin-(1-7) (Ang-(1-7)) (89; 99; 307; 399; 446). Alternatively, neprilysin (NEP), a neutral endopeptidase, can generate Ang-(1-7) directly from Ang I (84). The loss of this single residue from Ang II dramatically alters ligand-receptor interactions. Consequently, Ang-(1-7) acts on a completely unique GPCR known as the Mas receptor (MasR), and possesses little ability to stimulate AT1R signaling (325; 376). Ang-(1-7) can also signal through the Mas-related G-protein coupled receptor (MrgD), which functions similarly to MasR (read more in section 1C.3).

Multiple signaling cascades are initiated upon the binding of Ang-(1-7) to MasR. *In vitro* studies that overexpress and activate MasR in HEK293 cells describe intracellular increases of cAMP, which suggests that MasR signals through the G α s signaling pathway (325). Though it is generally accepted that MasR activation promotes phosphorylation of Akt and upregulation of eNOS, reports of other downstream signaling pathways are varied and model dependent (322). Nonetheless, Ang-(1-7)'s activity through MasR appears to oppose Ang II signaling through AT1R at molecular, cellular, and physiological levels; both systemically and within the CNS. Just as Ang II can

increase MAPK signaling, promote immune cell proliferation, and increase blood pressure, Ang-(1-7) can reduce MAPK signaling, attenuate immune cell proliferation, and lower blood pressure (53; 132; 157; 345; 367).

Ang-(1-7) signals through MasR in many different cell types, and in each of these cell types the specific signaling pathway is dependent on a multitude of factors (78; 381). However, both *in vitro* and *in vivo* studies suggest MasR signaling induces a neuroprotective phenotype through direct and indirect actions on neuronal and non-neuronal cells of the CNS, respectively (431). Furthermore, MasR and AT2R may form heterodimers with AT1R that result in its inactivation (3; 181). Thus, there is a possibility that some of the observed neuroprotective benefits of MasR and AT2R activation are due, at least in part, to their ability to inactivate AT1R (3; 64). Similarly, AT2R signaling can increase ACE2, and ACE2 can decrease AT1R expression (159). Thus, there are various ways the extended RAS can promote a neuroprotective phenotype.

MasR signaling can be exogenously stimulated by 1) administration of Ang-(1-7), 2) administration of the ACE2 activator, diminazene aceturate (DIZE), which functions to increase Ang-(1-7) and reduce Ang II, or 3) over-expressing ACE2 (54; 187). In humans, MasR is expressed highly in the brain, testis, retina, kidneys, and throughout the cardiovascular system (64; 92; 305; 393). When assessing expression of MasR in the murine forebrain, MasR is highly expressed in cortical and hippocampal neurons, and in cerebrovascular endothelial cells (107; 163; 229; 437). It is also found on microglia and astrocytes (133; 203; 216; 245; 304). Additionally, ACE2 and Ang II have been reported globally in the brain and specifically in neurons and glia (90; 111; 138; 212; 219; 246; 293; 416; 427).

1C.1.2 - Neurons and Cognition

MasR signaling in neurons directly promotes their viability (200; 304). This is demonstrated *in vitro* by assays of neuronal viability in glucose-deprived media (200). Glucose deprived neurons treated with the selective, small molecule MasR agonist AVE0991, have a 40 percent greater survival rate than untreated glucose deprived neurons (200). Another study showed that MasR signaling decreases oxidative stress in dopaminergic neurons of non-human primates and rodents (64). Many *in vivo* studies demonstrate the neuroprotective benefits of MasR agonism though they do not distinguish between direct action on neurons versus neuroprotection afforded from MasR action on non-neuronal cells. Mecca et al. assessed neuronal density in the peri-infarct brain region following middle cerebral artery occlusion (MCAO) in rats who were pre-treated with intracerebroventricular (ICV) Ang-(1-7) (238). Some rats also received the MasR antagonist, A779, simultaneously with Ang-(1-7) or independently. Indeed, neuronal density was higher, and infarcted lesion volume was lower, in Ang-(1-7) treated rats than in those co-administered A779 or treated with vehicle. However, MasR antagonism with A779 alone did not alter lesion volume, suggesting that endogenous MasR signaling does not play a protective role following stroke (238).

In a separate assessment of MCAO in rats, Mecca et al. administered intraperitoneal injections (IP) of DIZE as opposed to ICV Ang-(1-7) (238). Similar to treatment with Ang-(1-7), DIZE-treated rats developed significantly smaller lesion volumes and reduced neurological deficits compared to vehicle-treated controlled animals. MasR antagonism also attenuated the observed neuroprotective phenotypes, again demonstrating MasR-dependent benefits (26; 163). Just as DIZE-induced ACE2

activation is neuroprotective, transgenic mice with neuronal over-expression of ACE2 are protected against ischemic insult (54). Following MCAO in mice, neuronal ACE2 over-expression reduces infarct volume and neurological deficits, and increases cerebral microvascular density and cerebral blood flow in the peri-infarct area (54). Even in the absence of noxious CNS stimuli, the presence of MasR influences behavioral phenotypes in rodents. Though the presence of MasR is not required for spatial learning in uninjured, the lack of MasR promotes anxiety-like behavior in otherwise naive mice. Oddly, the anxiety-like behavior is significantly higher in male versus female MasR-KO mice (406; 408). Indeed, the overall benefits of increasing Ang-(1-7)/MasR signaling following non-traumatic CNS injury have been verified *in vivo*. However, whether the benefits of Ang-(1-7) on non-traumatic CNS insult are conserved in traumatic cases has only recently been assessed.

1C.1.3 - Microglia and Inflammation

Following noxious stimuli, such as LPS stimulation or PRO exposure, activated microglia proliferate and secrete pro-inflammatory cytokines, such as IL-1 β and TNF- α , that can promote deleterious effects (85). Similar pro-inflammatory changes occur in the CNS following infusion with Ang II, or after TBI (191). Treatment of LPS or PRO-stimulated microglia, *in vitro*, with Ang-(1-7) results in an overall reduction of ROS and pro-inflammatory cytokine production. Furthermore, Ang-(1-7) is able to attenuate NO production in LPS-stimulated primary rat microglial cultures (216; 303).

In mammals, cerebral inflammation is a hallmark characteristic of TBI that is shared with ischemic and hemorrhagic stroke (36). Rodent studies that induce cerebral inflammation with LPS can focus on a compound's anti-inflammatory properties *in vivo*

(50). This is in contrast to complex disease states such as stroke or TBI where both inflammatory and non-inflammatory pathological properties occur concurrently. One such study showed that genetic deletion of MasR exacerbates LPS-induced cerebral inflammation in mice, suggesting that endogenous Ang-(1-7) plays a neuroprotective role in pathologies with inflammatory etiologies (273).

Many of Ang-(1-7)'s anti-inflammatory effects on microglia are conserved *in vivo*. In spontaneously-hypertensive rats prone to hemorrhagic stroke, pretreatment with ICV injections of Ang-(1-7) decreases microgliosis in hemorrhage-prone regions and reduces overall mortality (303; 304). Pretreating with ICV Ang-(1-7) prior to ischemic stroke in rats also reduces infarct volume and lowers the stroke-induced increase of CD11b, IL-1 α , iNOS, CXCR4 and IL-6 mRNA expression, and iNOS protein expression, in the infarcted tissue 24 hours after insult. Additionally, ICV Ang-(1-7) prevents significant induction of TNF α and IL-1 β mRNA expression following insult (303). Clearly, stimulation of MasR combats various neuroinflammatory processes; many of which are observed following TBI (128; 415).

1C.1.4 - Astrocytes

The role of MasR signaling in astrocytes has only been assessed *in vitro*. AT2R and MasR mRNA expression have been confirmed in mouse astrocytes. Interestingly, in order for MasR or AT2R signaling to occur within mouse astrocytes, both receptors must be present and functional. Using this information, coupled with fluorescence cross correlation spectroscopy (FCCS) and fluorescence resonance energy transfer efficiency (FRET) assays, Leonhardt et al. showed not only that AT2R and MasR heterodimerize, but that doing so is required for either receptor to signal in astrocytes (203). A separate

study showed that Ang-(1-7) treatment of astrocytes after radiation attenuates radiation-induced inflammation by reducing MAPK activation (245).

1C.1.5 - Cerebrovasculature

Studies in cultured cerebrovascular endothelial cells demonstrate that Ang-(1-7) reduces Ang II-induced apoptosis by modulating NADPH oxidase 2 (Nox2)/ROS and phosphoinositide 3-kinase (PI3K)/nitrous oxide (NO) pathways (428). These highlight not only the reduction of noxious ROS and inflammatory mediators by Ang -(1-7), but also Ang-(1-7)'s potential for preserving the cerebrovasculature. In vivo studies have demonstrated that pre-treatment with ICV Ang-(1-7) in rats reduces cerebrovascular loss following ischemic stroke through an angiogenic, rather than anti-apoptotic, mechanism (163).

1C.1.6 - Blood-brain barrier

The effects of Ang-(1-7) on BBB function further demonstrate potential benefits of this peptide. One study showed that Ang-(1-7) administration increases expression of Claudin-5 and zonula occludens 1 (ZO-1) (cell adhesion molecules involved in tight junction formation of brain endothelium) in cell culture and in rats during glioma formation (208). In the same rat glioma model, Ang-(1-7) treatment reduces tumor edema and Evans blue leakage into the brain; thus highlighting Ang-(1-7)'s ability to ablate glioma-induced BBB damage (208). In a rat models of cerebral ischemia, Ang-(1-7) increases Claudin-5 and ZO-1 expression within brain tissue, and attenuates both the damage and the BBB permeability associated with cerebral ischemia reperfusion injury (426). Utilizing co-culture of astrocytes and endothelial cell lines, Wu et al. showed that

Ang-(1-7) increases Claudin-5 and ZO-1 by inducing expression of Tissue inhibitor of matrix metalloprotease 1 (TIMP-1) and hence downregulation of MMP-9 (426). Thus, Ang-(1-7) shows promise to decrease BBB permeability in various models of CNS insult, which may be conserved after TBI.

Investigator	Insult	Route of Administration	Effects
Mecca et al., 2011 (238)	Endothelin-1-induced middle cerebral artery occlusion in rats	ICV (pre-treatment)	• Reduced infarct volume • Increased perinfarct density of NeuN+ neurons • Reduced behavioral deficits
Regenhardt et al., 2013 (303)	Endothelin-1-induced middle cerebral artery occlusion in rats	ICV (pre-treatment)	• Reduced brain infarct volume • Decreased brain mRNA expression of CD11b, CXCL12, IL-6, and IL-1α • Decreased brain iNOS protein
Jiang et al., 2014 (163)	Permanent middle cerebral artery occlusion in rats	ICV (pre-treatment)	• Reduced brain infarct volume • Increased brain CD31+ capillary density • Increased cerebral blood flow • Reduced neurological deficits
Regenhardt et al., 2014 (304)	Hemorrhagic stroke in spontaneously hypertensive rats	ICV (two weeks after start of high salt diet)	• Reduced frequency of hemorrhagic stroke • Reduced mortality • Reduced microgliosis

Table 1. Benefits of Ang-(1-7) on various brain pathologies in rodents

1C.2 - AT4R

A far less discussed component of the extended RAS is Angiotensin IV (Ang IV) and its associated Angiotensin IV receptor (AT4R). Ang IV is formed following enzymatic cleavage of Ang II by two peptidases (301). First, the cleavage of Ang II by aminopeptidase A (APA) forms Angiotensin III (Ang III) (301). Second, the cleavage of Ang III by aminopeptidase N (APN) forms the hexapeptide Ang IV (301). Ang IV signals through AT4R, an insulin-regulated aminopeptidase (IRAP) (52). However, the mechanism by which it signals is up for debate (185; 423). Evidence suggests that Ang IV signaling increases transport of the glucose transporter GLUT4 to neuronal membranes, thereby increasing glucose uptake. Nonetheless, Ang IV signaling affects many targets within the CNS and promotes benefits similar to those of AT2R and MasR signaling. As such, AT4R signaling is anti-inflammatory, and enhances cerebral blood flow (185; 423). A study by Faure et al. demonstrated the neuroprotective potential of intravenous Ang IV administration on ischemic stroke in rats. Their results revealed a dose dependent decrease in neurological deficits, cerebral infarct, and overall mortality by 24 hours after insult. Additionally, they showed that Ang IV promotes a redistribution of cerebral blood flow to ischemic regions within minutes of arterial occlusion (98).

Ang IV's ability to modulate rodent behavior was first characterized 30 years ago and has been demonstrated by many others since. A study in rats showed that ICV administration of Ang IV stimulates exploratory locomotive behavior, and improves both acquisition and recall during passive avoidance task when compared to naive rats, suggesting that Ang IV possess cognitive enhancing characteristics (37). Ang IV's cognitive enhancing effects are preserved in models of CNS insult. Indeed, ICV

administration of Ang IV, or similarly acting analogues, reverses the spatiotemporal memory deficits associated with scopolamine treatment, ischemia, and bilateral knife cuts to the brain (8; 283; 421; 425). Most recently, it was shown that that subcutaneous administration of Ang IV in DBA/2 mice increases the amount of time they spend investigating novel objects (118). Even while utilizing less invasive routes of administration, the cognitive benefits of Ang IV are conserved. Thus, as AT4R stimulation promotes various beneficial phenotypes within the CNS, it too should be considered a potential target for pre-clinical TBI studies.

1C.3 - MrgD

The extended RAS also includes the angiotensin peptides Angiotensin A (Ang A) and Alamandine (Alam), and the GPCR Mas-related G-protein coupled receptor (MrgD) (377). Through an unclear mechanism, decarboxylation of the N-terminal aspartate residue on Ang II is often rendered to an alanine (162). This alanine-substituted octapeptide is known as Ang A. Ang A has similar affinity towards AT1R as Ang II. Though results vary regarding Ang A's affinity for AT2R, it is clear that its actions parallel Ang II and are mediated through AT1R. However, its potency is far less than Ang II (150; 162; 435). Thus, decarboxylation of Ang II's terminal aspartate may serve as a mechanism to reduce Ang II's actions.

Removal of Ang A's terminal phenylalanine forms the heptapeptide known as Alam. Decarboxylation of Ang-(1-7)'s N-terminal aspartate serves as another mechanism to produce Alam (194). Tetzner et al. showed that A779 and D-pro⁷ (MasR antagonist; Ang-(1-7) with a D-pro substitute for residue number seven) both attenuate Ang-(1-7) signaling in HEK293 cells overexpressing MasR (377). However, MrgD signaling

parallels that of MasR in response to Ang-(1-7) (377). Thus it is not clear whether Ang-(1-7) signaling is mediated by MasR or MrgD. One way to differentiate this is to utilize ligands that differentiate between the two receptors. D-pro blocks both MasR and MrgD whereas A779 more specifically blocks MasR (377). Furthermore, Alam signaling is only observed in MrgD overexpressing cells, not in those overexpressing MasR, and A779 does not block this signaling (377; 403). Thus, while Ang-(1-7) can signal through both MasR and MrgD, Alam signaling only occurs through MrgD. An illustrative summary of the RAS/extended RAS ligands and their respective receptors is shown in Figure 1.

As Ang-(1-7) signaling through MrgD has only recently been characterized, the relative expression levels of MrgD within the CNS have not been well studied. However, its expression has been confirmed on neurons (66; 168). As MrgD signaling is similar to that of MasR, activation of MrgD signaling may provide similar benefits to MasR or AT2R activation within the CNS and potentially to TBI.

1D - SUMMARY

Various components of the RAS, including ARBs and AT2R agonists, can reduce pathophysiological progression of TBI *in vivo* (160; 387; 388; 400; 402). However, the great majority of RAS and extended RAS studies for brain related pathologies focus on neurodegenerative disease, ischemic insult, or broader categories such as neuroinflammation, gliosis, or general neuroprotection. To our knowledge, no studies have investigated the therapeutic value of targeting the extended RAS to treat TBI. As we hypothesize that administration of Ang-(1-7) will benefit recovery in a CCI model of TBI in mice, we follow with an experiment designed to determine the effects of post-injury subcutaneous Ang-(1-7) administration on various CCI-related neurological

consequences; ultimately to assess whether Ang-(1-7) treatment holds potential as a therapeutic for the brain-injured patient.

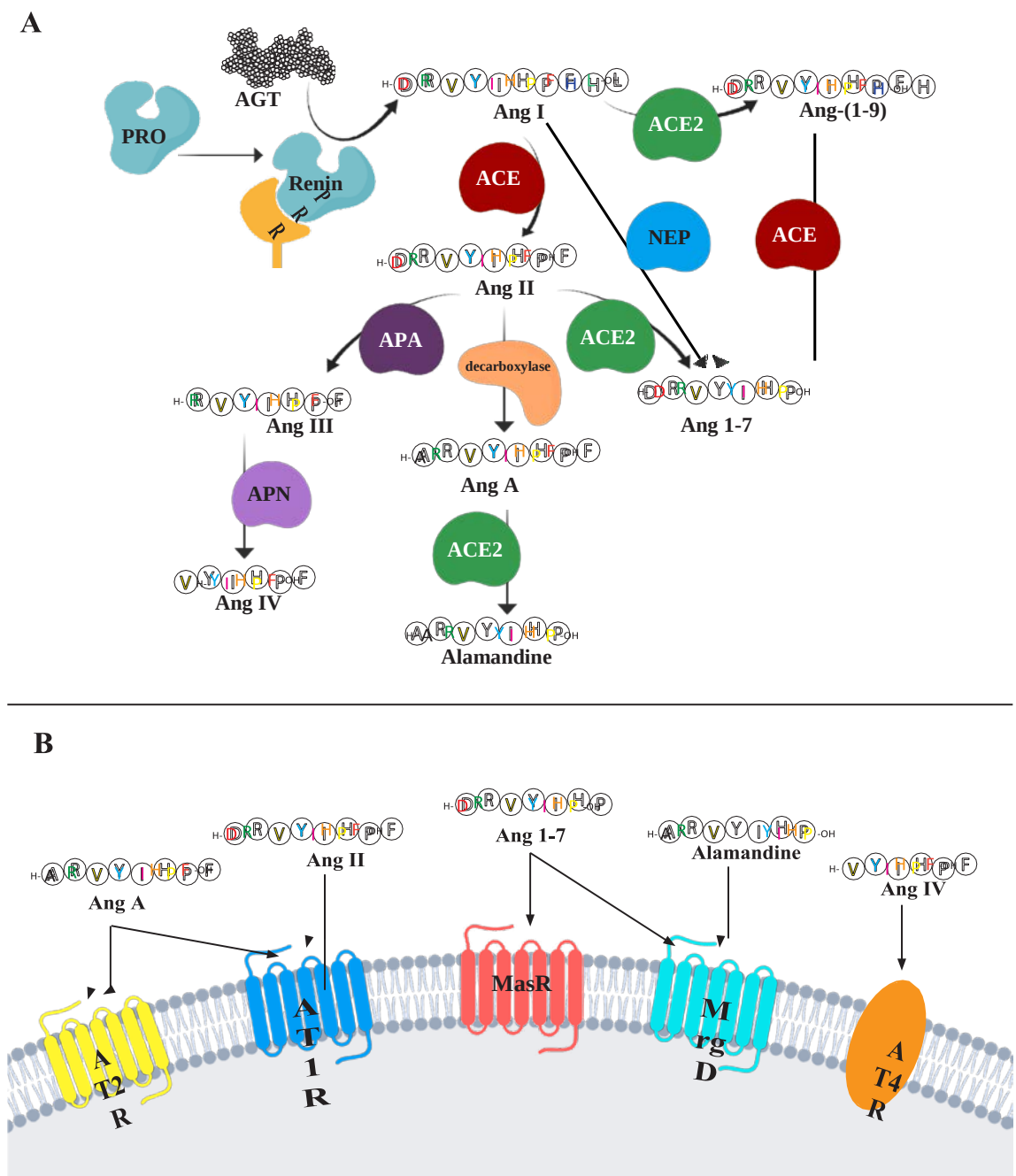


Figure 1. Overview of the renin-angiotensin system (RAS).
 A) Generation of various RAS peptides. B) Ligand-receptor interactions of RAS peptides.

CHAPTER 2: Methods

2A - ANIMALS, SURGICAL PROCEDURES, DRUG TREATMENTS AND EXPERIMENTAL DESIGN

2A.1 - *Animals*

All animal studies were approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee and were conducted in accordance with the NRC guide to the Care and Use of Laboratory Animals.

C57BL/6NCr male mice (NCI, Frederick, MD), ranging in age from 8-10 weeks and weighing 20–25 g, were utilized for each experiment. All mice were kept under 12:12 h light and dark cycle with access to food and water ad libitum. Five mice were housed in each cage. After arrival, mice were acclimatized for one week before use.

2A.2 - *Severe controlled cortical impact*

Mice were anesthetized with isoflurane (3% induction, 2% maintenance) and their heads mounted in a stereotaxic frame. Body temperature (37 ° C) was maintained for the duration of surgery using an isothermal heating pad (Stoetling, IL). Scalps were shaved over the designated area, cleansed with 70% ethanol followed by Betadine. Eyes were protected with ointment (Optixcare Eye Lube). A 1 cm midline scalp incision was made to expose Bregma, the pericranium was reflected, and a 3 mm craniectomy was made (2 mm lateral (left), 2 mm caudal to Bregma). An electromagnetically driven CCI injury device (Impact One stereotaxic impactor, Leica Microsystems, Germany) was used to deliver a severe CCI (impact depth 2 mm, speed 3.6 m/s, dwell time 100 ms) at a 12° angle to the dura mater. The scalp was closed using 6-0 nylon simple interrupted sutures. Sham-animals received the same anesthesia and scalp incision without craniectomy or impact injury.

Coordinates of CCI were set to maximize injury to the ipsilateral somatosensory cortex (S1BF), anterior thalamic nuclei, and hippocampus (106).

2A.3 - Drug treatment

All treatments were administered either by subcutaneous injection (daily 250 μ L bolus) or continuous infusion via subcutaneously implanted micro-osmotic pumps (Alzet, Model 1004, delivering 0.11 μ L/h). Angiotensin-(1-7) (Tarix Orphan, TXA-127) was suspended in bacteriostatic saline (Hospira, Lake Forrest, IL, RL-4471) at concentrations of 10.0mg/mL for micro-osmotic pumps and 0.1mg/mL for injections, ensuring 1.0mg/kg/d by either form of administration. Initially, mice that were sacrificed at 3 dpi received daily injections starting at 1 hour post-injury (hpi). Mice that were sacrificed at 29 dpi received a one-time injection at 6 hpi followed by micro-osmotic pump implantation at 24 hpi. The pumps were primed with Angiotensin-(1-7) for 48 hrs before implantation. Mice used to assess whether Ang-(1-7)'s benefits were MasR-dependent underwent implantation with micro-osmotic pumps filled with MasR-antagonist A-779 (Tocris Bioscience, Bristol, UK, 5937) suspended in bacteriostatic saline (Hospira, Lake Forrest, IL, RL-4471) at 25.0mg/mL, ensuring administration of 2.5mg/kg/d, starting 2 d prior to CCI. They then received daily injection of Ang-(1-7) or saline, starting at 6 hpi, until sacrifice at 3 dpi.

2A.4 - Experimental design and randomization

The overall experimental design is presented in Figure 1. For histology experiments, we used between 5-10 mice/group. For behavioral studies we included 18-20 mice/group. Less than 2% of the injured mice died prematurely after surgery. Three mice were excluded post hoc during lesion volume analysis due to predetermined measurement

criteria (see histological lesion volume calculations). Treatment of drug or vehicle was randomly assigned and administered to each mouse, with the investigator blinded to the treatment. Blinding was not undone until post hoc data analysis concluded. The investigator treated control mice similarly.

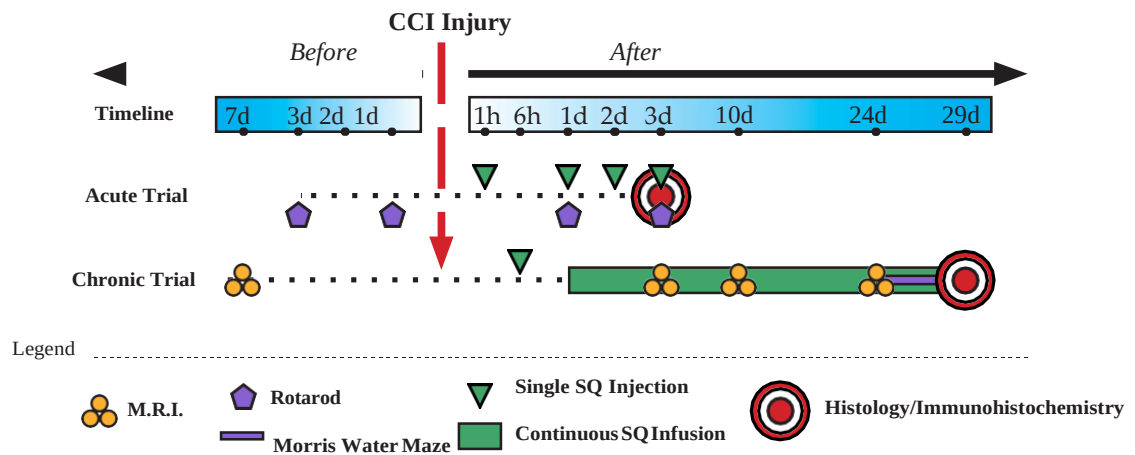


Figure 2. Experimental timeline.

Different cohorts of mice were subjected to CCI injury and sacrificed at either 3 or 29 days post-injury (dpi) for immunohistochemical and histological analysis of brain sections. Motor function after injury was assessed by rotarod in the acute trial, and cognitive function by Morris water maze in the final week of the chronic trial. Lesion volume was tracked longitudinally during the trial via magnetic resonance imaging.

2B - ASSESSMENT OF RECOVERY

2B.1 - Functional Recovery

2B.1.1 - Rotarod Performance Test

Motor function and/or motor coordination deficits in mice were determined by assessing ability to stay on a rotating rod (rotarod) using the Rota-Rod For Mice, 47600 (Ugo Basile, PA). Mice were trained for 3 consecutive days starting 3 days prior to injury, and tested again at 1 and 3 days post-injury. The Rota-Rod For Mice 46700 (Ugo Basile, PA) has 5 separate lanes, thus cagemates were tested simultaneously. During both training and testing, mice were placed on the rotarod set to 4 rpm. Once each mouse demonstrated an ability to remain on the rotarod at 4 rpm, acceleration was initiated simultaneously with a timer. The rotarod was accelerated from 4 to 40 rpm over 2 min and held at 40 rpm for an additional 2 min. Upon falling off the rotarod, mice would land on a switch, thus stopping the timer. The time the mice were able to stay on the rotarod (latency to fall) was recorded in seconds. Mice who did not fall received a maximum time of 240 s. Additionally, the timer was stopped when mice who clung to the rotarod underwent three continuous revolutions without an attempt to resume walking. This was repeated 3 times each day, and each mouse was allowed at least 20 min between each round of testing.

2B.1.2 - Morris water maze

Spatiotemporal learning and memory deficits were assessed daily from 24 to 28 dpi via the Morris Water Maze (MWM). A 1.5 m diameter Stoelting Morris Water Maze tank (Stoelting Co., IL) was filled to 30 cm with 26°C tap water as to submerge a 6 cm diameter Plexiglas platform in the upright position under 2 cm of water. Unique visual

cues were placed on each of the four walls visible from within the tank. Four 60 s training trials were performed each day for each mouse, with 4 min of rest between each trial, for five consecutive days.

For each training trial, mice were placed in 1 of 4 quadrants within the tank and allowed 60 s to find the platform. Each day, mice performed a training trial started from each of the four quadrants. However, the order that the quadrants were presented to the mice varied each day. The time to reach and stay on the submerged platform (always located in the northwest tank quadrant) for 15 consecutive seconds was recorded as latency to reach the platform. Mice who did not find the platform after 60 s were gently guided to the platform and allowed to remain in place for 15 s before their removal. Upon removal from the tank, mice were gently towel dried and allowed 4 minutes on dry bedding under a heat lamp before subsequent training trials.

On training day 5, 1 h after the last training trial, a probe trial was performed where mice were placed into the southeast quadrant of the tank, and remained in the tank for 60 s with the Plexiglass platform removed. The time spent in the northwest quadrant (where the platform had previously been found) , and average swim speed were recorded using ANY-maze video tracking software (Stoelting Co., IL).

2B.2 - Histology and Immunohistochemistry

2B.2.1 - Tissue preparation for histological/immunohistochemical analysis

The mice were sacrificed at 3 and 29 days after CCI injury. Mice were anaesthetized with ketamine/xylazine (intraperitoneal injection, 100mg/kg ketamine, 12.5mg/kg xylazine) and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde overnight

at 4°C. The brains were then transferred into 10% sucrose in phosphate buffered saline (PBS) and again stored overnight at 4°C. Brains were sequentially incubated overnight at 4°C with 20% and 30% sucrose in PBS. Following overnight storage in 30% sucrose solution, brains were frozen in -78.5°C isopropyl alcohol and transferred into a -80°C freezer for storage until sectioning. Once ready to section, frozen brains were completely concealed in Tissue-Tek O.C.T. (Sakura Finetek USA Inc, CA) and allowed 15 min to equilibrate to -22°C. Brains were then cut into 20 µm coronal sections by cryostat. Brain sections were stored in Olmo's cryopreservant at -20°C. Brain sections spanning the lesion site rostral-caudally were selected for histological and immunohistochemical analysis (approximately 0.5 mm caudal to 3.5 mm caudal from Bregma).

2B.2.2 - Cresyl-violet staining

Cresyl-violet (0.1%, Sigma-Aldrich) was dissolved in deionized water and filtered. Every 12th brain section was mounted on gelatin-coated slides. Sections were sequentially hydrated for 2 min with 100, 95, 70, and 50% ethanol, and then stained for 10 min with 0.1% cresyl-violet solution. Subsequently, sections were sequentially dehydrated for 2 min with 50, 70, 95, and 100% ethanol, and then cleared in 99.5% xylene for another 2 minutes. Slides were covered with Cytoseal 60 (Richard-Allan Scientific, Ft. Washington, PA, Cat. # 18007) and protected with a cover slip.

2B.2.3 - Histological lesion volume analysis

Bright-field images were acquired with the x4 objective on the ZEISS Axioscan.Z1 utilizing Zeiss ZEN microscope software. Lesion area was assessed on 7-15 20 µm sections per mouse brain spaced every 240 µm (every 12th section) from the rostral to caudal edge of the visible lesion using the area assessment tool in Zeiss ZEN

microscope software (Zeiss). Lesion volume was calculated by multiplying the total distance between sections by the average cross-sectional area of the lesion. Lesion was defined as any asymmetrical morphology in the ipsilateral hemisphere with respect to the contralateral. If a brain section had an uneven coronal cut and the lesion spanned into the lateral ventricle, the section was excluded. If 50% or more of sections for any mouse was excluded, no lesion area data was included from that mouse.

2B.2.4 - Immunohistochemical staining

Sections were washed three times in phosphate-buffered saline with 0.1% Triton™ X-100 (PBST) for 5 min each, and blocked with 10% normal goat serum (NGS) in PBST for 1 h. The following primary antibodies were incubated at 4°C overnight in PBST/5% NGS: anti-GFAP, chicken polyclonal (1:1000, Abcam, Ab4647); anti-Iba1, mouse monoclonal (1:1000, Wako, 019-19741); anti-NeuN, mouse monoclonal (1:1000, Milipore, NG1876252); and anti-CD31, rat monoclonal (1:300, BD Biosciences, 550274). Sections were washed three times in PBST and incubated with either Alexa Fluor® 568-conjugated donkey anti-rabbit (red), Alexa Fluor® 488-conjugated goat anti-chicken (green), or Alexa Fluor® 647-conjugated goat anti-rat (far-red) IgG secondary antibody (1:1000, Invitrogen) overnight at 4°C. Sections were then rinsed with PBST and deionized water, mounted on gelatin coated slides, covered with ProLong® Gold antifade reagent with DAPI (Invitrogen), and protected with coverslips.

2B.2.5 - Immunohistochemical quantification

Fluorescent images were acquired with the x10 and x20 objective, respectively, on the ZEISS Axioscan.Z1 utilizing Zeiss ZEN microscope software (Zeiss). Fluorescent images were also acquired with the x20 objective on an Olympus BX61 with attached

qImaging Retiga EXi Aqua CCD camera, and iVision software (BioVision Technologies). Following microscopy, image files were exported from Zeiss ZEN microscope software (Zeiss) and their files types converted from CZI to JPEG.

Quantitative image analysis was performed on at least four lesion-including sections per mouse, from between 2.50 mm and 1.50 mm caudal to bregma. For each neuroanatomical region in question, immunoreactivity or cell count was assessed in a total of five viewing fields per region, per section. Iba1+ microglia counts were acquired using an intensity-thresholded and size-gated automated counting method in ImageJ and were divided by the total area in the image field (mm²). The intensity-thresholded and size-gated automated counting method applied the same pre-determined “average optimal size range” and “average optimal intensity” to all images assessed. “Average optimal size range” and “average optimal intensity” thresholds were determined by averaging the optimal size range and optimal intensity thresholds required to maximize visibility of IBA-1+ microglia for five random images. Average IBA-1+ cell size was assessed following the same thresholding parameters in ImageJ. Similar methods were used for NeuN+ neuron counts and CD31+ capillary density. No size gating was required for GFAP immunoreactivity quantification as GFAP immunoreactivity was quantified by dividing the number of post-threshold pixels with the area of the image field. However, determination of the “average optimal intensity” threshold to apply to each image was performed as described for IBA-1+ microglia. The investigator was blinded to the treatment throughout the quantification process.

2B.2.6 - Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed on a 7 Tesla small animal (20-cm horizontal bore) Bruker BioSpec scanner (20-cm horizontal bore) with 120-mm diameter, 670 mT/m gradient coils and 4-channel phased array MR coils (Bruker Biospin, Inc., Billerica, MA). Mice were anesthetized with isoflurane (3% induction, 2% maintenance) and placed on a specialized small animal holder atop a precision controlled-heating pad. Proton density weighted imaging (PDW), T2-weighted imaging (T2W), and T2-mapping (T2map) was performed utilizing multi echo 2D Rapid Acquisition with Relaxation Enhancement (2D-RARE) with the following parameters: repetition time — 4500 ms; echo time — 10, 30, 50, 70, 90, and 110 ms; echo train length — 2; number of averages — 4; field of view — 13.8 x 112.0 mm²; in-plane resolution — 150 x 150 μ m²; slice thickness — 600 μ m. Each mouse underwent MRI 7 days prior to injury, and 3, 10 and 24 days post injury. Respiration, heart rate, SpO₂, and temperature were recorded every 10 minutes during imaging.

2B.2.7 - MRI Lesion volume calculation

Lesion was defined as any asymmetrical signal or morphology in the ipsilateral hemisphere with respect to the contralateral hemisphere. Structural or signal abnormalities that appeared unrelated to injury were compared to baseline images. The investigator was blinded to the treatment throughout the quantification process.

2B.2.8 - Statistical analysis

All data in this study are expressed as mean $\pm$ standard error of the mean (SEM). $P < 0.05$ was considered statistically significant. All experiments that simultaneously compared vehicle to Ang-(1-7) treatment and sham to injury were evaluated using a two-

way ANOVA with Tukey's multiple comparisons corrections. Reported p-values were overall effect of injury (two-way ANOVA) or group versus group (two-way ANOVA+ Tukey's multiple comparisons test). Experiments that compared treatment start times were evaluated with a one-way ANOVA with Tukey's multiple comparisons testing. Experiments that simultaneously compared vehicle to Ang-(1-7) over time were evaluated using a repeated-measure two-way ANOVA with Sidak's multiple comparison test. Experiments that solely compared vehicle to Ang-(1-7) were evaluated using an unpaired, parametric, two-tailed t-test. All previously mentioned statistics were analyzed with Graphpad Prism 7 software (GraphPad Software, CA). To assess spatiotemporal learning during the MWM, latency to platform was compared with time and treatment by three-way ANOVA in Statistical Package for the Social Sciences (SPSS) (IBM, NY).

CHAPTER 3: Results

3A - ANG-(1-7) TREATMENT REDUCES LESION VOLUME AFTER CCI

3A.1 - Histology

To determine if subcutaneous administration of Ang-(1-7) was neuroprotective after CCI, we treated mice with 1mg/kg/day of Ang-(1-7) or vehicle, starting 1 h after injury, and sacrificed the mice at 3 dpi (Figure 2). Quantification of the lesion size on cresyl violet stained brain sections showed that Ang-(1-7) treatment reduced ($p < 0.01$) lesion volume in comparison to that of vehicle treated injured mice (Figure 3A, 3B). To determine whether this neuroprotective effect was detectable over a longer duration of treatment, we repeated the experiment, starting treatment 6 hours after injury and maintaining continuous Ang-(1-7) infusion (1mg/kg/day) until sacrifice at 29 dpi (Figure 2). Quantification of the lesion volume in brains taken from these mice showed a similar protective effect, with Ang-(1-7) treatment leading to a smaller ($p < 0.05$) lesion volume after 29 days of treatment (Figure 3A, 3C).

3A.2 - Magnetic Resonance Imaging

To assess whether lesion size at time of sacrifice accurately represented changes of lesion volume *in vivo*, we performed CCI on a different cohort of mice, and treated them with Ang-(1-7) or vehicle, for 29 days, starting 6 hrs after CCI. We utilized magnetic resonance imaging (MRI) to visualize changes in the lesion size with time by measuring the lesion at 3, 10 and 24 dpi. Using T2-weighted MRI to calculate lesion volume (Figure 3D), Ang -(1-7) treatment led to reduction ($p < 0.05$) in lesion volume at 3, 10, and 24 dpi (Figure 3E). Thus, Ang-(1-7) treatment leads to a smaller lesion after CCI injury.

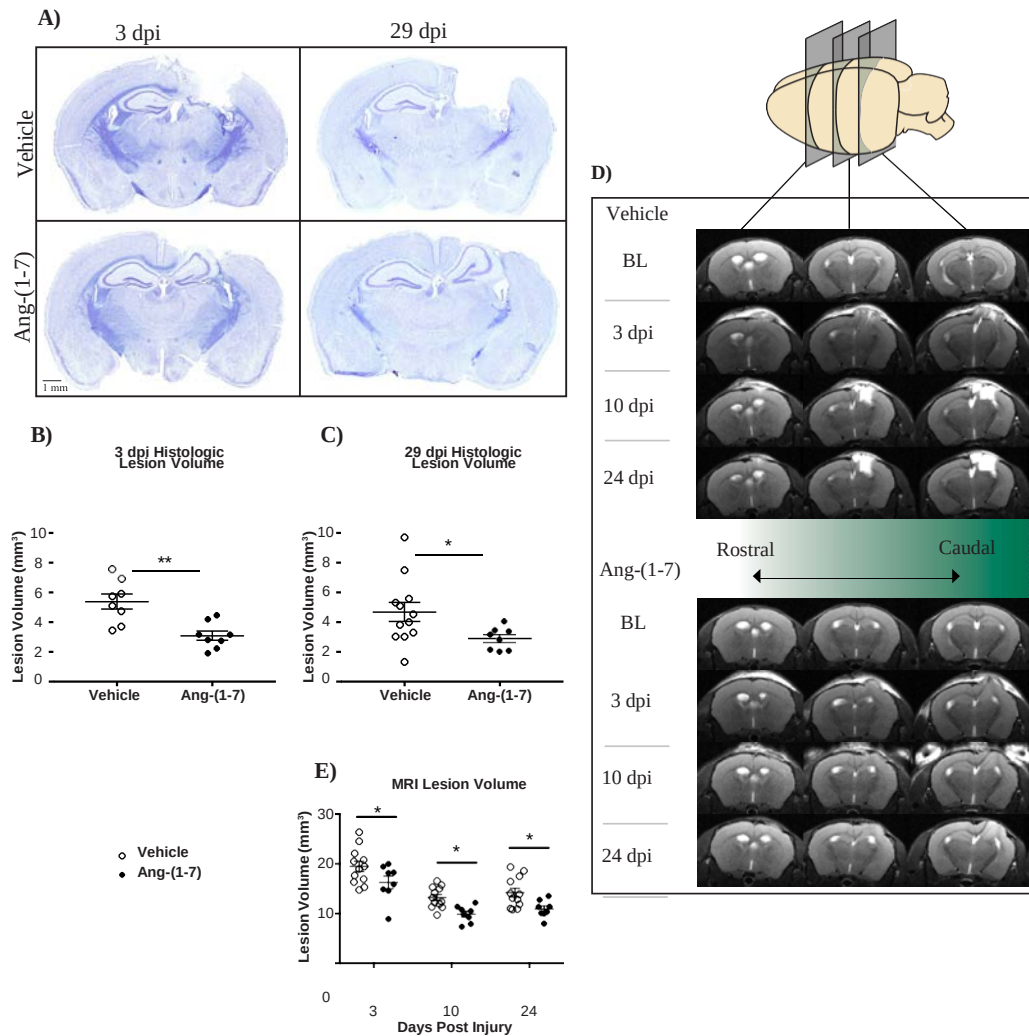


Figure 3. Lesion volume assessed by histology and MRI at different time points after CCI.

(A) Representative cresyl violet stained brain sections from CCI injured mice treated with either vehicle or Ang- (1-7) (1mg/kg) for either 3 or 29 days. Graph of lesion volume in brain sections sacrificed at (B) 3 or (C) 29 dpi n = 8-13. Data are mean $\pm$ SEM, **p < 0.01, *p < 0.05. Mice brains were assessed using T2-weighted MRI before injury (baseline - BL), and at 3, 10, and 24 days post injury (dpi) for cohorts treated with either vehicle or Ang-(1-7) after CCI. (D) Representative images from different mice showing the variation in lesion size (hyperintense regions) from rostral to caudal over time and with treatment. (E) Graph of lesion volume at 3, 10 and 24 dpi. n = 8-12, Mean $\pm$ SEM, *p < 0.05. p-values represent vehicle-to-Ang-(1-7) comparison (two-way ANOVA + Sidak's multiple comparisons test).

3B - ANG-(1-7) TREATMENT REDUCES ACUTE MICROGLIOSIS FOLLOWING CCI

To determine whether Ang-(1-7) administration altered the activation or survival of glial cells after CCI, we assessed staining for glial specific markers in brain sections taken from mice treated with Ang-(1-7) or vehicle and sacrificed at 3 dpi. We examined staining in the perilesional cortex, ipsilateral thalamus (centered around the anterior thalamic nuclei), and contralateral hippocampus (centered around CA3). We assessed the contralateral hippocampus as the ipsilateral hippocampus was often absent following injury. Additionally, others have noted changes of microgliosis in the contralateral hippocampus after CCI (262). However, we anticipated less robust alterations in the contralateral hippocampus compared to the ipsilateral brain regions examined as this is further away from the impact. We found a robust increase ($p < 0.01$) of Iba1+ microglia as well as GFAP+ astrocytes in each brain region after CCI (Figure 4). We found that Ang-(1-7) treatment reduced ($p < 0.05$) the number of Iba1+ microglia in each brain region examined, but did not reduce ($p > 0.9$) the level of GFAP + astrocytic staining in any of the same brain regions. Microglia in vehicle treated mice after CCI displayed an activated phenotype with a dramatic increase ($p < 0.0001$) in soma size and overall amoeboid morphology at 3 dpi (Figure 4D, K, S). Ang-(1-7) treatment reduced ($p < 0.01$) microglia soma size and promoted a spindle morphology, indicating an overall reduction in microglial activation (Figure 4A-D, AE).

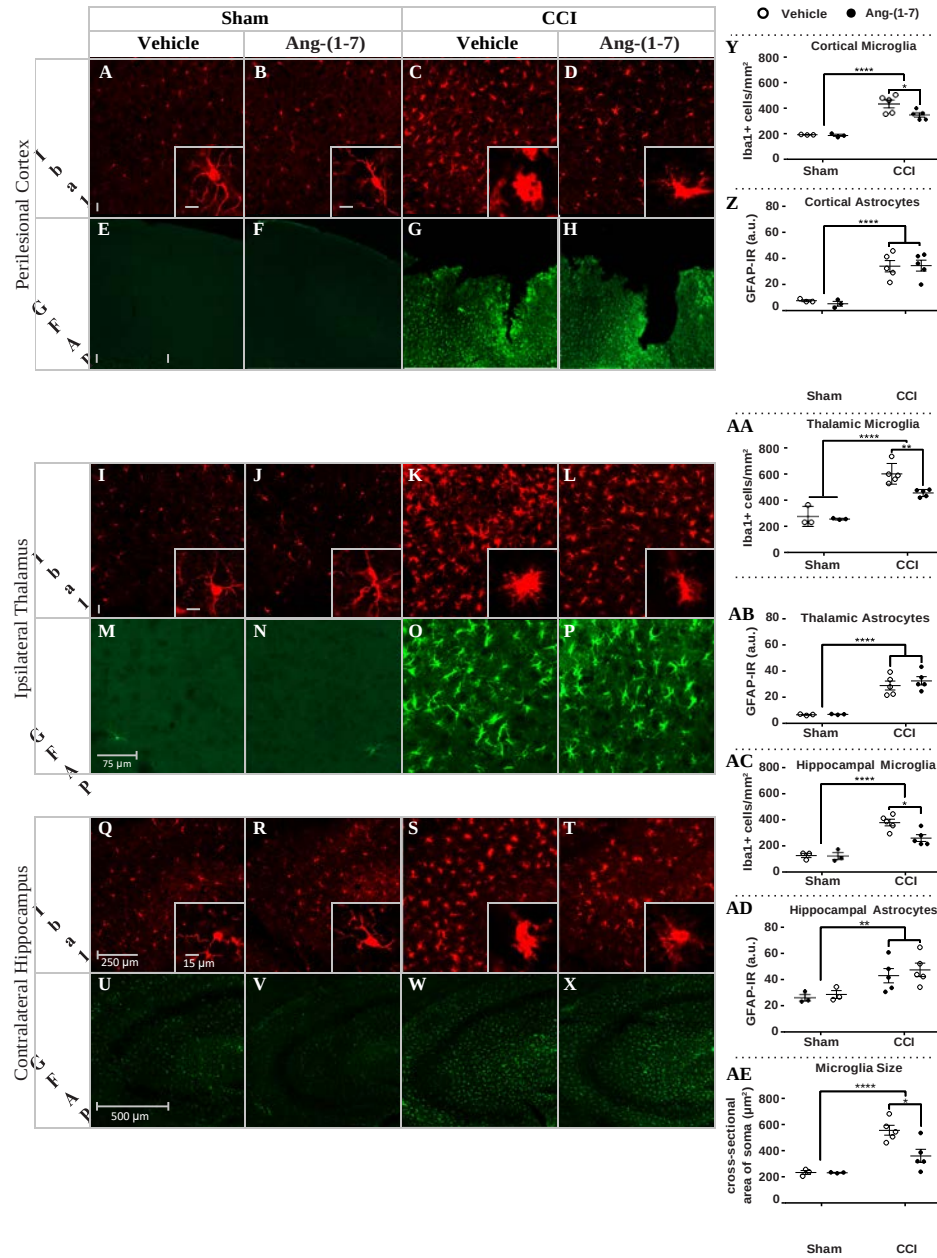


Figure 4. Acute gliosis at 3 days after CCI.

(A-X) Representative immunohistochemistry of brain sections from CCI injured or sham mice treated with vehicle or Ang-(1-7) sacrificed at 3dpi. Microglial reactivity was determined in the perilesional cortex (A-D, Y), ipsilateral thalamus (I-L, AA), and contralateral hippocampus (Q-T, AC), by counting Iba1+ cells/mm². Microglia soma size was assessed in the perilesional cortex (AE). Insets show morphology of individual microglia in each brain region following indicated treatment. Reactive astrocytes were assessed by quantifying GFAP immunoreactivity (IR) in the perilesional cortex (E-H, Z), ipsilateral thalamus (M-P, AB), and contralateral hippocampus (U-X, AD). n = 3-5 mice/ group, Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. p-values represent either overall effect of injury (two-way ANOVA) or injury + vehicle versus injury + Ang-(1-7) comparison (two-way ANOVA + Tukey's multiple comparison test).

(a.u.) = arbitrary unit. Scale bar = 250 μm for A-D, I-L, and Q-T. Scale bar = 500 μm for E-H, M-P, and U-X. Scale bar = 15 μm for inlets.

3C - ACUTE CAPILLARY AND NEURONAL CHANGES FOLLOWING CCI

Traumatic insults to the CNS can rupture both healthy neurons and capillary networks formed by cerebrovascular endothelial cells. We therefore assessed the effect of Ang-(1-7) treatment on CD31+ capillary density and NeuN+ neuronal density in brains taken from mice sacrificed at 3 dpi (Figure 5). Immunohistochemical assessment demonstrated a decrease ($p < 0.001$) of both capillary and neuronal density in the perilesional cortex and ipsilateral thalamus following CCI. However, neither CD31+ or NeuN+ staining was altered by Ang-(1-7) treatment ($p > 0.9$) at 3 dpi (Figure 5A-L, Y-AB). We did not detect any injury-induced change in capillary or neuronal density ($p > 0.9$) in the contralateral hippocampus (Figure 5Q-X, AC, AD).

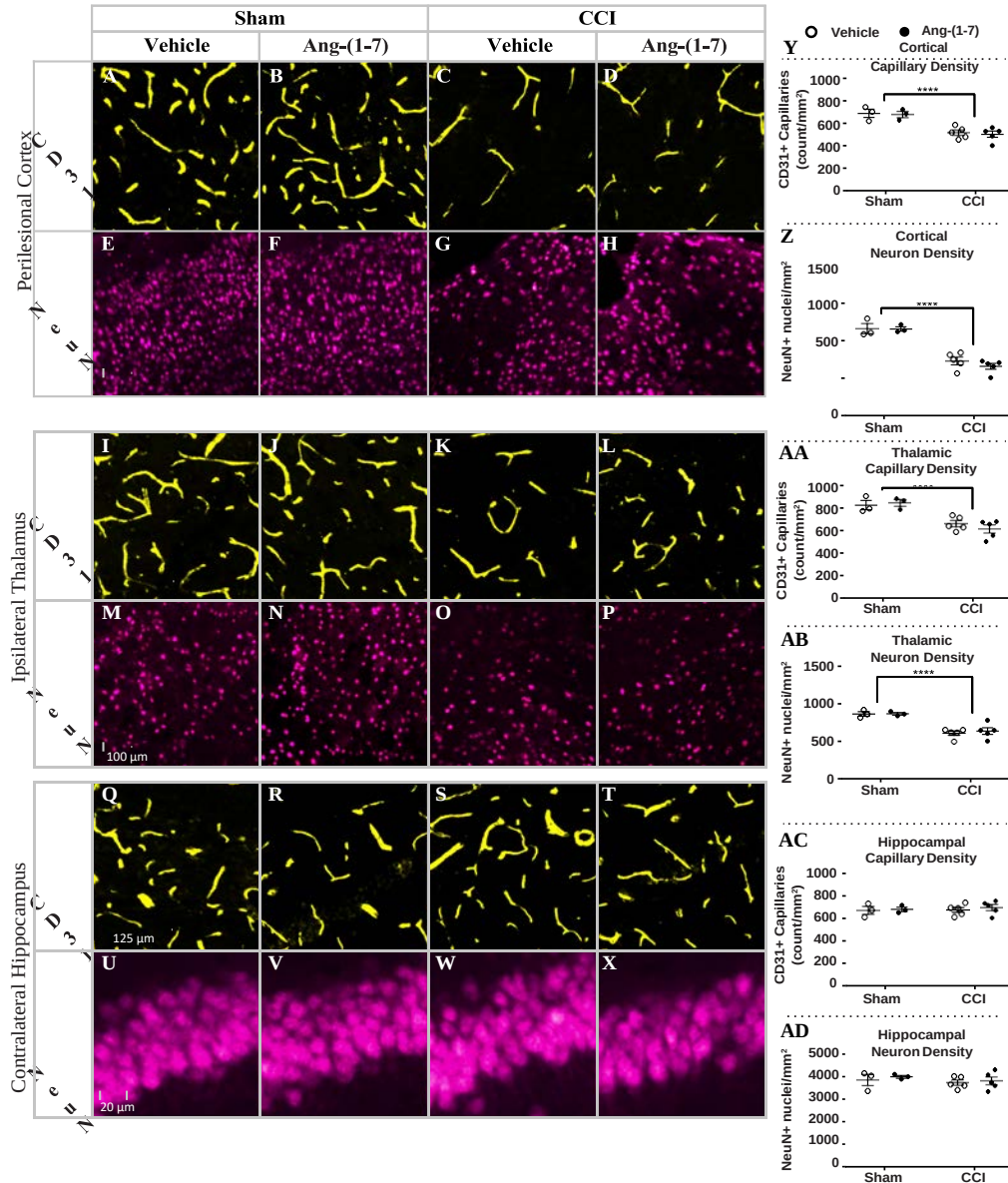


Figure 5. Effect of Ang-(1-7) treatment on neurons and microvasculature in different brain regions at 3dpi.

(A-X) Representative immunohistochemistry of sections from different brain regions taken from CCI injured or sham mice treated with vehicle or Ang-(1-7) sacrificed at 3dpi. Capillary density was determined in the perilesional cortex (A-D, Y); ipsilateral thalamus (I-L, AA); and contralateral hippocampus (Q-T, AC); by counting CD31+ structures/mm². Density of neurons was assessed by counting NeuN+ nuclei/ mm² in the perilesional cortex (E-H, Z), ipsilateral thalamus (M-P, AB), and contralateral hippocampus (U-X, AD) n = 3-5 mice/ group, Mean $\pm$ SEM, ***p<0.001, ****p<0.0001. p-values represent either overall effect of injury (two-way ANOVA) or injury + vehicle versus injury + Ang-(1-7) comparison (two-way ANOVA + Tukey's multiple comparison test). Scale bar = 125 μ m for A-D, I-L, and Q-T. Scale bar = 20 μ m for E-H, M-P, and U-X.

3D - ANG-(1-7) REDUCES ASTROGLIOSIS AND MICROGLIOSIS ONE MONTH AFTER CCI

As Ang-(1-7) treatment altered the lesion volume over several different time points, we wanted to determine the effects that longer Ang-(1-7) treatment had on glial activation beyond 3 dpi. We therefore determined whether continuous treatment with Ang-(1-7) for 29 days altered the number of Iba1+ microglia, or the amount of GFAP+ astrocytic staining, in brains of mice (Figure 6). Robust astrogliosis was present in the perilesional cortex, ipsilateral thalamus, and contralateral hippocampus ($p < 0.001$) 29 days after CCI (Figure 6E-H, Z; M-P, AB; U-X, AD). Ang-(1-7) treatment resulted in significant reduction ($p < 0.05$) of GFAP+ astrocytic staining in all three brain regions at 29 dpi (Figure 6E-H, M-P, U-X). In the cortex, the glial scar is visible at the lesion edge, and appears thinner after Ang-(1-7) treatment. The number of Iba1+ cells after CCI was greater ($p < 0.001$) than in brains from sham mice in the perilesional cortex and ipsilateral thalamus; but not the contralateral hippocampus ($p > 0.9$) at this time point (Figure 6A-D, Z; I-L, AA; Q-T, AC). Microglia in the contralateral hippocampus had also reverted to the resting morphology, in contrast to the ameboid shape of microglia in the ipsilateral thalamus and perilesional cortex (Figure 6C, K, S). Interestingly, Ang-(1-7) treatment reduced ($p < 0.05$) the number of Iba1+ microglia in the ipsilateral thalamus, but did not alter ($p > 0.8$) Iba1+ cells in the perilesional cortex (Figure 6I-L, AA; A-B, Z).

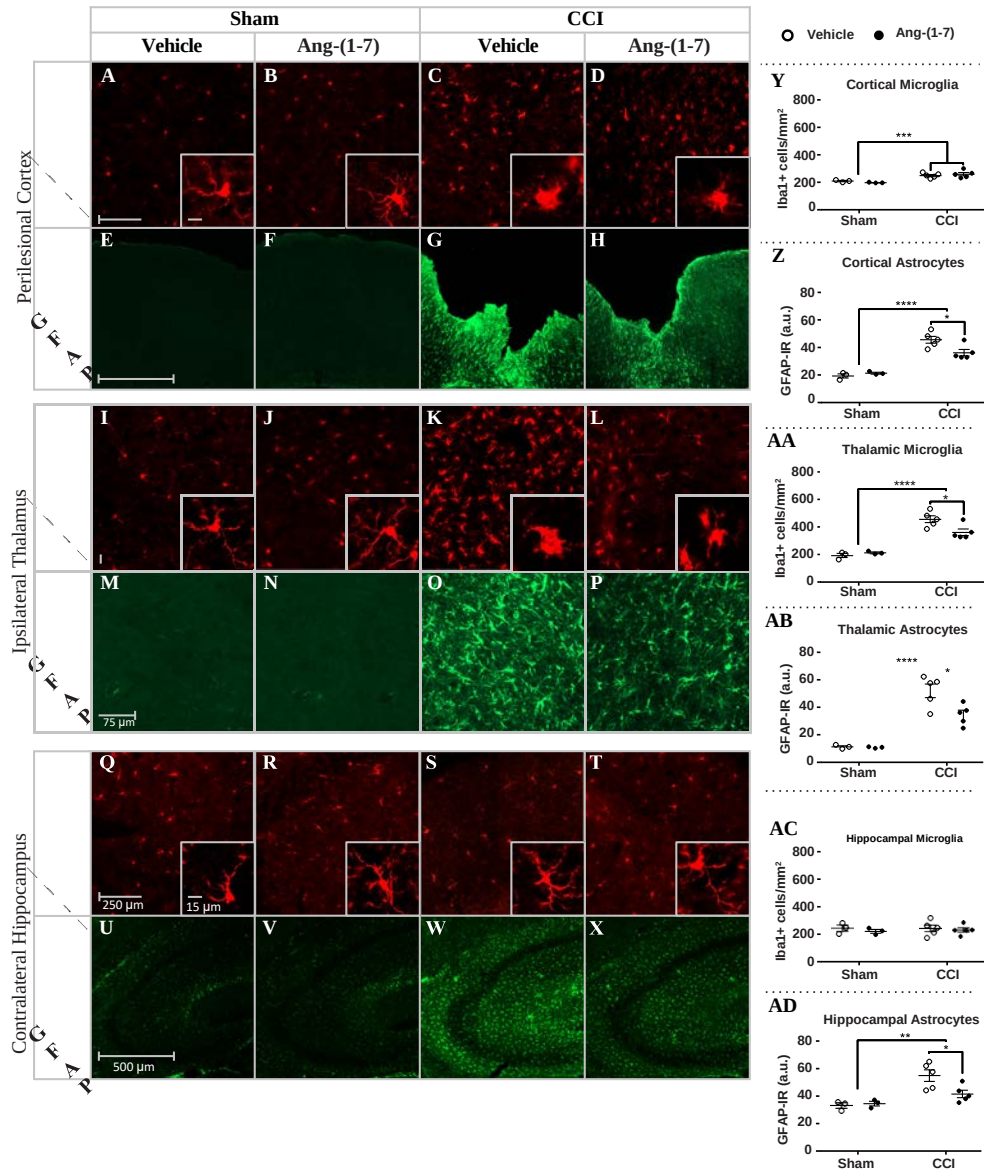


Figure 6. Glial reactivity at 29 days after CCI.

(A-X) Representative immunohistochemistry of brain sections from CCI injured or sham mice treated with vehicle or Ang-(1-7) sacrificed at 29 dpi. Microglial reactivity was determined in the perilesional cortex (A-D, Y), ipsilateral thalamus (I-L, AA), and contralateral hippocampus (Q-T, AC), by counting Iba1+ cells/mm³. Insets show morphology of individual microglia in each brain region following indicated treatment. Reactive astrocytes were assessed by quantifying GFAP immunoreactivity (IR) in the perilesional cortex (E-H, Z), ipsilateral thalamus (M-P, AB), and contralateral hippocampus (U-X, AD). n = 3-5 mice/group, Mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (a.u.) = arbitrary unit. p-values represent either overall effect of injury (two-way ANOVA) or injury + vehicle versus injury + Ang-(1-7) comparison (two-way ANOVA + Tukey's multiple comparison test). Scale bar = 250 μm for A-D, I-L, and Q-T. Scale bar = 500 μm for E-H, M-P, and U-X. Scale bar = 15 μm for

inlets.

3E - ANG-(1-7) TREATMENT PRESERVES CAPILLARY AND NEURONAL DENSITY ONE MONTH AFTER CCI

To determine the effect of longer treatment with Ang-(1-7) on capillary and neuronal density, we examined brains taken from mice after 29 days of treatment. Brain sections from Ang-(1-7) treated injured mice had more ($p < 0.05$) neurons in the ipsilateral thalamus than sections from vehicle treated injured mice (Figure 7O, P, AB). However, Ang-(1-7) treatment did not alter ($p > 0.8$) the injury induced reduction in neuronal density in the perilesional cortex (Figure 7G, H, Z). The contralateral hippocampus did not exhibit any change ($p > 0.9$) in neuron density with injury (Figure 7W, X, AD). The effect of Ang-(1-7) treatment on CD31+ capillary density mirrored that of neuronal density. Prolonged Ang-(1-7) treatment led to an increase ($p < 0.05$) in the injury-reduced density of CD31+ capillaries in the ipsilateral thalamus (Figure 7K, L, AA). Ang-(1-7) treatment did not alter ($p > 0.3$) the injury-induced reduction in capillary density in the perilesional cortex (Figure 7A-H, Y, Z), and there was no injury effect ($p > 0.9$) on the density of CD31+ capillaries in the contralateral hippocampus (Figure 7S, T, AC).

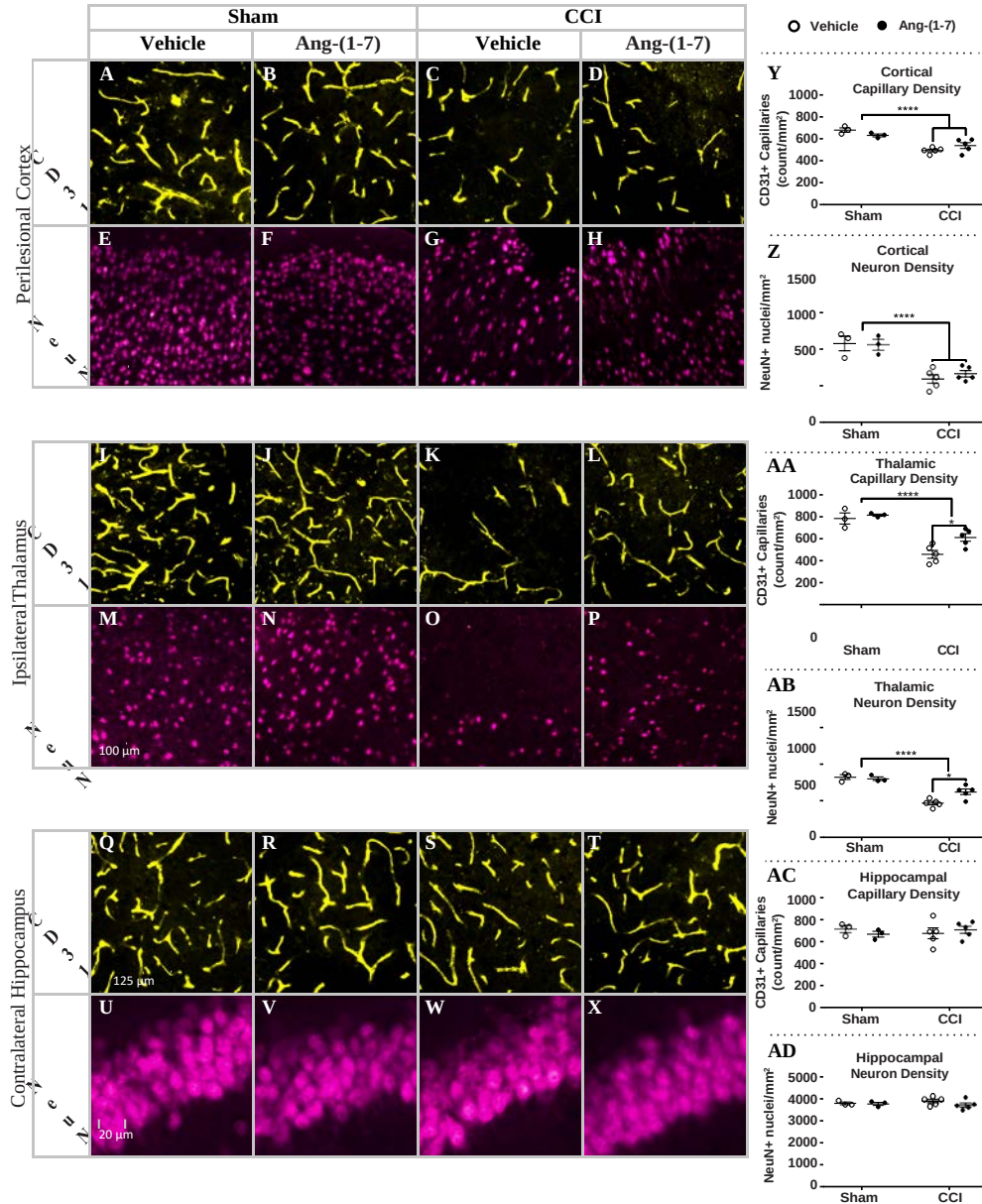


Figure 7. Effect of Ang-(1-7) treatment on neurons and microvasculature in different brain regions 29 days after CCI.

Representative immunohistochemistry of sections from different brain regions taken from CCI injured or sham mice treated with vehicle or Ang-(1-7) sacrificed at 29 dpi. Capillary density was determined in the perilesional cortex (A-D, Y), ipsilateral thalamus (I-L, AA), and contralateral hippocampus (Q-T, AC) by counting CD31+ structures/mm³. Density of neurons was assessed by counting NeuN+ nuclei/ mm³ in the perilesional cortex (E-H, Z), ipsilateral thalamus (M-P, AB), and contralateral hippocampus (U-X, AD). n = 3-5 mice/ group, Mean ± SEM, *p<0.05, ****p<0.0001. p-values represent either overall effect of injury (two-way ANOVA) or injury + vehicle versus injured + Ang-(1-7) comparison (two-way ANOVA + Tukey's multiple comparison test). Scale bar = 125 μm for A-D, I-L, and Q-T. Scale bar = 20 μm for E-H, M-P, and U-X.

3F - ANG-(1-7) BENEFITS FUNCTIONAL RECOVERY FOLLOWING CCI

3F.1 - Motor Coordination

To determine if Ang-(1-7) treatment altered functional recovery from injury, we assessed mice for motor coordination deficits at various time points after CCI. We assessed motor function by measuring latency to fall from a rotating rod at baseline, 1 dpi, and 3 dpi. At both 1 and 3 dpi vehicle-treated injured mice developed motor deficits ($p = 0.01$ and $p < 0.05$), falling off the rotating rod earlier than uninjured mice. The latency to fall of Ang-(1-7) treated injured mice was not different than either vehicle treated sham mice ($p > 0.1$ for 1 dpi; $p > 0.2$ for 3 dpi), or vehicle-treated injured mice ($p > 0.8$ for 1 dpi; $p > 0.7$ for 3 dpi), indicating an intermediate motor phenotype at both 1 and 3 dpi (Figure 8A). Ang- (1-7) treatment had no effect on sham treated mice.

3F.2 - Spatiotemporal learning

To determine whether Ang-(1-7) altered CCI-induced spatiotemporal learning and memory deficits, we assessed performance of the mice in the Morris water maze (MWM) from 24 to 28 days post-injury. During training, the vehicle treated injured mice took longer to find the submerged platform than either sham mice ($p < 0.05$), or Ang-(1-7) treated injured mice ($p < 0.05$ at day 3, 4, and 5 of training), indicating that Ang-(1-7) treatment was beneficial during the training period (Figure 8B). In the probe trial performed at 28 dpi, injured mice spent less time ($p < 0.0001$) in the quadrant where they had been trained to find the platform than did uninjured mice. Ang-(1-7) treated injured mice spent more time ($p < 0.01$) in the correct quadrant than did vehicle-treated injured mice (Figure 8C). Thus, Ang-(1-7) treatment not only prevented motor function deficits

from developing early after CCI, it improved spatiotemporal learning and memory up to 1 month after injury.

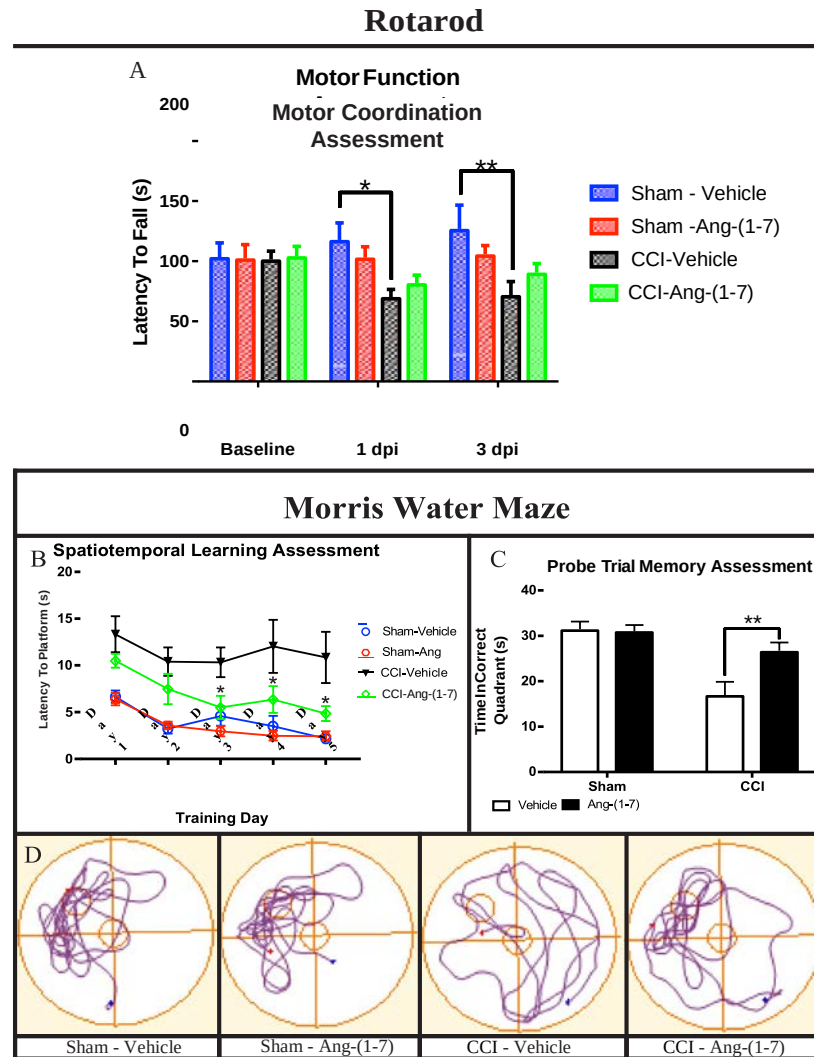


Figure 8. The effect of Ang-(1-7) treatment on motor performance and spatial memory after CCI.

Representative cresyl violet stained brain sections from CCI injured mice treated with either vehicle or Ang- (1-7) (1mg/kg). Recovery of motor function after CCI in mice treated with either Ang-(1-7) or vehicle was assessed by performance on the rotarod from 1 to 3 dpi (A) $n = 8-9$. Rates of spatial learning were assessed using the Morris Water Maze from 24 to 28 days post injury (days 1-5). Average time taken to reach the platform each day was recorded. $*p < 0.05$ when analyzing CCI-Vehicle and CCI-Ang-(1-7) in three-way ANOVA by drug pairwise comparisons (B). On the fifth day (28dpi), mice were assessed for spatial memory retention during a 60 second probe trial 1 hour after the final training phase (C). Representative track plots of swimming patterns during the Morris Water Maze probe trial (D). $n = 18-19$ mice/group, Mean $\pm$ SEM, $*p < 0.05$, $**p < 0.01$. p-values represent injury + vehicle to injury + Ang-(1-7) comparison (two-way

ANOVA + Tukey's multiple comparison test).

3G - EXTENDING THE THERAPEUTIC WINDOW OF TREATMENT

As we have shown that Ang-(1-7) treatment starting 6 hours after injury reduced lesion volume, we wanted to determine whether we could extend the therapeutic window to initiate Ang-(1-7) treatment. We therefore injured mice by CCI, and initiated Ang-(1-7) treatment by a single SQ injection (1mg/kg) at either 6, 24, or 72 hours after injury. 24 hours after the initial injection, we implanted a SQ micro-osmotic pump to dispense Ang-(1-7) by continuous infusion until sacrifice at 29 dpi. As in our previous experiments, starting subcutaneous administration of Ang-(1-7) 6 hours after CCI reduced lesion volume ($p < 0.05$) when assessed by histology at 29 dpi (Figure 9). Starting administration of Ang-(1-7) 72 hours after injury did not reduce lesion volume ($p > 0.1$) relative to vehicle treated injured mice. However, initiating Ang-(1-7) treatment at 24 hours after injury resulted in a lesion size that was not different from that found after initiating treatment at 6 hours ($p > 0.7$), or at 72 hours ($p > 0.2$), suggesting an intermediate effect (Figure 9).

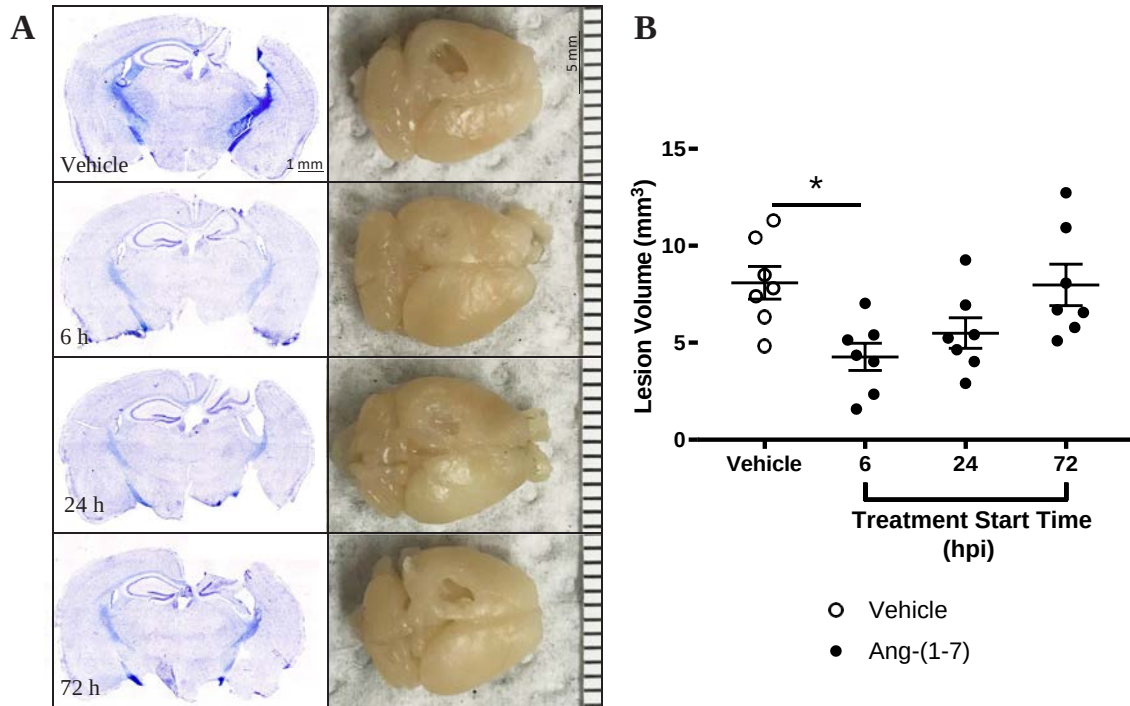


Figure 9. Determination of the therapeutic window for Ang-(1-7) treatment after CCI. Ang-(1-7) treatment was initiated by SQ injection starting at 6, 24, or 72 hpi after CCI. One day after the initial injection, a micro-osmotic pump was implanted to allow continuous Ang-(1-7) treatment (1mg/kg/day) until sacrifice at 29 dpi. Representative whole perfused brains and cresyl violet stained brain sections from CCI injured mice treated with either vehicle or Ang-(1-7) (A). Measurement of lesion volume was assessed by cresyl violet histology and plotted (B). $n = 7$ mice/group. Mean $\pm$ SEM, $*p < 0.05$. p-values represent vehicle to treatment-start-time comparison (one-way ANOVA + Tukey's multiple comparison test). Scale bar = 1 mm

3H - NEUROPROTECTION BY ANG-(1-7) FOLLOWING CCI IS MASR MEDIATED

The major receptor through which Ang-(1-7) signals is the MasR (325). However, other receptors have also been proposed to mediate Ang-(1-7) signaling (377). We therefore sought to determine whether Ang-(1-7) efficacy in reducing lesion volume was MasR-mediated, through the use of the MasR antagonist, A779. We initiated continuous SQ infusion of MasR antagonist (A779) through a micro-osmotic pump implanted two days prior to injury and continued treatment until the time of sacrifice. Mice were injured by CCI as before, and Ang-(1-7) treatment was initiated 6 h after injury, and continued by daily SQ injection until sacrifice at 3 dpi. As expected, determination of lesion volume on cresyl violet stained sections showed that brains from Ang-(1-7) treated mice had a smaller lesion size ($p < 0.05$) compared to that in brain sections from vehicle treated mice (Figure 10). However, in mice pre-treated with A779, the protective effect of Ang-(1-7) treatment was not detectable. The lesion volume in brains taken from mice treated with both Ang-(1-7) and A779 was larger ($p < 0.05$) than that of injured mice treated with Ang-(1-7) alone (Figure 10). Furthermore, A779 treatment alone did not alter lesion volume ($p > 0.9$) after CCI (Figure 10). Thus, the ability of Ang-(1-7) to reduce lesion volume is dependent, at least in part, on MasR signaling.

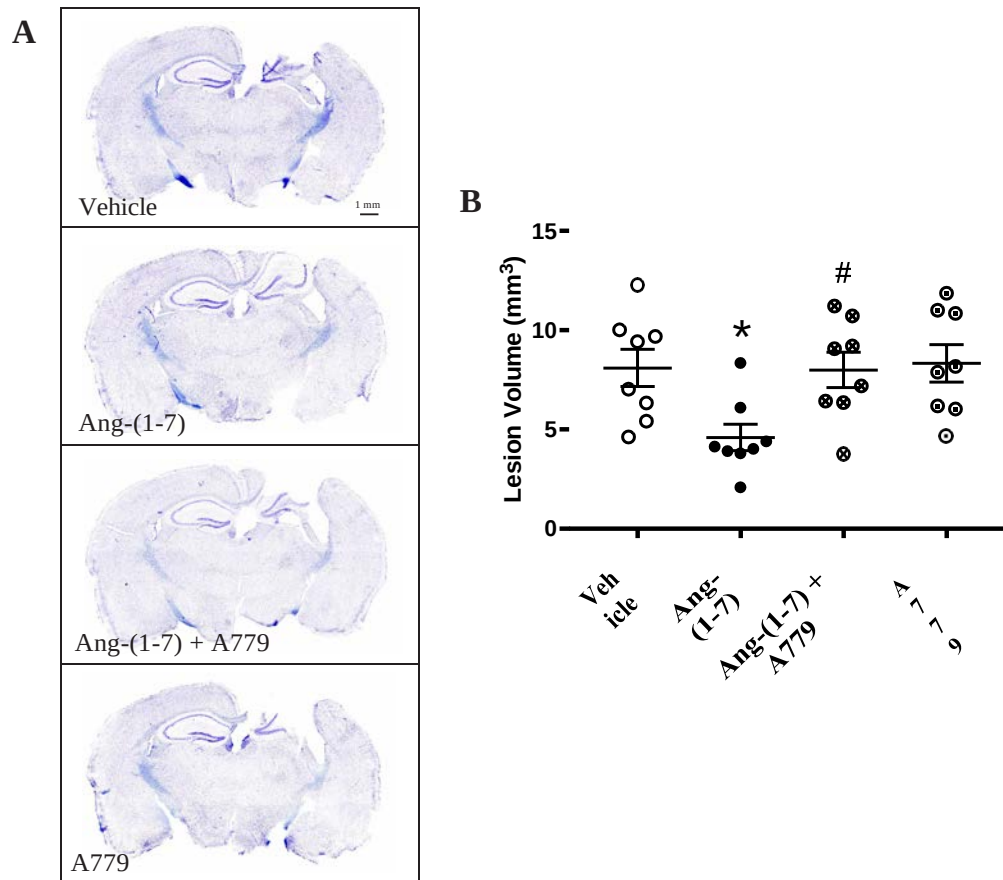


Figure 10. Dependence of Ang-(1-7) protective effect on MasR signaling. Mice were treated with a continuous subcutaneous infusion of MasR antagonist, A-779 (dose), starting two days prior to injury. Ang-(1-7) (1mg/kg/day) or vehicle administration was initiated 6 hrs after CCI. Mice were sacrificed at 3 dpi. Representative cresyl violet stained brain sections showing lesion size (A). Quantification of lesion volume in brain sections from all treatment groups (B). n = 8 mice/ group. Mean ± SEM *p< 0.05 compared to vehicle, #p<0.05 compared to Ang-(1-7). p-values represent group-to-group comparison (one-way ANOVA + Tukey's multiple comparison test). Scale bar = 1 mm

CHAPTER 4: Discussion

Our study shows that subcutaneous administration of Ang-(1-7) improved physiological, morphological, and functional recovery when administered up to 6 h following TBI in the mouse. The beneficial effects of Ang-(1-7) treatment included reduction of lesion volume, reduction of activated microglia and astrocytes, neuroprotection, and preservation of microvascular capillary density (Figure 11). We were able to track the reduction in lesion volume over time *in vivo* with MRI, and correlate these morphological neuroprotective effects of Ang-(1-7) with improved functional and cognitive recovery. Specifically, Ang-(1-7) prevented acute motor function deficits and attenuated long term learning and memory deficits associated with CCI. Mechanistically, our data indicate that Ang-(1-7) neuroprotection is mediated in a MasR-dependent fashion. Thus, these preclinical data suggest that Ang-(1-7) may be a promising therapeutic for the treatment of TBI.

MasR was found to be a receptor for Ang-(1-7) in 2003 (325). However, although MasR signals through G α s and increases intracellular cAMP, there is still a knowledge gap in understanding broader intracellular changes in response to MasR signaling (25). More recently Ang-(1-7) was shown to also signal through the MrgD in cell culture (354; 377). The antagonist that we utilized, A779, has greater specificity for MasR over MrgD (377). Thus, our finding that the Ang-(1-7) mediated reduction in lesion volume can be blocked with A779 (Figure 10), suggests that this neuroprotective effect of Ang-(1-7) is mediated by MasR signaling.

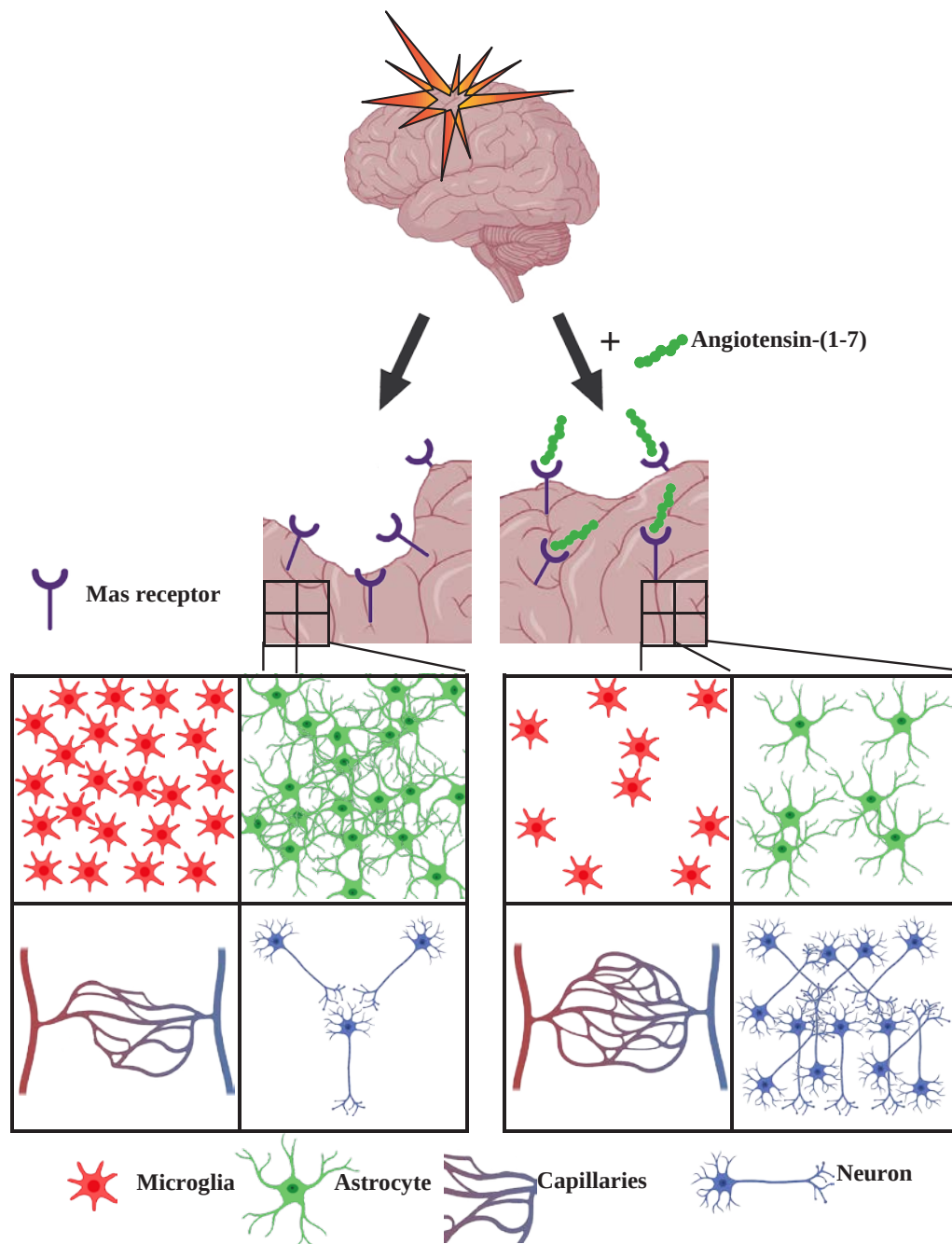


Figure 11. The effects of Ang-(1-7) are neuroprotective after traumatic brain injury. Subcutaneous administration of Ang-(1-7) promotes morphological and functional recovery in a mouse model of traumatic brain injury by reducing gliosis and preserving both capillary and neuronal density around the site of injury.

Our data indicate that CCI caused microgliosis, astrogliosis, capillary loss, and neuronal loss at both 3 and 29 dpi. At 3 dpi, microgliosis, but not astrogliosis, was attenuated by Ang-(1-7) treatment in each neuroanatomical region assessed. By 29 dpi the thalamus was the only region assessed where Ang-(1-7) treatment continued to attenuate microgliosis. Ang-(1-7) treatment did not attenuate the injury induced microgliosis in the perilesional cortex at 29 dpi and at this time point there was no longer a detectable microgliosis in the contralateral hippocampus. At 29 dpi, the overall microglial response to injury appears reduced in comparison to that at 3 dpi, and the thalamic microglia response is the strongest of the three brain regions examined. Therefore, the residual effect of Ang-(1-7) on thalamic microglia, but not the cortical microglia, could simply reflect the ability of the assay to detect a larger reduction in Iba1+ cells. Ang-(1-7) treatment of cultured primary rat microglia can reduce both basal and activated expression of pro-inflammatory cytokines such as IL-1 β and TNF- α , and iNOS (216; 303; 344). These anti-inflammatory effects of Ang-(1-7) on microglia are conserved *in vivo* following both ischemic and hemorrhagic stroke (303). After TBI, microglial secretions of various pro-inflammatory cytokines coupled with unregulated phagocytosis can exacerbate the initial injury (175; 218). Thus, the Ang-(1-7) reduction of microgliosis is likely, at least in part, to be a direct effect of the peptide on microglia.

The astroglial response to injury changed over time. There was increased astrogliosis at both 3 and 29 dpi in all three brain regions examined. There were also clear signs of glial scar formation in the perilesional cortex at 29 dpi (Figure 6). Interestingly, and in contrast to the effect on microgliosis, Ang-(1-7) treatment did not affect the degree of reactive astrogliosis at 3 dpi, but significantly reduced it at 29 dpi in

each neuroanatomical region assessed. The differential ability of Ang-(1-7) to reduce reactive astrogliosis at 29 dpi, but not 3 dpi, could also be a result of the accumulated treatment time required for an effect on this histopathological measure; or, it may indicate that different pathways contribute to GFAP induction at different time points. Thus, Ang-(1-7) may only have efficacy attenuating pathways involved at the later time point. It would be interesting to determine whether other markers of astrocyte activation are similarly affected by Ang-(1-7) treatment.

CCI injury led to significant reduction in neuronal and capillary density in the ipsilateral cortex and thalamus, but not in the contralateral hippocampus, at 3 and 29 dpi (Figures 5 and 7). At 29 dpi, Ang-(1-7) treatment led to greater neuronal and capillary density in injured mice than did treatment with vehicle; though only in the ipsilateral thalamus. MasR is expressed on neurons and acts to promote neuronal survival. Indeed, MasR activation increases neuronal survival *in vitro* during glucose deprivation studies, and in Ang-(1-7) treated mice subject to stroke (54; 200; 442). Exogenous Ang-(1-7) has also been shown to induce vascular repair in a diabetic mouse model, and to enhance the production of endothelial progenitor cells after myocardial infarction (395; 411). It can additionally act on endothelial cells to promote angiogenesis following ischemic stroke in rats, and on cerebrovascular cells in culture to reduce apoptosis (163). Thus, there are direct beneficial effects of Ang-(1-7) action on endothelial progenitor cells, endothelial cells, and neurons; and each may have contributed to the neuronal and cerebrovascular protection that occurred with Ang-(1-7) treatment after CCI.

In our study we demonstrated that Ang-(1-7) preserved thalamic capillary density. However, we did not address whether this preservation was from anti-apoptotic effects of

Ang-(1-7) on endothelial cells, or due to an angiogenic effect. Ang-(1-7)'s accessibility to the brain through the BBB is likely limited (159). Though Ang-(1-7) treatment did not increase brain capillary density in uninjured mice, this could be due to a lack of brain access in uninjured animals rather than a lack of angiogenic properties. However, we are planning to assess whether the angiogenic and/or anti-apoptotic effects of Ang-(1-7) are conserved in mice after CCI.

BrdU is an analog of the nucleoside thymidine that gets incorporated into DNA during cellular proliferation (313). Antibodies directed against BrdU can be used for immunohistochemistry in order to detect BrdU incorporation into DNA, and thus, extent of proliferation. Co-localization of BrdU with CD31 allows for assessment of endothelial proliferation (287; 314). For future experiments, treatment with BrdU at various time points following injury and Ang-(1-7) treatment will allow us to determine whether Ang-(1-7) affects endothelial proliferation and at what time points it may do so. However, the attenuation of thalamic capillary loss by Ang-(1-7) may also be due to a decrease in apoptosis. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a technique that labels the 3'-hydroxyl termini that are exposed when DNA fragments during apoptosis (221). When terminal deoxynucleotidyl transferase catalyzes the attachment of a fluorochrome-tagged deoxynucleotide to the exposed 3'-hydroxyl termini, fluorescent microscopy can be used to quantify the extent of fluorescence, and thus, total DNA breaks (221). Co-localization of CD31 with signal from TUNEL will allow us to determine whether Ang-(1-7) treatment affects apoptosis of the endothelial cells that make up cerebrovascular capillaries (40; 45; 382). After assessing TUNEL and BrdU co-localization with CD31 endothelial cells, we will have a better understanding of

the cellular mechanism underlying the preservation of capillary density in the ipsilateral thalamus of injured Ang-(1-7) treated mice at 29 dpi.

We showed that thalamic neuronal loss was also reduced in Ang-(1-7)-treated injured mice at 29 dpi. In our experiment, we associated loss of the neuron-specific nuclear protein, NeuN, with a loss of neurons. Though sensitive to neuronal loss following TBI, inability to visualize the presence of NeuN is not necessarily specific for neuronal death (38; 389). A previous study showed that loss of NeuN following ischemic stroke in rats was reversed following antigen retrieval (389). This suggests that changes in NeuN reactivity may also be affected by metabolic/intracellular changes following ischemia, independent from neuronal death. Thus, there is possibility that the observed neuronal loss following CCI was due to a disturbance in NeuN antigenicity, rather than neuronal loss. As such, we plan to assess NeuN-TUNEL co-localization to assess whether TBI-induced changes in neuronal apoptosis are affected by administration of Ang-(1-7).

Interestingly, the thalamic-specific Ang-(1-7) rescue of neuronal and capillary density at 29 dpi corresponds with the Ang-(1-7) mediated attenuation of microgliosis in the same neuroanatomical region at the same time point (Figure 6). Thalamic astrocytic activation is also reduced at 29 dpi (Figure 6). Activation of astrocytes and microglia can affect neuronal and cerebrovascular viability (6; 62; 91; 170). Therefore, the beneficial effects of Ang-(1-7) on endothelial cells and neurons could be a combination of direct and indirect effects. Taken together, it is likely that Ang-(1-7) promotes its neuroprotective phenotype through both direct action on neurons and cerebrovascular endothelial cells, as well as by paracrine signaling through astrocytes and microglia.

We do not understand why Ang-(1-7) reduced the cellular consequences of CCI at

29 dpi specifically in the thalamus. Although we were unable to accurately measure immunohistochemical changes in the ipsilateral hippocampus, it is probable that Ang-(1-7) treatment also had similar beneficial effects in this region. In fact, various studies have demonstrated a requirement for intact CA3 and CA1 hippocampal fields, as well as intact anterior thalamic nuclei, for full-functioning spatiotemporal memory in the MWM (302; 361; 412; 413). Thus, the Ang-(1-7) mediated recovery of spatiotemporal learning and memory deficits after TBI, as assessed by the MWM (Figure 8), suggests a preservation or recovery of both thalamic and hippocampal integrity.

We chose our coordinates of CCI to maximize injury to the ipsilateral somatosensory cortex, anterior thalamic nuclei, and hippocampus (106). Though the motor cortex serves to control planning and execution of movement, sampling through various sensory modalities, to include tactile feedback processed in the somatosensory cortex, influence proper movement (34). The anterior thalamic nucleus and hippocampus both play active roles in memory formation, though the thalamus as a whole is involved in the integration and execution of many cortical processes (96; 161; 324). Thus, due to overlapping outcomes from unique neuronal processes, the rotarod and MWM assays are sensitive to injury in particular brain regions, but are not specific to any one region. Disruption to the somatosensory cortex, for instance, can hinder an animal's ability to ambulate or swim, thus confounding the results observed in the MWM (71). However, when we assessed average swim speed during the MWM, there were no changes associated with injury or treatment (data not shown). Thus, in our experiment, results of the MWM demonstrated that Ang-(1-7) treatment preserves spatiotemporal memory after CCI, and was not confounded by a motor deficit.

4A - FROM STROKE TO TBI

Within the CNS MasR expression is strongest in cortical and hippocampal neurons, but it is also expressed in vascular endothelium, microglia and astrocytes (107; 203; 216; 228; 437). MasR signaling serves to induce vasodilation, reduce inflammation, enhance neuronal survival, and promote angiogenesis (163; 273; 431). The benefit of these effects after CNS insult has been demonstrated in various rodent stroke studies where exogenously increased Ang-(1-7), either by increased ACE2 activity or direct ICV administration, reduces neurological deficits and lesion volume (26; 163; 303; 304). In contrast, Ang II signaling through AT1R induces vasoconstriction, increases inflammation, and subsequently exacerbates lesion volume following ischemic stroke (179; 264; 407). Ischemic stroke and TBI share a variety of pathological features (36). However, features such as direct cell membrane damage, contusion formation, and primary axotomy are all unique to TBI (36). Thus, our finding that Ang-(1-7) administration improves recovery after CCI injury in the mouse extends the potential clinical applications of this peptide.

4B - CLINICAL RELEVANCE

We designed our study to optimize the translational relevance of these pre-clinical experiments. We found that Ang-(1-7) administration could be started a minimum of 6 hours after the injury, a clinically relevant therapeutic time window, and still have a beneficial effect when evaluated at 3, 10, 24 or 29 dpi (Figures 3, 6, 7, 8, 9). Evaluation of later times of administration showed that a 24 hour window between injury and drug administration produced an intermediate effect on lesion volume that was not

significantly different from either the 6 hour Ang-(1-7) or the saline treatment group (Figure 9). It may therefore be possible to lengthen the beneficial therapeutic window past 6 hours with a more detailed, highly powered study. We also administered the Ang-(1-7) peptide through subcutaneous (SQ) administration. To our knowledge, ours is the first study using SQ administration of Ang-(1-7) to treat a CNS injury, instead of an ICV route of administration (25). Clinically, SQ administration is preferable to ICV administration. Indeed, SQ administration of Ang-(1-7) is currently being used in two clinical trials to improve cognitive function following cardiovascular stress (NCT03252093, NCT03159988). Drugs administered SQ will need to cross the BBB to act within the CNS. The significant BBB disruption after CCI will allow access into the CNS early after injury (10; 22; 340). However, in mouse models of TBI the BBB is reformed relatively quickly, such that by 29 dpi the BBB has majorly healed (22). Whether or not Ang-(1-7) needs to cross the BBB to influence the CNS is uncertain. Nonetheless, some of the neuroprotective effects of Ang-(1-7) could be directly mediated through the vasculature, to which Ang-(1-7) readily gains access (370). Furthermore, in contrast to rodent studies, recent reports suggest that TBI-induced BBB permeability may persist in humans for months to years following injury; thus, drug access to the CNS in human subjects may remain at later stages of injury (143).

We did not monitor the blood pressure of mice in these experiments even though Ang-(1-7) is a known anti-hypertensive (158). It could be postulated that part of the beneficial effects of Ang-(1-7) may be mediated by a reduction in blood pressure. However, blood pressure is often reduced as a consequence of CCI in mice, and there is a deleterious association with severely decreased blood pressure (hypotension) and

neurological outcome following TBI in rodents (333; 402). More so, the most recent update from the 26,873-participant Impact of Implementing the EMS Traumatic Brain Injury Treatment Guideline clinical trial (NCT01339702) shows a significant increase in mortality in normoxic hypotensive TBI patients when compared to normoxic, normotensive TBI patients, even after controlling for multiple potential confounders (359). Extreme increases or decreases of blood pressure can both exacerbate the consequences of TBI, and there is clearly an optimal range for beneficial outcomes (186). These data suggest that a reduction in blood pressure from Ang-(1-7), in addition to the reduction caused by CCI, would not be beneficial to mice. Thus, it is unlikely that the anti-hypertensive properties of Ang-(1-7) are confounding factors in its mechanism of neuroprotection. However, as blood pressure monitoring via tail-cuff methods is noninvasive, it would be interesting to see whether Ang-(1-7) further reduces blood pressure subsequent to CCI.

Various studies have shown discrepancies between histology and MRI (83; 197). Our histologic analysis was in agreement with that of our MRI, although MRI provided a different, and probably more accurate, assessment of the lesion. The cavitating brain lesion observed under histologic assessment appeared as a herniating lesion *in vivo* (Figure 3). This was likely due to increased ICP following post-injury brain swelling within the skull (320). Interestingly, although we did not replace the bone flap after the craniotomy, the small craniotomy window may worsen the lesion by promoting further brain damage in the herniated tissue. This is, at least partially, why current guidelines for the management of severe TBI recommend large (12 x 15 cm), rather than small, decompressive craniectomy for emergency cranial decompression (49). Nonetheless,

Ang-(1-7) administration significantly reduced lesion volume by histologic and MRI analysis at all time points examined. The ability to utilize a clinically approved imaging modality to measure Ang-(1-7) efficacy shows potential for use as a clinical readout.

4C - FROM BENCH TO BEDSIDE

Revised in 1991, Title 45 Code of Federal Regulations 46, also known as “The Common Rule,” is a U.S. law that protects vulnerable patients (patients with compromised ability to give consent) from inclusion within clinical trials (385). Patients who have sustained moderate, severe, or penetrating TBI are often unconscious or experiencing an altered state of consciousness (164; 384). Thus, patient consent is not often possible (164; 384). However, in 1996, “The Final Rule” (21 DFR 50.24) was implemented in order to provide means for inclusion of patients in emergency settings into clinical research (386). In order to receive an exception from the informed consent requirements for inclusion into emergency research, patients are required to be:

- unable to give consent due the nature of their injury (386)
- in a life-threatening situation where available treatments are unsatisfactory (386)
- in a life-threatening situation the necessitates treatment (386)
- unable to be prospectively enrolled in the study (386)

Furthermore, an appropriate number of animal studies must have been performed to provide evidence that supports intervention; and the risks associated with treatment need to be reasonable with respect to what is known about the potential benefits of treatment (386).

Patients who have sustained moderate to severe TBI are often unable to consent to emergency treatment due to the nature of their injury (164; 384). TBI can be life

threatening, often requires immediate treatment, has extremely limited treatment options, and is not conducive to prospective studies due to the spontaneity of its occurrence (49; 309). As SQ administration of Ang-(1-7) for non-brain related ailment is tolerated in humans and has made it passed Phase I and through Phase 2 of clinical trials, pre-clinical data regarding the efficacy of Ang-(1-7) as a therapeutic for TBI is needed to bridge the bench to bedside gap (292; 329). Due to the fact that we are the first laboratory to demonstrate the value of Ang-(1-7) as a therapeutic for TBI, demonstrations of similar findings by others are critical for consideration of use in a clinical trial of TBI.

In summary, we have shown that subcutaneous administration of Ang-(1-7) 6 hours after CCI injury in the mouse is neuroprotective and able to improve functional recovery; likely through a reduction in glial activation and promotion of greater microvascular density. These pre-clinical data show the pleiotropic neurorestorative effects of Ang-(1-7) after TBI and, together with previous studies on the efficacy of ARBs in improving recovery after TBI, suggest that manipulation of the renin angiotensin system has real potential for the clinical treatment of patients suffering from TBI (400; 402).

4D - CONCLUDING REMARKS

From what was once understood only as a modulator of blood pressure, the RAS has developed into a field with implications in more human conditions than ever predicted. Expression of RAS components throughout the mammalian body provide many possibilities for targeted therapeutics and creates a promising system to explore for treating several different conditions. Today, few pathologies exists where their prevalence in society and number of treatment options are as opposite in abundance as

that observed with TBI. Thus, the need for efficacious treatments for acute and chronic TBI is great.

Inhibition of the traditional ACE/Ang II/AT1R axis or stimulation of the alternate ACE2/Ang-(1-7)/MasR axis share similar mechanisms of neuroprotection: direct neuroprotective action on neurons, reduction of inflammation and gliosis, increased cerebral blood flow, and preservation of both cerebrovascular and BBB integrity. As each axis perturbs the system uniquely, each holds promise as an area to target for TBI therapy. However, as with all emergency treatments, advancement to clinical trial presents ethical roadblocks on a frequent basis. Furthermore, an abundance of pre-clinical data must exist to provide rationale for human testing. Thus, RAS manipulation after TBI must be studied in greater depth and in different scenarios in order to push these molecules towards clinical trials.

We feel that manipulation of the extended RAS holds promise as a future therapy for TBI and propose the following questions as those most important to answer: 1) Does TBI perturb the endogenous ACE2/Ang-(1-7)/MasR axis? 2) Does alamandine exert the same neuroprotective benefits of Ang-(1-7) after TBI? 3) Does genetic deletion of MasR, MrgD, or AT4R alter pathophysiological progression of TBI? 4) Are the beneficial actions of ARBs and/or Ang-(1-7) mediated by action within the CNS, or perhaps through peripheral effects? Indeed, answers to any of the aforementioned questions will bring us one step closer to providing better care for the brain-injured patient.

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