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Harnessing Blood Clot Removal Mechanisms after Germinal Matrix Hemorrhage

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Harnessing Blood Clot Removal Mechanisms after Germinal Matrix Hemorrhage

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

FPR2	N-formyl peptide receptor 2
CSF	Cerebrospinal Fluid
DUSP1	Dual-Specificity Protein Phosphatase 1
GMH	Germinal Matrix Hemorrhage
ICP	Intracranial Pressure
AnxA1	Annexin A1
IVH	Intraventricular Hemorrhage
NIH	National Institute of Health
PHH	Post-hemorrhagic Hydrocephalus
CD36	Cluster of Differentiation 36
PPARγ	Peroxisome Proliferator Receptor Gamma
ERK	Extracellular-Signal-Regulated Kinase
SD	Standard Deviation
SEM	Standard Error of Mean
ICP	Intracranial Pressure
TNFα	Tumor Necrosis Factor Alpha
M1	Activated Microglia
M2	Alternately-Activated Microglia

ABSTRACT OF THE DISSERTATION

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by

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Dr. John H. Zhang, Chairperson

Germinal matrix hemorrhage (GMH) is one of the leading causes of morbidity, mortality, and acquired infantile hydrocephalus in preterm infants in the United States, with little progress made in its clinical management. Blood clots have been shown to elicit secondary brain injury after GMH, by disrupting normal cerebrospinal fluid circulation and absorption after germinal matrix hemorrhage causing post-hemorrhagic hydrocephalus development. Current evidence suggests that rapid hematoma resolution is necessary to quickly improve neurological outcomes after hemorrhagic stroke. N-formyl peptide receptor 2 (FPR2), a G-protein-coupled receptor, has been shown to be neuroprotective after stroke. FPR2 activation has been associated with the upregulation of phagocytic macrophage clearance, yet its mechanism has not been fully explored. Recent literature suggests that FPR2 may play a role in the stimulation of scavenger receptor CD36. Scavenger receptor CD36 plays a vital role in microglia phagocytic blood clot clearance after GMH. FPR2 has been shown to activate extracellular-signal-regulated kinase 1/2 (ERK1/2), which promotes the transcription of the dual-specificity protein phosphatase 1 (DUSP1) gene. Our data suggests that FPR2 activation enhances hematoma resolution and improves neurological deficits. Our central hypothesis is that FPR2 stimulation enhances microglia induced hematoma resolution through the

activation of the p-ERK(1/2)/DUSP1/CD36 signaling pathway, thereby improving short- and long-term neurological outcomes. Aim 1 investigated the role of FPR2 in enhancing hematoma resolution, thereby improving neurological function following GMH. Aim 2 investigated FPR2-induced activation of the p-ERK/DUSP1/CD36 signaling pathway after GMH. The long-term goal of this proposal is to provide a basis for clinical translation of FPR2 stimulation as an effective non-invasive therapeutic strategy to protect against acute and chronic complications in the GMH patient population. FPR2 stimulation improved short-term hematoma resolution and motor coordination, which was reversed by FPR2 antagonist Boc2 and FPR2 CRISPR. FPR2 stimulation attenuated long-term neurocognitive deficits and post-hemorrhagic hydrocephalus, which was reversed by pharmacological inhibition. Furthermore, FPR2 stimulation increased the expression of the proposed signaling pathway, which was then inhibited by pharmacological inhibition and gene knock-down of FPR2. Thus, our study presents a non-invasive therapeutic target for the treatment of GMH.

CHAPTER ONE

A COMPREHENSIVE REVIEW OF THERAPEUTIC TARGETS THAT INDUCE MICROGLIA/MACROPHAGE-MEDIATED HEMATOMA RESOLUTION AFTER GERMINAL MATRIX HEMORRHAGE

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Abstract

Currently, there is no effective treatment for germinal matrix hemorrhage and intraventricular hemorrhage (GMH-IVH), a common and often fatal stroke subtype in premature infants. Secondary brain injury after GMH-IVH is known to involve blood clots that contribute to inflammation and neurological deficits. Furthermore, the subsequent blood clots disrupt normal cerebrospinal fluid circulation and absorption after GMH-IVH, contributing to posthemorrhagic hydrocephalus (PHH). Clinically, GMH-IVH severity is graded on a I to IV scale: Grade I is confined to the germinal matrix, grade II includes intraventricular hemorrhage, grade III includes intraventricular hemorrhage with extension into dilated ventricles, and grade IV includes intraventricular hemorrhage with extension into dilated ventricles as well as parenchymal hemorrhaging. GMH-IVH hematoma volume is the best prognostic indicator, where patients with higher grades have worsened outcomes. Various preclinical studies have shown that rapid hematoma resolution quickly ameliorates inflammation and improves neurological outcomes. Current experimental evidence identifies alternatively activated microglia as playing a pivotal role in hematoma clearance. In this review, we discuss the pathophysiology of GMH-IVH in the development of PHH, microglia/macrophage's role in the neonatal CNS, and established/potential therapeutic targets that enhance M2 microglia/macrophage phagocytosis of blood clots after GMH-IVH.

K E Y W O R D S

germinal matrix hemorrhage, hematoma resolution, neonatal brain hemorrhage, phagocytosis, microglia/macrophages, posthemorrhagic hydrocephalus.

1) Introduction

Germinal matrix hemorrhage and intraventricular hemorrhage (GMH-IVH) is a leading cause of morbidity and mortality in premature and or low birthweight infants, occurring in approximately 3.5 per 1,000 live births. Clinically, GMH-IVH severity is graded on a I–IV scale: Grade I is confined to the germinal matrix, grade II includes intraventricular hemorrhage (IVH), grade III includes intraventricular hemorrhage with extension into dilated ventricles, and Grade IV includes intraventricular hemorrhage with extension into dilated ventricles as well as parenchymal hemorrhaging (Roland & Hill, 2003). Debilitating consequences of GMH-IVH include the formation of posthemorrhagic hydrocephalus (PHH) (Ballabh, 2010; Heron et al., 2010). The GMH-IVH grades are the best prognostic indicator, where patients with higher grades (III and IV) have worsened outcomes and increased incidence of PHH and patients with lower grades (I and II) often resolve without detrimental long-term consequences (Roland & Hill, 2003). The expansion of the cerebroventricular system may lead to mechanical compression and consequent injury of the surrounding brain tissue, causing neurological deficits in patients that survive the initial bleed. Intraventricular blood clots have been identified as causative factors of hydrocephalus formation since they directly impair the circulation and absorption of cerebrospinal fluid (CSF) (Stein et al., 2010; Whitelaw, Cherian, Thoresen, & Pople, 2004). Current clinical management of hydrocephalus relies on the surgical insertion of shunts that drain excess CSF from the ventricles into the peritoneum, where it can be absorbed by the vasculature (Woernle et al., 2013). Unfortunately, this procedure can cause postsurgical complications, including infection, and shunts obstruction, seizures, over/under drainage, and shunt replacement (Woernle et

al., 2013). Therefore, a safe and noninvasive treatment that reduces the burden of clot after GMH-IVH could lessen the incidence of PHH and lead to improved long-term outcomes.

Macrophage populations of the CNS consist of several cell groups: microglia, perivascular macrophages (PVM), meningeal macrophages (MM), macrophages of the circumventricular organs, and the macrophages of the choroid plexus (Perry & Teeling, 2013). Microglia activation and macrophage recruitment within the CNS has been perceived as harmful in neurodegenerative conditions in adults, yet the role that these cells play in hemorrhagic brain injury in neonates differs (Mallard, Tremblay, & Vexler, 2018). Within the neonatal brain, microglia cells play a pivotal role in blood–brain barrier development, postnatal brain development and connectivity, and have been shown to be neuroprotective after stroke (Flores et al., 2016; Mallard et al., 2018). Microglia and recruited macrophages can be both harmful and protective following CNS injury, depending upon their activation states. After CNS insult, microglia are activated and circulating macrophages are recruited to the site of injury, becoming virtually indistinct from resident microglia (London, Cohen, & Schwartz, 2013). Although advanced gene sequencing techniques are available to better distinguish microglia from infiltrated macrophages, these have not been applied extensively in most brain injury models. Herein, we refer to microglia and recruited macrophages interchangeable as one population. Classically activated M1 microglia are pro-inflammatory and drive the innate defense mechanism following neural tissue injury, while alternatively activated M2 microglia are anti-inflammatory and remove damaged tissue while also driving tissue repair mechanisms. Thus, augmenting M2 activation may enhance tissue repair and

recovery following CNS insult. Microglia depletion by intracerebral injection of liposome-encapsulated clodronate in postnatal day 7 rats with neonatal focal stroke led to the enhancement of injury by removing endogenous protective mechanisms (Faustino et al., 2011). Similar to microglia/macrophages in adult rodent models of intracerebral hemorrhage (ICH), these cells participate in the phagocytosis of red blood cells (RBCs) and consequent hematoma resolution after GMH-IVH in neonatal rats, ultimately reducing PHH in the long term (Flores et al., 2016).

Currently, no clinical trial has investigated the removal of hematoma in the GMH-IVH patient population. Clinical studies conducted in adult hemorrhagic stroke have shown that surgical evacuation of blood clots is insufficient in improving neurological outcomes (Wilkinson, Keep, Hua, & Xi, 2018). It is speculated that the surgical evacuation of blood alone after adult hemorrhagic stroke was inadequate as the developed techniques do not remove the entire clot, failing to ameliorate cytotoxicity and neuronal death associated with the initial insult (Wilkinson et al., 2018). Various strategies have been conducted that investigate the body's clearance mechanisms that lead to the enhancement of microglia phagocytic clearance of extravasated RBCs in adult ICH models, which would avoid the release of cytotoxic products (Zhao, Sun, Ting et al., 2015; Zhao et al., 2007). Yet, these therapeutic targets are greatly understudied in GMH-IVH. In this review, we discuss the pathophysiology of GMH-IVH in the development of PHH, microglia/macrophage's role in the neonatal CNS after GMH-IVH and established/potential therapeutic targets that enhance M2 microglia/macrophage phagocytosis of blood clots after GMH-IVH.

2) THE PATHOPHYSIOLOGY OF GMH-IVH AND THE DEVELOPMENT OF PHH

Abrupt fluctuations in cerebral blood flow in the inherently frail germinal matrix vasculature, due to hemodynamic and cardiorespiratory instability in premature and or low birthweight infants, often results in spontaneous bleeding (Ballabh, 2014). The consequent hematoma applies mechanical pressure to glia and neurons, resulting in cytotoxicity and necrosis, as well as evokes an inflammatory response, leading to secretion of destructive proteases and oxidative species from innate immune cells, especially activated microglia and infiltrated macrophages. It has been postulated that PHH is caused by blood clots obstructing the cerebral aqueduct or formanina of Luschka and Magendi or by microthrombi obstructing small CSF outflow passages in the subarachnoid space (Jun Tang et al., 2016; Strahle et al., 2012; Whitelaw, Thoresen,& Pople, 2002). When blood invades the ventricles, it mixes with cerebrospinal fluid (CSF) and circulates toward the subarachnoid space, leading to noncommunicating/obstructive PHH (Daou, Hasan, & Jabbour, 2017). After hemorrhage, erythrocyte lysis releases hemoglobin and iron into the surrounding tissue. Iron is released from metabolized hemoglobin, consequently causing iron overload. Iron overload has been associated with CSF overproduction and consequent PHH development (Klebe et al., 2014). Experimental neonatal models of GMH-IVH have shown that hemoglobin metabolites were found in the CSF and contributed to significant acute ventricular dilation (Lee et al., 2010; Savman, Nilsson, Blennow, Kjellmer, & Whitelaw, 2001; Strahle et al., 2014). A detailed schematic demonstrating the pathophysiology of GMH and the consequences of the hematoma blood clot can be found on Figure 1.1.

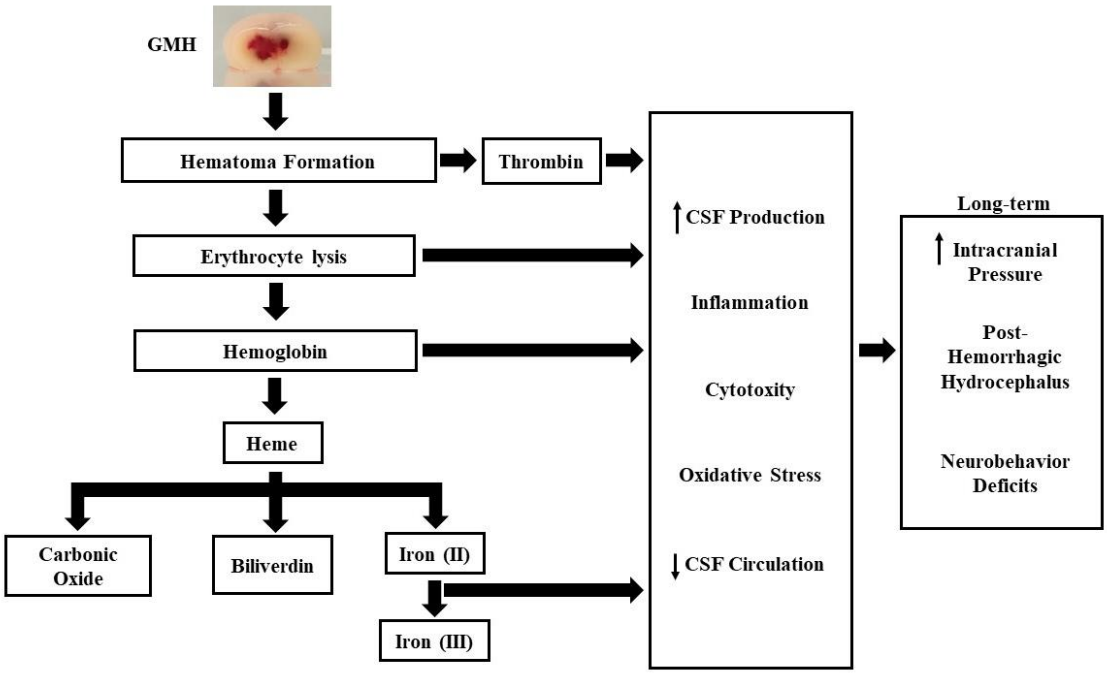

Figure 1.1. Germinal Matrix Hemorrhage Pathophysiology. The Rupture of blood vessels in the germinal matrix leads to the hematoma formation. Hematoma is the primary causative factor of secondary brain injury after GMH, which results in increased CSF production, inflammation, cytotoxity, oxidative stress, and decreased CSF circulation that ultimately leads to long-term neurological deficits.

3) MICROGLIA AND THEIR ROLE IN THE CNS AFTER HEMORRHAGE

Microglia are resident macrophages of the CNS and play a primary role in brain development and homeostasis in the neonatal brain. At 4.5 gestational weeks, microglia cells infiltrate the brain through the choroid plexus, meninges, and ventricles (Pierre et al., 2017). Within the healthy neonatal CNS, microglia participate in immune surveillance, neuronal network development, myelination, and cerebral and retinal angiogenesis/vascularization (Eyo & Dailey, 2013). Microglia are vital to the maturation of the neonatal CNS. CSF1 receptor, which is prominent in microglia and a key marker of neuro–immune interaction, knockout mice had abnormal postnatal brain development including enlarged ventricles and regionally compressed parenchyma, and microglia depletion by intracerebral injection of liposome-encapsulated clodronate in postnatal day 7 rats with neonatal focal stroke led to the enhancement of injury by removing endogenous protective mechanism (Erblich, Zhu, Etgen, Dobrenis, & Pollard, 2011; Faustino et al., 2011).

Microglia respond rapidly to hemostatic imbalances and have been shown to respond, migrate to, and form bulbous microglial process tips near the site of injury within 60 s (Nimmerjahn, Kirchhoff, & Helmchen, 2005). The function of activated microglia varies on the environmental stimuli and can adopt an activated proinflammatory phenotype (M1) associated with neurotoxicity or alternatively activated, anti-inflammatory phenotype (M2) associated with wound healing (Kanazawa, Ninomiya, Hatakeyama, Takahashi, & Shimohata, 2017; Pierre et al., 2017; Savman, Heyes, Svedin, & Karlsson, 2013). Literature suggests that macrophages and microglia can switch phenotypes depending upon the microenvironment (Stout & Suttles, 2004).

Under normal conditions, microglia act as resident macrophages of the CNS and help maintain the brain microenvironment by pruning neuronal synapses, removing plaques, and monitoring for infectious agents. When activated in response to injury and or infection by lipopolysaccharides, interferon-γ, TNF-α, or stimulation of Nod-like receptors (NLR) or Toll-like receptors (TLR), primarily TLR-4, activated M1 macrophages and microglia are responsible for the innate defense mechanism (Klebe, McBride, Flores, Zhang, & Tang, 2015; Martinez, Helming, & Gordon, 2009). Activated M2 macrophages and microglia are responsible for tissue repair, which can be induced by IL-4, IL-10, TGF-β, and IL-13 (Martinez et al., 2009). A detailed schematic of microglia polarization and microglia subtypes can be found in Figure 1.2.

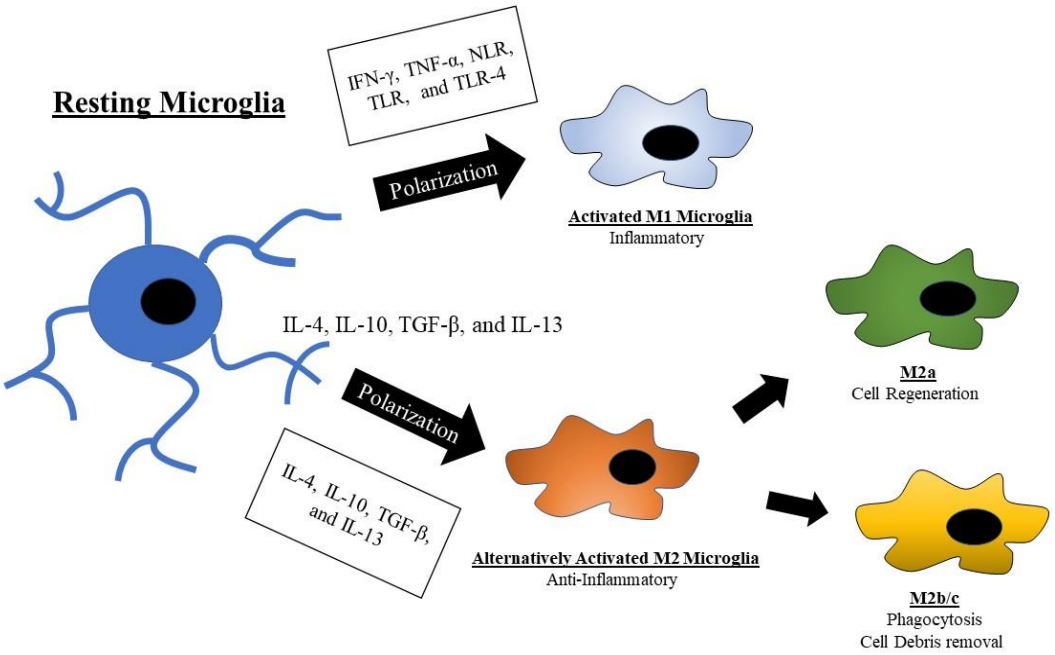

Figure 1.2. Microglia Polarization. Microglial phenotype switching is induced by pro- or anti-inflammatory cytokines. M1 microglia are inflammatory, whereas M2 microglia are anti-inflammatory and can have multiple phenotype subtypes.

The time course of changes in M1 and M2 microglia populations was determined in adult models of cerebral hemorrhage. After insult, M1 microglia/macrophage population peak almost immediately at 6 hr then steadily decline up to 14 days post-ictus, but M2 microglia/ macrophages steadily increase up to 14 days after injury (Kanazawa et al., 2017). After hemorrhage, conceivably, RBCs enter the brain tissue and immediately stimulate microglial TLRs and NLRs, triggering their activation into a pro-inflammatory M1-state and eventually recruiting circulating macrophages to the site of injury. M1 microglia destroy potential pathogens by secreting reactive oxygen species and extracellular proteases, which further injures the surrounding tissue (Klebe et al., 2015; Martinez et al., 2009). Over time, microglia increasingly shift toward an anti-inflammatory M2-state to remove dead tissue and RBCs by phagocytosis and promote tissue repair (Martinez et al., 2009). The time course of changes in M1 and M2 microglia populations has not yet determined in any preclinical model of GMH-IVH.

The increase in the severity of the hemorrhage has been associated with greater microglia increases in GMH-IVH patients (Supramaniam et al., 2013). Activated microglia have been shown to play a significant role in the formation of hydrocephalus after GMH-IVH, through the release of inflammatory cytokines and the formation of glial scarring (Olopade, Shokunbi, & Siren, 2012). Scar tissue formation has led to the reduction of CSF reabsorption, which has been shown to contribute to the development of hydrocephalus (Khasawneh, Garling, & Harris, 2018). Additionally, microglia activation has been shown to contribute to ventriculomegaly in hydrocephalic rats (Olopade et al., 2012). The suppression of activated microglia attenuated inflammatory cytokines, ventricular dilation, and PHH after GMH-IVH (Feng et al., 2017; Tang et al., 2016;

Zhang, Xu et al., 2018). Yet, much more studies need to be conducted to fully characterize M1's role in the formation of PHH after GMH-IVH.

M2 microglia polarization has been shown to be neuroprotective by decreasing inflammation, oxidative stress, reduction in cytotoxic iron, and phagocytosis of blood clots after GMH-IVH (Flores et al., 2016; Zhang, Ding et al., 2018; Zhang, Xu et al., 2018). M2 microglia/macrophages are recruited to the site of injury and engulf blood clots and damaged surrounding tissues (Flores et al., 2016; Zhao et al., 2007). M2 microglia are divided into three subtypes—M2a, M2b, and M2c which all have different cell surface markers and functions. Both M2b and M2c participate in phagocytosis and removal of tissue debris, while M2a plays a role in cell regeneration (Lan, Han, Li, Yang, & Wang, 2017; Roszer, 2015). Recent studies in GMH-IVH evaluated therapeutics that shift microglia/macrophages into the M2 phenotype, which leads to an enhancement of blood clot clearance, attenuation of short- and long-term neurological deficits, and reduce PHH (Flores et al., 2016; Zhang, Ding et al., 2018; Zhang, Xu et al., 2018). Furthermore, M2 microglia are phagocytes that play a crucial role in regulating iron as the cell phenotype can contain large intracellular labile iron pools, which effectively take up and spontaneously release iron at low concentrations away from the site of injury to avoid iron overload (Chavez-Sanchez et al., 2014).

4) MEDIATORS OF PHAGOCYTOSIS IN GMH-IVH

4.1 Cluster of Differention 36's (CD36) role in hematoma clearance

Scavenger receptor CD36, a transmembrane glycoprotein, is known to be located on the cell surface of several cell types, including monocytes, endothelial cells, and microglia. CD36 plays an essential role in microglial phagocytosis by binding to

erythrocytes, thrombospondin, collagen, lipids, and fatty acids, and upregulating its expression beneficially increases hematoma resolution in hemorrhagic stroke (Chen et al., 2017; Zhao, Grotta, Gonzales, & Aronowski, 2009). When goat mammary gland epithelial cells are transfected with CD36-expressing gene, they acquire phagocytic functionality, demonstrating CD36's important role in phagocytosis (Cao, Luo et al., 2016). The M2 phenotype has increased phagocytic activity, and CD36 stimulation has been shown to mediate microglia/macrophage M2 polarization (Woo, Yang, Beltran, & Cho, 2016). CD36 is vital to the immune response within the neonatal CNS, as CD36 deletion attenuated the removal of apoptotic cells which lead to worsened injury after neonatal stroke (Woo et al., 2012). Additionally, our research group has knocked down CD36 in a neonatal rat GMH-IVH model, via CD36 siRNA, which resulted in the inhibition of CD36- induced hematoma resolution (Flores et al., 2016).

4.2 PPAR-γ's role in CD36 stimulation

Peroxisome proliferator-activated receptor gamma (PPAR-γ), a member of the nuclear hormone receptor superfamily, has been shown to play a role in upregulating CD36 expression (Zhao, Grotta et al., 2009; Zhao et al., 2007). In adult hemorrhagic stroke models, PPAR-γ stimulation has been directly associated with CD36 upregulation and consequent augmented microglia/macrophage phagocytic clearance of hematoma, ameliorated secondary brain injury, and improved functional recovery (Zhao et al., 2007). Our lab has recently demonstrated that CD36 stimulation, via PPAR-γ agonist 15d-PGJ2, enhanced hematoma resolution, as hematoma volume was significantly reduced at 3- and 7-days post-ictus in treated groups when compared to nontreated and inhibitor groups. In the same study, the resulting reduction of hematoma volume in the short-term reduced

long-term white matter loss, incidence of posthemorrhagic ventricular dilation, and intracranial pressure, which was met with improved neurofunctional outcomes. Inhibition of PPAR-γ reversed the therapeutic effects of the agonist, which was also met with reduced CD36 expression.

Furthermore, PPAR-γ stimulation increased CD206 (M2 marker) and significantly increased M2 microglia/macrophages at the site of hematoma, but quantification of M1 microglia was not determined (Flores et al., 2016). PPAR-γ agonist presents great promise in hematoma resolution after hemorrhagic stroke, yet many of these agonists have not been evaluated in GMH-IVH. The most notable treatment options/strategies for PPAR-γ stimulation are rosiglitazone, pioglitazone, monascin, and cilostazol (G. Wang, Wang, Sun, & Tang, 2018). In an adult mouse ICH model, rosiglitazone treatment increased the population of M2 microglia/macrophages around the injured site and enhanced hematoma resolution (Chang et al., 2017). Pioglitazone is being evaluated as a means for increasing hematoma resolution after ICH in clinical trials (Gonzales et al., 2013). Monascin treatment reduced blood–brain barrier permeability, brain edema, and hematoma volume in adult rats after ICH (J. Wang et al., 2017). Cilostazol protected blood–brain barrier integrity after intracerebral hemorrhage in mice (Takagi et al., 2017).

4.3 BVRA's role in CD36 stimulation

Biliverdin reductase A (BVRA), also known as Biliverdin-IX-alpha reductase, is an enzyme responsible for converting Biliverdin-IX alpha into the antioxidant molecule bilirubin-IX-alpha (Kapitulnik & Maines, 2009; Maines, 2005). BVRA has been well documented as having anti-inflammatory properties, most notably the inhibition of pro-

inflammatory receptor Toll-like receptor-4 (TLR4) (Wegiel et al., 2011). However, BVRA's role in the microglia/macrophage phagocytic blood clot removal has been understudied in stroke and GMH-IVH. Our most recent study suggests that BVRA treatment upregulates CD36 expression 72-hr post-ictus (Zhang, Ding et al., 2018). Additionally, in the same study, BVRA accelerated hematoma resolution, as hematoma volume was significantly decreased in the treated group when compared to vehicle and inhibitor groups. Similar to PPAR-γ, the reduction in hematoma volume was met with a reduction in PHH and improved neurofunctional outcomes in the long term. Accordingly, it was also found that BVRA increased the population of M2 microglia/macrophages around the site of injury (Zhang, Ding et al., 2018). The underlying mechanisms of how BVRA upregulates CD36 remains to be elucidated.

4.4 Cluster of Differentiation 163 (CD163)

Adult hemorrhagic stroke preclinical models have shed light on various other targets that enhance microglia/macrophage phagocytic hematoma resolution. CD163, a class B glycoprotein scavenger receptor, is involved in hemoglobin clearance through microglia/macrophage phagocytosis (Thomsen, Etzerodt, Svendsen, & Moestrup, 2013). CD163 has been shown to be elevated in children with traumatic brain injury, suggesting that this receptor may play a role in the CNS (Newell et al., 2015). The haptoglobin-CD163-heme oxygenase-1 (HO-1) pathway is noted as the first line of defense in preventing hemoglobin-induced toxicity, as CD163 mediates intracellular uptake of hemoglobin via phagocytic means following ICH (Madsen, Graversen, & Moestrup, 2001; Thomsen et al., 2013; Zhao, Song et al., 2009). Similar to CD36, CD163 is primarily

expressed in M2 microglia/macrophages (Garton, Keep, Hua, & Xi, 2017). Potential therapeutic options for the upregulation of CD163 are Hb-haptoglobin (Hp) complex (Nantasenamat, Prachayasittikul, & Bulow, 2013), glucocorticoids (Vallelian et al., 2010), and deferoxamine (Hatakeyama, Okauchi, Hua, Keep, & Xi, 2013; G. Wang et al., 2018). Recently it has been demonstrated that the upregulation of CD163 was associated with the resolution of hematoma after GMH (Liu, Flores et al. 2021).

4.5 Nuclear factor erythroid 2-related factor 2 (Nrf2)

PPAR-γ has recently been shown to be stimulated indirectly, via nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a ubiquitous pleiotropic transcription factor involved in the induction of CD36 through PPAR-γ activity (Hsu, Lee, Chang, Hsu, & Pan, 2013). In ICH, Nrf2 has been shown to reduce oxidative damage, ameliorate inflammation, and improve blood product detoxification. In addition to the aforementioned effects, Nrf2 also augments microglia/macrophage-mediated phagocytosis (Zhao, Sun, Zhang et al., 2015). Chemerin treatment, which is a chemoattractant protein that promotes microglia chemotaxis and upregulates Nrf2 via ChemR23 receptor stimulation, suppressed neuroinflammation and reduced PHH in a neonatal rat GMH-IVH model. These neuroprotective effects are contributed to Nrf2-induced accumulation and proliferation of M2 microglia (Zhang, Xu et al., 2018). Although this study evaluated Nrf2's anti-inflammatory effects. The most notable treatment options/strategies for Nrf2 agonism are carnosol (Martin et al., 2004), monascin (Lee et al., 2013), dimethyl fumarate (Brennan et al., 2015), chemerin (Zhang, Xu et al., 2018), and ankaflavin (Lee et al., 2012; Wang et al., 2018; Zhao, Sun, Zhang et al., 2015). Dimethyl fumarate reduced blood–brain barrier permeability, diminished brain

edema, and ameliorated neurological deficits in adult ICH mouse models (Iniaghe et al., 2015; Zhao, Sun, Zhang et al., 2015). Monascin treatment reduced blood–brain barrier permeability, brain edema, and hematoma volume in adult rats after ICH (Wang et al., 2017). Carnosol and ankaflavin have not been evaluated in any preclinical ICH or GMH-IVH model. In a recent publication by our research group, we demonstrated that Nrf2 upregulation via IL-19, part of the IL-10 subfamily, improved hematoma clearance and attenuated neurological deficits induced by GMH which was mediated through the actions of Nrf2 on scavenger receptor CD163 (Liu, Flores et al. 2021).

5) POTENTIAL THERAPEUTIC TARGETS THAT IMPROVE BLOOD CLOT CLEARANCEAFTER GMH-IVH VIA MICROGLIA/MACROPHAGE PHAGOCYTOSIS

5.1 Cluster of differentiation 47 (CD47)

CD47, an integrin-associated protein and well known “don't eat me” signal, is expressed on erythrocytes and is described as aregulator of microglia/macrophage phagocytosis initiation (Ni, Mao, Xi, Keep, & Hua, 2016; Ravichandran, 2011). CD47 inhibits phagocytosis via CD47 interactions with signal regulatory protein-α (SIRPα) expressed on microglia/macrophages (Ni et al., 2016). Erythrocytes deficient in CD47 are more readily phagocytized and removed from tissue then wild-type cells when injected into mice, which was corroborated in in vitro macrophage phagocytosis experiments (Olsson, Nilsson, & Oldenborg, 2007). In a mouse ICH model, it has been found that injecting CD47 knockout blood promoted M2 microglia/macrophage recruitment in the perihematomal region and enhanced phagocytosis of RBCs (Ni et al., 2016). Unlike in adults, in whom expression is high, CD47 expression is low in rodent neonatal erythrocytes, which allows for avid phagocytic activity by monocyte-derived

macrophages in vitro (Lawrence, King, Frazier, & Koenig, 2009). Inhibition of CD47 has yet to be investigated in GMH-IVH but shows great promise. No notable treatment options/strategies that inhibit CD47 have been identified and investigation in any preclinical ICH or GMH-IVH models, although deferoxamine and clodronate liposomes have been used as tools to investigate CD47 and its role in hematoma clearance (Ni et al., 2016; Wang et al., 2018). Deferoxamine treatment inhibited erythrocyte CD47 loss in an autologous blood-injection pig ICH model, reducing hematoma clearance as a result (Cao, Zheng et al., 2016). Clodronate liposomes depleted phagocytes, resulting in more severe brain swelling and reduced clot clearance in mice with CD47 knockout erythrocytes injected into their brain (Cao, Zheng et al., 2016).

6) Conclusion

Our understanding of microglia/macrophage-mediated hematoma resolution after GMH-IVH is still minimal. Clinically, hematoma volume after GMH-IVH is a prognostic indicator of future neurologic outcomes, and patients who have large hematoma volumes have worsened neurologic deficits (Keep, Xi, Hua, & Hoff, 2005; Xi, Keep, & Hoff, 2006). Various preclinical models of stroke have shown that rapid hematoma clearance after hemorrhagic stroke ameliorated inflammation and improved neurological deficits in the short and long term (Zhao et al., 2007). Studies in adult hemorrhagic stroke have shed light on the many mechanisms involved in hematoma resolution, yet many of these strategies need to be corroborated in GMH-IVH since neonatal microglial population and brain physiology differ from adults. Current literature demonstrates the effectiveness of microglia/macrophage-mediated blood clot clearance via PPAR-γ stimulation and consequent CD36 upregulation in the treatment of GMH-IVH and reduction of PHH in

preclinical animal models (Figure 1.3). Additionally, more investigations are needed to evaluate microglia/ macrophage subtypes and their role in preventing iron overload. Currently, our research group is investigating the time course of microglia activation and polarization after GMH-IVH in a neonatal rat model. Further exploration of the fundamental molecular mechanisms of phagocytic signaling [and microglia function] may reveal potential targets for innovative, noninvasive interventions to treat GMH-IVH and prevent PHH in neonates.

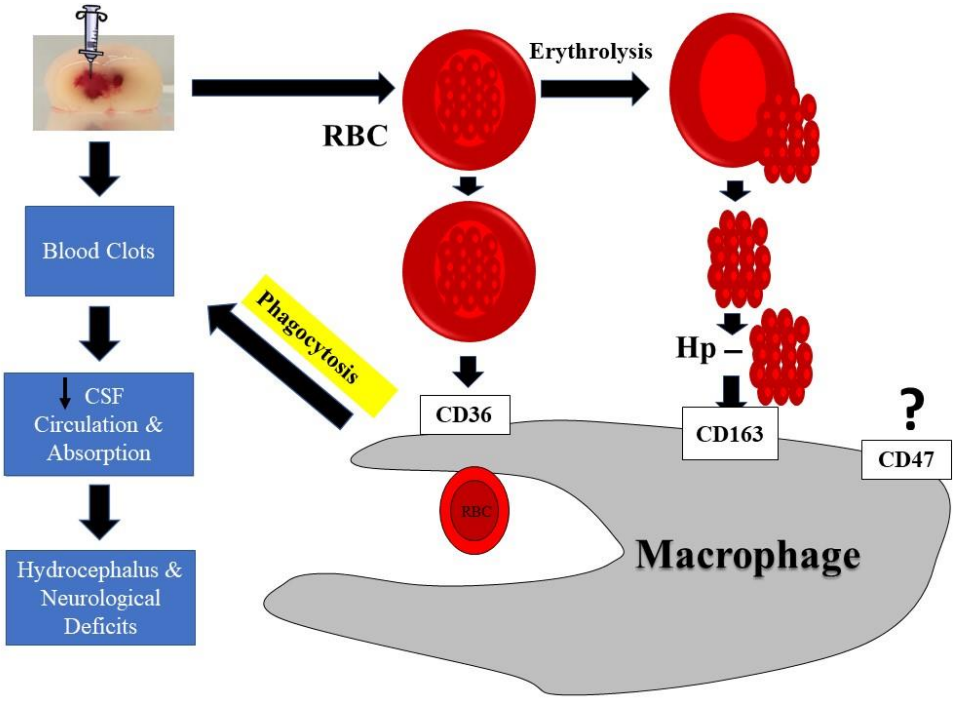

Figure 1.3. Schematic of previously investigated scavenger receptors after GMH. The actions of CD36 and CD163 after GMH have been previously investigated, yet the actions of CD47 are unknown.

SPECIFIC AIMS

Germinal matrix hemorrhage (GMH), the leading cause of morbidity and mortality in premature infants, affects approximately 3.5 per 1,000 live births in the United States (Fanaroff, Stoll et al. 2007, Jain, Kruse et al. 2009). GMH is defined as the rupture of immature blood vessels within the subependymal (or periventricular) germinal matrix, where blood clots have been identified as a causative factor in the formation of post-hemorrhagic hydrocephalus (Ballabh 2010, Heron, Sutton et al. 2010). Current literature suggests that higher grades of GMH (large hematoma expansion) in patients have worsened outcomes (Keep, Xi et al. 2005, Xi, Keep et al. 2006, Mukerji, Shah et al. 2015). Preclinically, enhancing hematoma resolution after GMH has shown to improve short- and long-term outcomes (Flores, Klebe et al. 2016). Although much is known about the GMH pathophysiology, there is no effective treatment regimen in improving the acute and chronic outcomes following GMH; thus, reducing hematoma volume is expected to optimize recovery in this patient population (Zazulia, Videen et al. 2011).

N-formyl peptide receptor 2 (FPR2), a G-protein-coupled receptor, are expressed on microglia/macrophages in the central nervous system. FPR2 stimulation has been shown to improve outcomes after stroke (McArthur, Cristante et al. 2010, Bena, Brancaleone et al. 2012, Ding, Flores et al. 2019). Additionally, FPR2 upregulation promoted monocyte recruitment and increased phagocytic activity (Ramon, Bancos et al. 2014). Indeed, FPR2 has been associated with the activation of phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2), which in turn promotes the transcription of the dual-specificity protein phosphatase 1 (DUSP1) gene (Finelli, Murphy et al. 2013). Current literature suggests that DUSP1 may play a role in the upregulation of phagocytosis by macrophages, yet its importance in stroke has not been evaluated. Interestingly, FPR2 has been shown to upregulate scavenger receptor CD36, which plays a significant role in hematoma resolution (Cattaneo, Parisi et al. 2013, Ramon,

Bancos et al. 2014). Our research group has previously established the role of CD36 after GMH, which enhanced hematoma clot clearance and attenuated post-hemorrhagic hydrocephalus (Chavez-Sanchez, Garza-Reyes et al. 2014, Flores, Klebe et al. 2016). Although FPR2 has shown to reduce inflammation through the inhibition of MAPK/P38 pathway after stroke, the role of this receptor and its signaling pathway to induce hematoma resolution has not been explored after hemorrhagic stroke nor have strategies been developed to evaluate FPR2 as a potential therapeutic agent for the reduction of post-hemorrhagic hydrocephalus (Guo, Hu et al. 2016). Our preliminary data demonstrates that FPR2 agonism, via Annexin A1, Lipoxin A4, Resolvin D1, is associated with the reduction hematoma volume and improved neurological outcomes in the short- and long-term. Furthermore, our finding suggests that FPR2 receptor is present in the GMH neonatal brain. Figure 1.4 illustrates the proposed signaling pathway of FPR2 and proposed AIMs.

Lastly, FPR2 stimulation increased DUSP1 and CD36 expression, which indicated that this signaling pathway may play a role in hematoma clot clearance. Based existing literature and our preliminary data, we hypothesize that FPR2 stimulation enhances microglia induced hematoma resolution through the activation of the p-ERK (1/2)/DUSP1/CD36 signaling pathway, thereby improving short- and long-term neurological outcomes. We propose the following two specific aims to test our central hypothesis in well-established preclinical GMH rodent models:

Aim 1

Will investigate the role of FPR2 in enhancing hematoma resolution, thereby improving neurological function following GMH. We hypothesize that FPR2 activation will increase hematoma resolution after GMH and antagonism of FPR2 will abolish this protective effect. To enhance the clinical relevance, we will 1) compare different

therapeutic routes of administration of FPR2 agonist AnxA1 (interperitoneally or intranasally), 2) use both male and female specimen, and 3) study the role FPR2 on post-hemorrhagic hydrocephalus formation.

Aim 2

Will investigate FPR2-induced activation of the ERK/DUSP1/CD36 signaling pathway after GMH. We hypothesize that FPR2 stimulation will activate ERK1/2 and DUSP1, thereby upregulating CD36 following GMH. Whereas inhibition or gene knock-down of the signaling pathway will disrupt signaling proteins and FPR2 induced hematoma resolution.

The long-term goal of this proposal is to provide a basis for clinical translation of FPR2 stimulation as an effective non-invasive therapeutic strategy to protect against acute and chronic complications in the GMH patient population.

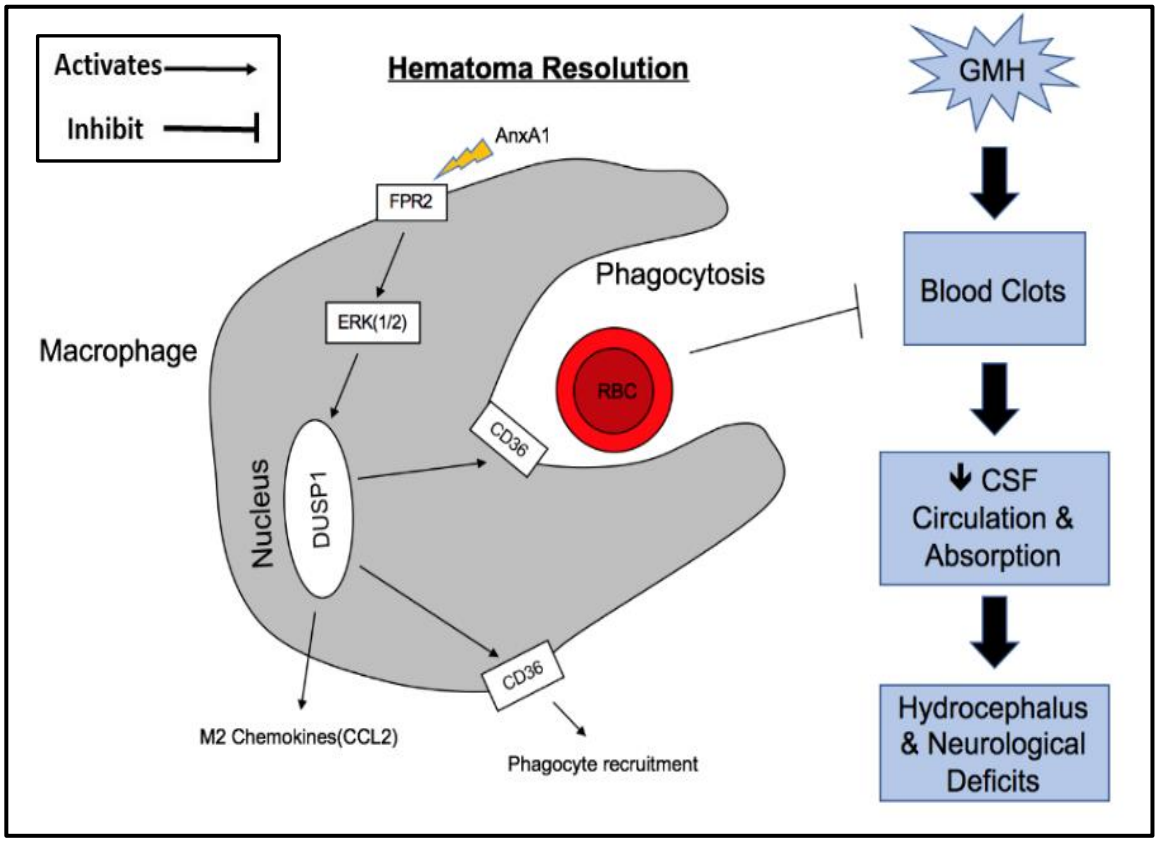

Figure 1.4: Schematic representation of the overall central hypothesis and research aims. Our Central Hypothesis is that FPR2 stimulation enhances microglia induced hematoma resolution through the activation of the p-ERK (1/2)/DUSP1/CD36 signaling pathway, thereby improving short- and long-term neurological outcomes. Will investigate the role of FPR2 in enhancing hematoma resolution, thereby improving neurological function following GMH. Will investigate FPR2-induced activation of the p-ERK/DUSP1/CD36 signaling pathway after GMH.

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CHAPTER TWO

N-FORMYL PEPTIDE RECEPTOR 2 ACTIVATION VIA ANNEXIN A1 UPREGULATES HEMATOMA RESOLUTION VIA THE FPR2/P-ERK(1/2)/DUSP1/CD36 SIGNALING PATHWAY AFTER GERMINAL MATRIX HEMORRHAGE

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Abstract

Germinal matrix hemorrhage (GMH) is one of the leading causes of morbidity and mortality in preterm infants in the United States, with little progress made in its clinical management. Blood clots disrupting normal cerebrospinal fluid circulation and absorption after germinal matrix hemorrhage are key contributors towards post-hemorrhagic hydrocephalus development. n-formyl peptide receptor 2 (FPR2), a G-protein-coupled receptor, has been associated with the activation of p-ERK1/2, which in turn promotes the transcription of the DUSP1 gene, which may play a role in CD36 signaling. CD36 scavenger, a transmembrane glycoprotein, plays an essential role in microglia phagocytic blood clot clearance after GMH. FPR2's role in blood clot clearance after hemorrhagic stroke is unknown. We hypothesize that FPR2 activation by Annexin A1 will enhance hematoma resolution via upregulation of the CD36 signaling pathway, thereby improving short- and long-term neurological outcomes. Bacterial collagenase (0.3 U) was infused intraparenchymally into the right hemispheric ganglionic eminence in P7 rat pups to induce GMH. Annexin A1 and FPR2 Inhibitor (Boc2) were given at 1-hour post-GMH via intranasal administration. FPR2 CRISPR was given 48-hours prior to GMH induction. Short-term neurological deficits were assessed using negative geotaxis test. Hematoma volume was assessed using hemoglobin assay. Protein expression was assessed using western blots. Long-term neurocognitive deficits and motor coordination were assessed using Morris water maze, rotarod, and foot fault tests. We have demonstrated that Annexin A1 treatment enhances hematoma resolution and improved short and long-term outcomes. Lastly, FPR2 agonism resulted in the upregulation of the FPR2/p-ERK(1/2)/DUSP1/CD36 signaling pathway.

1) Introduction

Germinal matrix hemorrhage and intraventricular hemorrhage (GMH-IVH) is a leading cause of morbidity mortality in premature and or low birthweight infants, especially those born before 32 weeks of gestation (Egesa, Odoch et al. 2021). The site of hemorrhage occurs in the fragile capillary network of the subependymal germinal matrix of the neonatal brain, where ruptures of these fragile capillaries may be due to abrupt fluctuations in cerebral blood flow due to hemodynamic and cardiorespiratory instability (Ballabh 2014). Debilitating consequences of GMH include the formation of post-hemorrhagic hydrocephalus (Ballabh 2010, Heron, Sutton et al. 2010). The expansion of the cerebroventricular system leads to mechanical compression and consequent injury of the surrounding brain tissue, causing neurological deficits in patients that survive the initial bleed. Intracerebroventricular blood clots have been identified as causative factors of hydrocephalus formation. Blood clots directly impair the circulation and absorption of cerebrospinal fluid (CSF) (Whitelaw, Cherian et al. 2004, Stein, Luecke et al. 2010, Li, Ding et al. 2018). Therefore, the quick removal of the blood clot after GMH may serve as a potential therapeutic target for patients with GMH.

The primary macrophages of the CNS are known as microglia, which have been shown to play a primary role in the innate immune response after injury. After hemorrhagic stroke, resident microglia and recruited macrophages can play either a harmful or protective role after CNS injury, which is dependent on the dominate microglia phenotype (London, Cohen et al. 2013). M2 phenotype, also known as alternatively activated M2 microglia, are noted as being recruited to the site of the hematoma, where the blood clot is removed through the process of phagocytosis

(Aronowski and Zhao 2011, Flores, Klebe et al. 2016). Quick microglia blood clot clearance after hemorrhagic stroke was shown to be beneficial after GMH (Flores, Klebe et al. 2016). The quick removal of the blood clot after GMH serves as a beneficial target for this patient population.

N-formyl peptide receptor 2 (FPR2), a G-protein-coupled receptor, is expressed on microglia/macrophages in the central nervous system. FPR2 stimulation has been shown to improve outcomes after stroke (McArthur, Cristante et al. 2010, Bena, Brancaleone et al. 2012, Ding, Flores et al. 2019). Additionally, FPR2 upregulation promoted monocyte recruitment and increased phagocytic activity (Ramon, Bancos et al. 2014). FPR2 has been associated with the activation of phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2), which in turn promotes the transcription of the dual-specificity protein phosphatase 1 (DUSP1) gene (Finelli, Murphy et al. 2013). Current literature suggests that DUSP1 may play a role in the upregulation of phagocytosis by macrophages, yet its importance in stroke has not been evaluated. Interestingly, FPR2 has been shown to upregulate scavenger receptor CD36, which plays a significant role in hematoma resolution (Cattaneo, Parisi et al. 2013, Ramon, Bancos et al. 2014). Additionally, FPR2 activation has also been found to polarize microglia into the M2 phenotype (Li, Cai et al. 2011), is primarily responsible for the mediation of wound healing and enhancement of macrophage phagocytosis of blood clots in GMH (Flores, Klebe et al. 2016). Yet the underlying role of FPR2 after GMH and its mechanism remains to be elucidated.

In this present study, we demonstrate that FPR2 agonism enhanced hematoma resolution, through the promotion of M2-like microglia which improved short- and long-

term neurological deficits after GMH through the p-ERK(1/2)/DUSP1/CD36 signaling pathway.

2) Materials and Methods

2.1 Animals

Animal usage, methods, and experimental design were approved by the Loma Linda University Institute Animal Care and Use Committee and in compliance with the NIH Guidelines for the use of animals in research. 252 p7 Sprague-Dawley Neonatal pups (weight 9-15g, Harlan, Livermore, CA) were used for this study.

2.2 Germinal Matrix Hemorrhage (GMH) Model

Germinal Matrix Hemorrhage (GMH) was induced by stereo-guided injection of sterile bacterial collagenase as described in (Lekic, Manaenko et al. 2012). Neonatal rat pups were be anesthetized with 5% isoflurane via isoflurane gas mixer, which were then adhered onto a stereotactic frame with a neonatal adaptor. An incision was made in the midline exposing the bregma, and a burr hole was made on the right side of the skill at coordinates of 1.6 mm (right-lateral), and 1.5 mm (rostral) from bregma. A 10 uL syringe with 0.3 units of collagenase V-II (VII-S, Sigma; 0.3 U in 0.5 μl of PBS) is then guided through the burr hole at a depth of 2.7 mm, where the infusion of the collagenase occurs by a microinfusion pump ((Harvard Apparatus, Hollison, MA). To minimize the backflow of the collagenase, the syringe was kept in place for 5 minutes after the completion of infusion and was withdrawn at a rate of 0.5 mm/min. Pups were observed for changes in skin color, temperature, respiratory rhythm and frequency, and heart rates. Once the needle is removed bone wax was used to seal the burrhole, followed by the suturing of the incision line. Pups were then placed in a 37ºC heated blanket for recovery

and then placed back with the dam. The sham group was only subjected to needle insertion without collagenase infusion.

2.3 CRISPR Intracerebroventricular Injections

CRISPR was administered 48 hrs prior to GMH by Intracerebroventricular injection to the rat pup brain. Animals were anesthetized with 5% isoflurane and a scalp incision will be made on the skull surface where the bregma was exposed. A selected group of rats were given different reagents according to the project design and will be injected with a 10-ul syringe (Hamilton, Nevada, USA) at the location of 1.3 mm posterior and 1.3mm lateral to the bregma, and 1.7 mm deep to the skull surface at the contralateral hemisphere 48 hrs before GMH (2ul/rat pup). The control rat pup was injected with a CRISPR dilutant (Santa Cruz, Dallas, TX). The injection was completed in 5 minutes, and the needle was maintained in the injection position for an additional 2 minutes. The needle was removed slowly out of the brain, and the incision line was sutured. After recovery from anesthesia, the rat pups were placed back into their respective cages. Control CRISPR plasmid (Santa Cruz Biotech, Dallas, Tx) and FPR2 CRISPR will be used. Sham animals will receive CRISPR Dilutant.

2.4 Drug Administration

Human recombinant Annexin A1 (AnxA1, R&D Systems) was administered intranasally (in 20 μL of saline) at 1 hr after GMH-induced induction and given for three consecutive days for the short-term studies and 7 consecutive days for the long-term time-points. The dosage concentrations for annexin A1 were modified from the following study (Ding, Flores et al. 2020). FPR2 CRISPR knockout (San Cruz Biotech, USA) plasmid or CRISPR control (Santa Cruz Biotech, USA) were given at a dose of 3 ug/pup

were intracerebroventricular administered 48 h prior to GMH induction. FPR2 inhibitor Boc-2 (MP Biomedical, Irvine, CA) was intranasally administered 1-hour after surgery or give for either 3 or 7 consecutive days depending on the time-point.

2.5 Experimental Design and Animal Groups

Both male and female P7 rat pups were randomly divided into the following groups: Sham-operated, GMH+Vehicle, GMH+AnxA1, GMH+AnxA1+Boc-2, GMH+FPR2 CRISPR, GMH+FPR2 CRISPR, GMH+CRISPR Plasmid Control, GMH+CLOD, GMH+AnxA1+CLOD. Figure 2.1 shows each animal group and experimental design.

Experiment 1: Was conducted to detect the endogenous expression levels of FPR2 and DUSP1. Western Blot (WB) was used to evaluate the expression of these proteins in whole-brain lysate at 12h, 24h, 72h, 5d, and 7d after GMH. Additionally, to confirm if FPR2 and DUSP1 proteins were found on microglia cells, the colocalization of FPR2 or DUSP1 on microglia cells was done through immunohistochemistry.

Experiment 2: Experiment 2 was performed to evaluate the best dosing regimen and route of administration of AnxA1. Animals were divided randomly into two groups, either received intraperitoneal or intranasal administration of AnxA1 at a low and high concentration. The following groups were made: Sham, GMH+Vehicle, GMH+AnxA1 (I.P, 0.5 µg/rat pup), GMH+AnxA1 (I.P 1.5 µg/rat pup), GMH+AnxA1(in 0.05 µg/rat pup), and GMH+AnxA1 (0.0150 µg/rat pup). Hemoglobin assay and short-term neurobehavior were used to evaluate the best dosing regimen at 72h post-ictus.

Experiment 3: Experiment 3 was executed to evaluate the effects of FPR2 agonism via AnxA1 on short- and long-term after GMH. Hemoglobin assay and short-term neurobehavior were conducted to evaluate the hematoma content and neurobehavioral deficits, respectively at 24 h, 72 hr, and 72 hours. Long-term behavior was conducted to assess long-term neurobehavioral deficits. Long-term animals were assessed for CSF pressure and Nissl staining was used to assess morphological changes (ventricular dilation).

Experiment 4: Experiment 4 evaluated FPR2's role in microglia cells and its ability to polarize M1 microglia to the M2 phenotype. Immunohistochemistry was conducted at 72h after GMH, where M1 marker (TNF-α) or M2 marker (Mannose Receptor) was colocalized with activated microglia (CD11b). Additionally, to determine the role of FPR2 in microglia cells, microglia cells were globally inhibited with liposomal clodronate. To determine if microglia cells were successfully inhibited, western blots and IHC were performed to measure the expression of Iba1 (microglia marker). At 72 hours, hemoglobin assay was performed to determine if FPR2 agonism mediated hematoma removal through microglia cells.

Experiment 5: Experiment 5 was conducted to investigate the mechanism of the proposed FPR2 signaling pathway. Expression Levels of FPR2, ERK (1/2), DUSP1, and CD36 were detected by WB. At 72 hours, hemoglobin assay was performed to determine if FPR2 CRISPR ameliorated the effects of AnxA1.

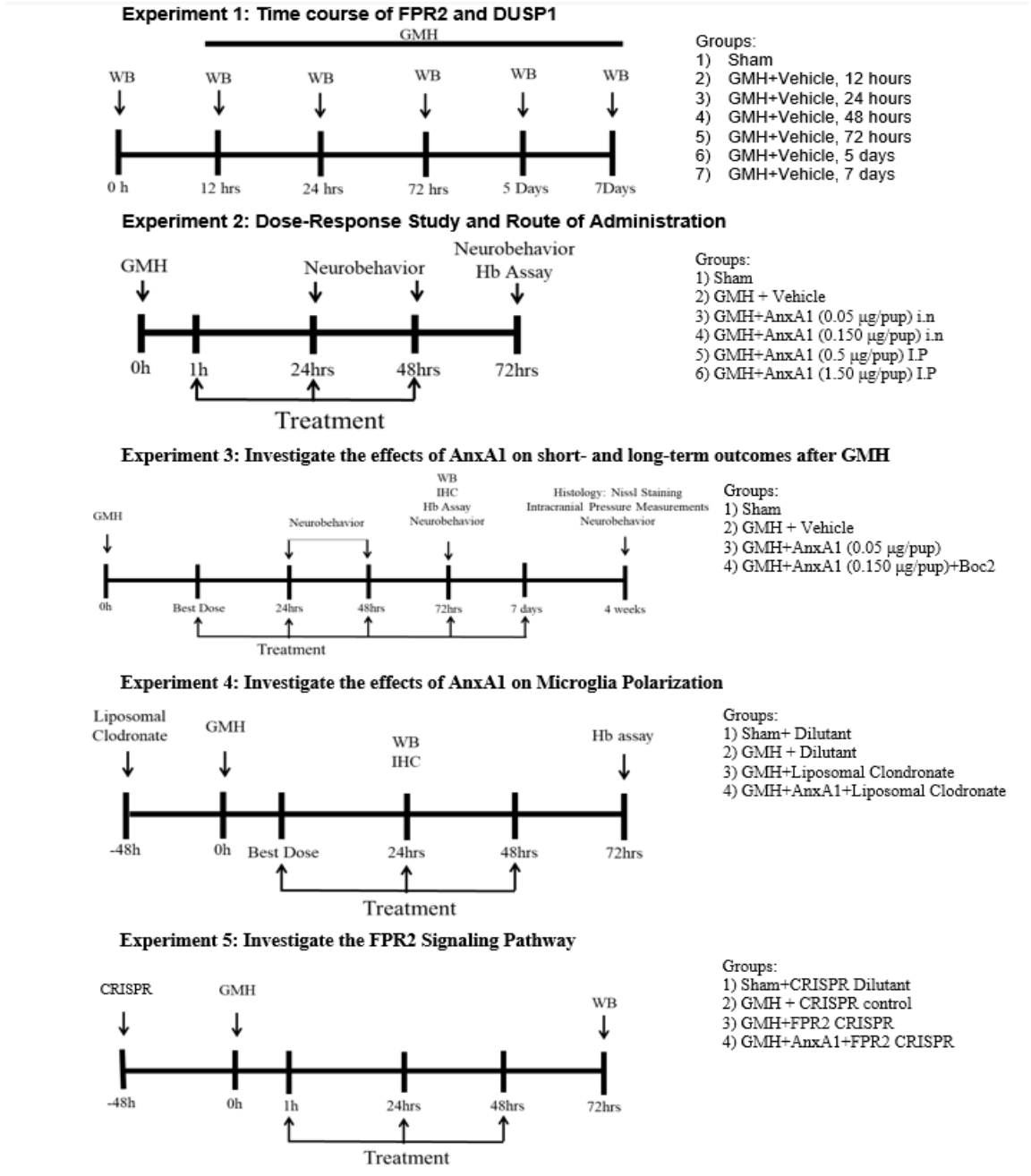

Figure 2.1: Experimental Design and Animal Groups. (GMH: Germinal Matrix Hemorrhage, WB: Western Blot, Hb Assay: Hemoglobin Assay, AnxA1: Annexin A1, IHC: Immunohistochemistry)

2.6 Animal Perfusion and Tissue Extraction

Euthanasia was dependent on the timeline of experiments for each animal group. Animals will be put under deep Anesthesia prior to tissue extraction. Brain tissues will be harvested for molecular biology, morphology, and physiology studies. The rats for histology & immunohistochemistry will be perfused with 10% formalin, under anesthesia with isoflurane. The tissues removed and placed in 10% formalin for tissue sectioning. These methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA) and have been approved by the Animal Care and Use Committee at Loma Linda University.

2.7 Western blot

Brain hemispheres were perfused with PBS, collected and snap frozen with liquid nitrogen and storage will occur at 80 degrees Celsius. Cytosolic and nuclear fractionation extract will be obtained from brain samples as described in (Lekic, Manaenko et al. 2012). Denatured protein extract (50 µg) will be electrophoresed and transferred to a nitrocellulose membrane and probed with antibodies. The following proteins will be detected by the primary antibodies for detecting Annexin A1 (1:1000, Cell Signaling), FPR2 (1:1000, Santa Cruz), DUSP1 (1:1000, Cell Signaling), CD206 (1:1000, Life Sciences), CD36 (1:1000, Santa Cruz), Iba-1 (1:1000, Abcam), ERK1/2 (1:1000, Cell Signaling), and p-ERK1/2 (Thr202/Tyr204) respectively as described in previous study (Ma, Huang et al. 2011). The optical densities of the bands will be visualized using ECL Plus or Li-Cor fluorescence technology and analyzed with Image J Software (NIH).

2.8 Immunohistochemistry

Immunohistochemistry was executed as previously described in (Flores, Klebe et al. 2016). Pups were perfused under deep anesthesia with PBS followed by 4% formaldehyde. The brains were removed for post-fixation in formalin and dehydrated with 30% sucrose solution. Paraffin-embedded brains would then be sectioned into 10-μm-thick slices via cryostat. Slices were stained with the following primary antibodies; FPR2 (1:1000, Santa Cruz), DUSP1 (1:1000, Cell Signaling), CD206 (1:1000, Life Sciences), CD36 (1:1000, Santa Cruz), Iba-1 (1:1000, Abcam), CD11b (1:1000, Abcam), TNF-α (1:2000, Abcam) at 4ºC overnight. Samples were then incubated with their respective fluorescence-conjugated secondary anti-bodies (1:200, Jackson ImmunoResearch Labs) for 2 hours at room temperature, which were then dried and stained with Hardset mounting solution with Dapi (Fischer Scientific). The perihemorrhagic area was imaged using a DMi8 fluorescent microscope (Leica Microsystems, Buffalo Grove, IL) under a 200c Fold field. To detect the proportion of CD206 and TNf-α positive activated microglia/macrophage cells, 4 fields surrounding the perihematomal region were used to count.

2.9 Neurobehavior Examination

Short-term Neurobehavior: For up to 7 consecutive days after GMH both the righting reflex test and negative geotaxis will be conducted to evaluate locomotor deficits. Long-term behavioral analysis: Long-term neurological outcomes were assessed with water maze, foot-fault and Rota rod tests at 4 weeks post GMH. The Rotarod assesses motor impairment, foot fault test assesses motor and proprioception function, and the Morris water maze test assesses learning, memory, and visual functions. All tests were conducted in a blinded manner.

Negative Geotaxis: Rat pups were oriented at a 45º downward angle, where the amount of time it takes the pup to recognize that it is on an incline and correct its positioning by making a 180-degree turn facing the top of the incline is recorded in 60-second durations. This exam was repeated three times for three consecutive days post-ictus. The Average of each day was taken for statistical analysis.

Morris Water Maze: Morris Water Maze was conducted at 4 weeks as described in (Lekic, Manaenko et al. 2012). In short, rats were released into a circular pool (110 diameters) filled with water. Spatial cues were placed on the pool walls where animals were then trained to find a submerged platform (11 cm diameter). Each animal went through 10 trials per day, where the time and distance it took the animals to find the platform was recorded. On the last day, Probe trial was conducted where the platform was removed, and the time spent in each quadrant was measured. An overhead camera in conjunction with Noldus Ethosvision (Noldus Ethovision; Noldus, Tacoma, WA tracking system) recorded the swim path and measured the swim distance, swim speeds, and time spent in probe quadrant.

Rotarod and Foot Fault: Rotarod and foot fault were conducted as described in (Flores, Klebe et al. 2016). Rats were placed into the beams in the rotarod (7 cm diameter) and tested at starting speeds of 5 RPM or 10 RPM with acceleration at 2 RPM per 5 seconds. Falling latency was recorded with the use of the Rotarod integrated photobeam (SD Instruments, San Diego, CA).

2.10 Hemoglobin Assay

Spectrophotometric measurements were used to assess hemorrhagic volume as

previously described in (Flores, Klebe et al. 2016). Preserved forebrains were placed into individual glass tube where 3 mL of PBS is inserted. The forebrains were then homogenized for 60 seconds each (Tissue Misder Homogenizer; Fisher Scientific, Pittsburgh, PA) and processed through ultrasonication for 1 min to lyse erythrocyte membranes. The supernatant was then mixed with Drabkin's reagent (Sigma Aldrich, St Louis, Mn) and were left to react for 15 minutes. Absorbance was measured using a spectrophotometer (540 nm; Genesis 10uv; Thermo Fisher Scientific, Waltham, MA), and hemorrhagic volume (μL) was calculated based on a standard curve as routinely performed as described by (Lekic, Manaenko et al. 2012).

2.11 Intracranial Pressure (ICP) Measurements

At 4 weeks post-GMH, animals were anesthetized and mounted onto an adult stereotaxic frame. A midline incision was made to expose the atlanto-occipital membrane and the cisterna magna was punctured with a 26G Hamilton needle attached to a low-pressure transducer (Digi-Med LPA 400-low pressure Analyzer, Med-Louisville, Kentucky, USA) as described in (Lackner, Vahmjanin et al. 2013).

2.12 Ventricular Volume

4-weeks after GMH, animals were anesthetized and euthanized, where the whole brains were extracted and prepared for histology similar to those prepared for IHC. Brains were embedded with optimal cutting temperature solution (OCT, Fisher Scientific, Waltham, MA) and sectioned into 24 μm thick slices. Samples were then stained with Cresyl violet solution (Fischer Scientific, Waltham, MA), where then optical dissector principles were used to delineate the cerebral structure borders as previously described in

(Lekic, Manaenko et al. 2012). Ventricular volume was analyzed using ImageJ software (NIH).

2.13 Rigor and Statistical Analysis

All animals used in this study were randomly numbered and sorted into each of the indicated animal groups using Excel. The following system was used to randomly assort the animals: 1) pups are sequenced vertically in Excel; 2) "RAND" function was used to generate a random number to each pup; 3) reranked the random numbers in an ascending or descending order; 4) lastly designate a certain number of pups into each animal group indicated in the experimental design. The assigned animal groups were unknown to researchers conducting the indicated methodology.

The number of animals needed per groups was determined using type 1 error at a rate of 0.05 and a power of 0.8 on a 2-sided test by power analysis. Data will be represented as mean ± SEM. After normality is confirmed, parametric data will be analyzed using one-way ANOVA with Tukey's post-hoc test. P values < 0.05 were considered as statistically significant. Graphpad Prism 7 was used for graphing and analyzing all data.

3) Results

3.1 The endogenous expression of FPR2 and AnxA1 increased after GMH

To determine how FPR2 and AnxA1 are affected by the GMH pathophysiology, a time-course study was conducted to examine these proteins by WB at 0 (Sham), 12-, 24-, and 72 hours, and 5- and 7-days post-ictus. Endogenous expression of FPR2 was significantly increased at 72 hours, where expression remained elevated at 7 days after

GMH (Figure 2.2 A and C). In Similar fashion, AnxA1 expression significantly elevated at 72 hours after GMH and remained elevated for 7 Days (Figure 2.2 A and Figure 2B). Both endogenous proteins demonstrated a similar trend in gradual increased expression after GMH. Based on these results, it can be suggested that endogenous AnxA1 may play a role in the upregulation of FPR2.

To determine if FPR2 receptor is present in the neonatal CNS, Double immunofluorescence staining was used for the cellular localization of both markers in microglia cells at 72 hours after GMH. FPR2 expression was more prominent in microglia cells around the site of peri-hematoma 72 hours after GMH (Figure 2.3).

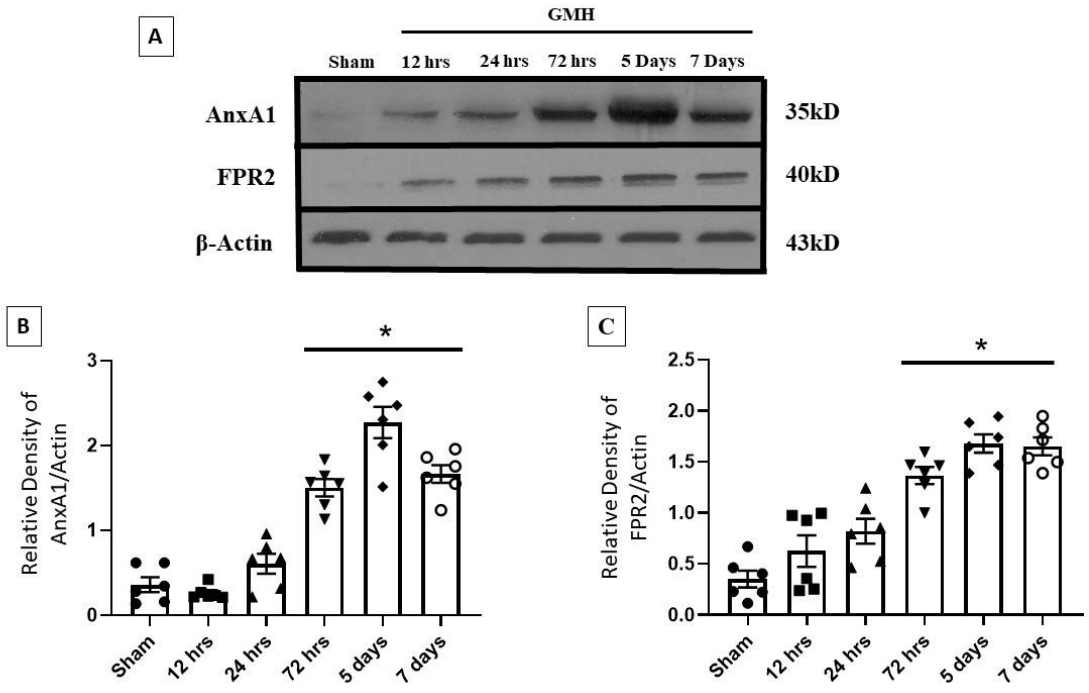

Figure 2.2. The time-course of endogenous levels of Annexin A1 and FPR2 after Germinal Matrix Hemorrhage. Western Blot was performed to detect endogenous expression of AnxA1 and FPR2 at 0 hr (sham), 12 hrs, 24 hrs, 72 hrs, 5 days, and 7 days. A) Representative Bands of AnxA1, FPR2, and β-Actin. B) The expression of AnxA1 throughout different time-points. C) The Expression of FPR2 at different time-points. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * $P<0.05$ vs. sham.

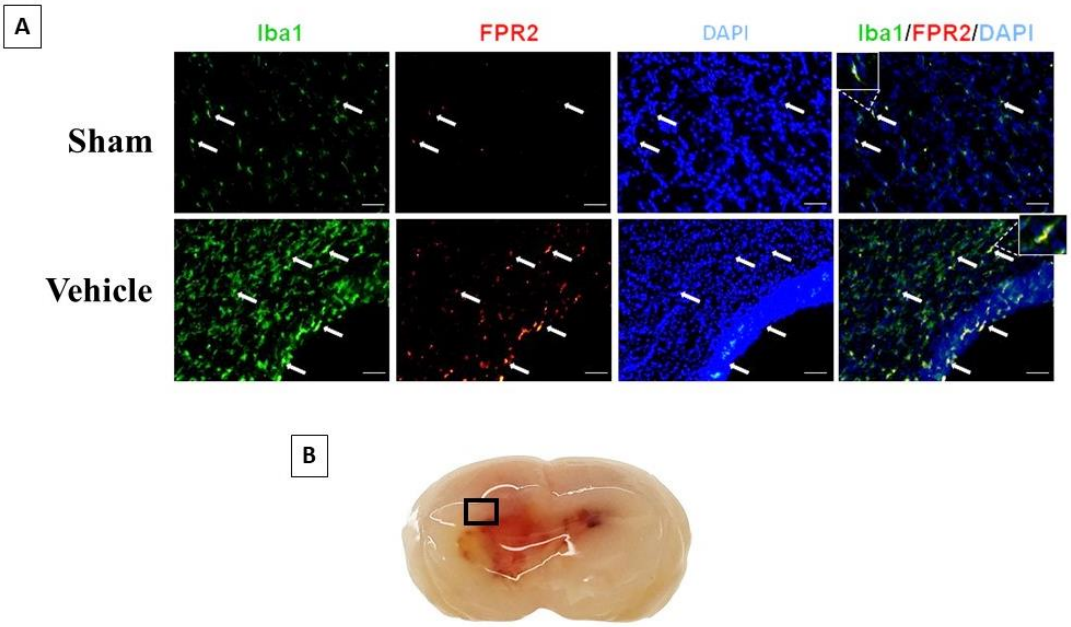

Figure 2.3. Colocalization of FPR2 and Iba1at 72 hours after GMH. Immunohistochemistry was performed to detect FPR2 (red) and Iba1 (green) in Sham and Vehicle at 72 hours after GMH induction. Positive FPR2 microglia cells are indicated by the arrows. Proteins are co-stained with DAPI (blue). Scale bar = 20μm.

3.2 High-dose Intranasal administration of Human Recombinant AnxA1 has the highest efficacy in decreasing hematoma content and improving short-term behavior

A dose-response study was conducted to determine the best dose and route of administration of AnxA1. AnxA1 was administered in low or high-dose and was given interpersonal (low, 0.5 µg/mg rat pup or High, 1.5 ug/mg rat pup) or intranasally (low, 0.05 ug or High, 0.150 ug per rat pup). Experimental design is described in experiment 2 (Figure 2.1). Negative Geotaxis was conducted for 3 consecutive days and hemoglobin assay was examined at 72 hours after GMH. At 24 hours when compared to sham, all groups had significant neurological impairment (Figure 2.4A). At 48 hours after GMH, all dosages and routes of administration showed a positive trend of improved neurobehavior at 48 and 74 hours (Figure 2.4A). Only the high dose intranasal AnxA1treatment significantly improved motor coordination when compared to vehicle at 48 and 72 hours (Figure 2.4A). Furthermore, when compared to sham, high dose intranasal AnxA1 was not significantly different (Figure 2.4A). At 72 hours when compared to the vehicle group, all dosing regiments and routes of administration were significantly efficient in reducing the hemoglobin content (Figure 2.4B). Additionally, High dose intranasal group was not significant to the sham group (Figure 2.4B). Based on these results we determined that the high dose of AnxA1 administered intranasally was the best dose and route of administration and will be used for the remainder of the experiments.

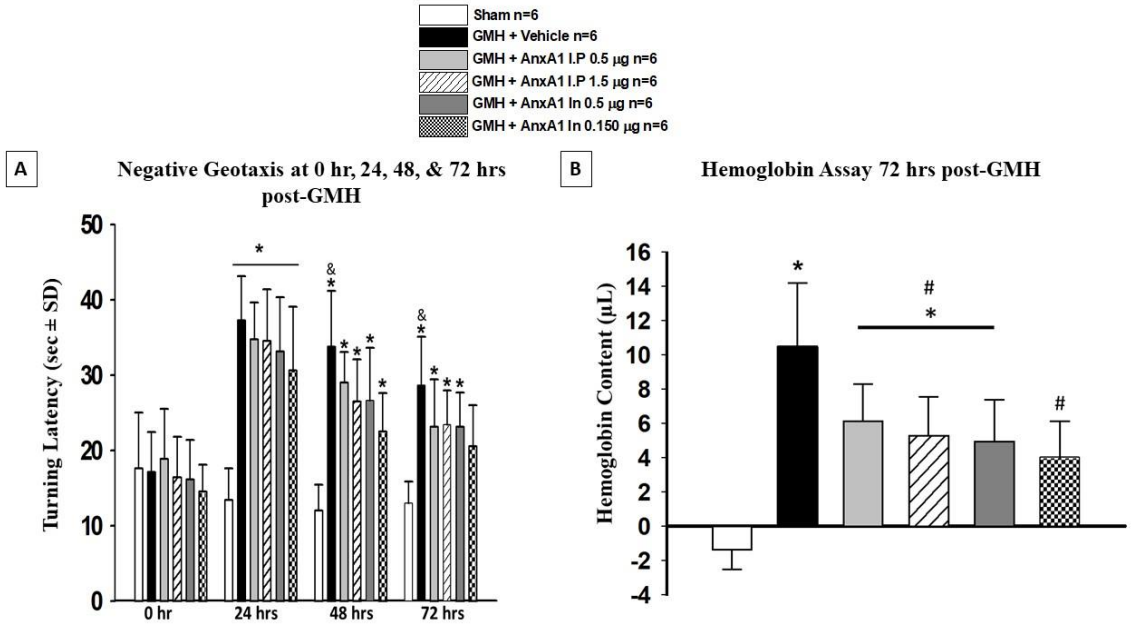

Figure 2.4. Dose Response study to determine the best effective dose and route of administration. A) Negative geotaxis was used to assess neurobehavioral function at 0, 24, 48, and 72 hours. B) hemoglobin assay was conducted at 72 hours post-GMH. All dosing regimens significantly reduced hemoglobin content at 72 hrs after GMH induction. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * P<0.05 Vs. sham, # p< .05. Vs. Vehicle.

3.3 Intranasal administration of Human recombinant AnxA1 improves Short- and Long-Term outcomes after GMH.

To determine if the neuroprotective effects of AnxA1 are through the activation of FPR2, FPR antagonist Boc-2 was given in combination with treatment. Animals were randomly divided into 4 groups as described in experiment 3 (Figure 2.1). At 48- and 72 hours, Boc-2 significantly reversed the protective effects of AnxA1 on motor coordination (Figure 2.5). Additionally, pharmacological inhibition of FPR2 significantly attenuated AnxA1's effects on the reduction of hematoma content at 72 hours and 7 days after GMH (Figure 2.6). No significance was seen at 24 hours post-GMH among treatment and inhibitor (Figure 2.6). Thus, this data demonstrates that AnxA1 effects on hematoma resolution were mediated through FPR2 agonism in the short-term.

We further assessed the effects of AnxA1 on long-term outcomes (long-term neurological function (spatial memory, reference memory, and motor coordination). Animals were assigned randomly to each group described in Experiment 3 (Figure 2.1). Neurological function was performed at 4 weeks after GMH for 5 consecutive days. During the Probe Trial of the Morris water maze, the vehicle group spent significantly less time in the platform quadrant when compared to the sham group, whereas AnxA1 treated animals spent more time in the platform quadrant when compared to Vehicle group (Figure 2.7C and D). AnxA1 treatment also significantly improved sensorimotor function in the foot fault test (Figure 2.7A) and motor coordination in the Rotarod test (Figure 2.7B) when compared to Vehicle group. Yet, these improvements in reference

memory, sensorimotor, motor coordination were reversed by FPR2 antagonist Boc-2 (Figure 2.7).

At the end of long-term neurobehavior test, animals were evaluated for intracranial pressure (ICP), as it is an indicator of the formation of hydrocephalus. At the end points of the long-term study, brain tissues were extracted and processed for histology to examine morphological changes in all animal groups. ICP was significantly decreased in the AnxA1 treated group when compared to vehicle and FPR2 antagonist groups (Figure 2.8C). For changes in brain morphology, ventricular volume was assessed at 4 weeks post-ictus. Additionally, ventricular volume was significantly increased in vehicle and FPR2 antagonist groups, yet AnxA1 treatment reduced post-hemorrhagic ventricular dilation (Figure 2.8A and B).

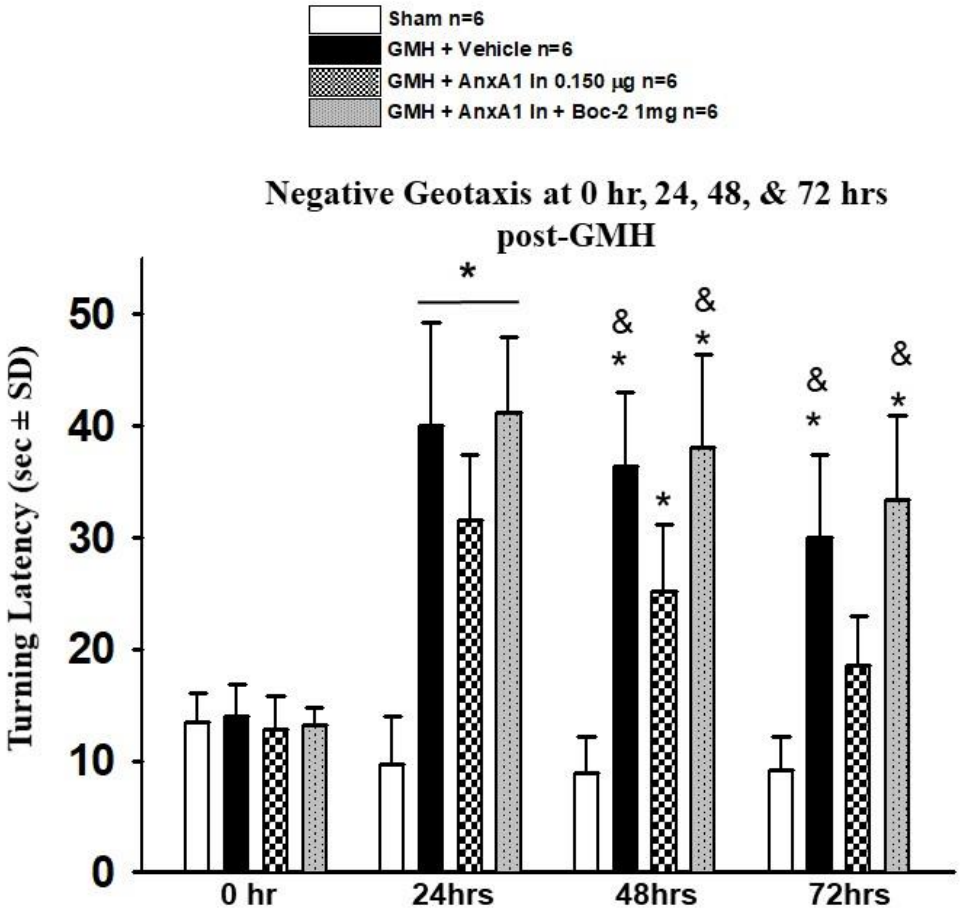

Figure 2.5. Pharmacological inhibition of FPR2 attenuated the protective effects of AnxA1 treatment at 2 and 3 days after GMH Induction. AnXA1 treated groups showed significant improvements in neurobehavior at 2 and 3 days. FPR2 inhibitor Boc-2 reversed the protective effects of Annexin A1 at 2 and 3 days after GMH. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * $P<0.05$ vs. Sham, & $P<0.05$ vs. AnxA1.

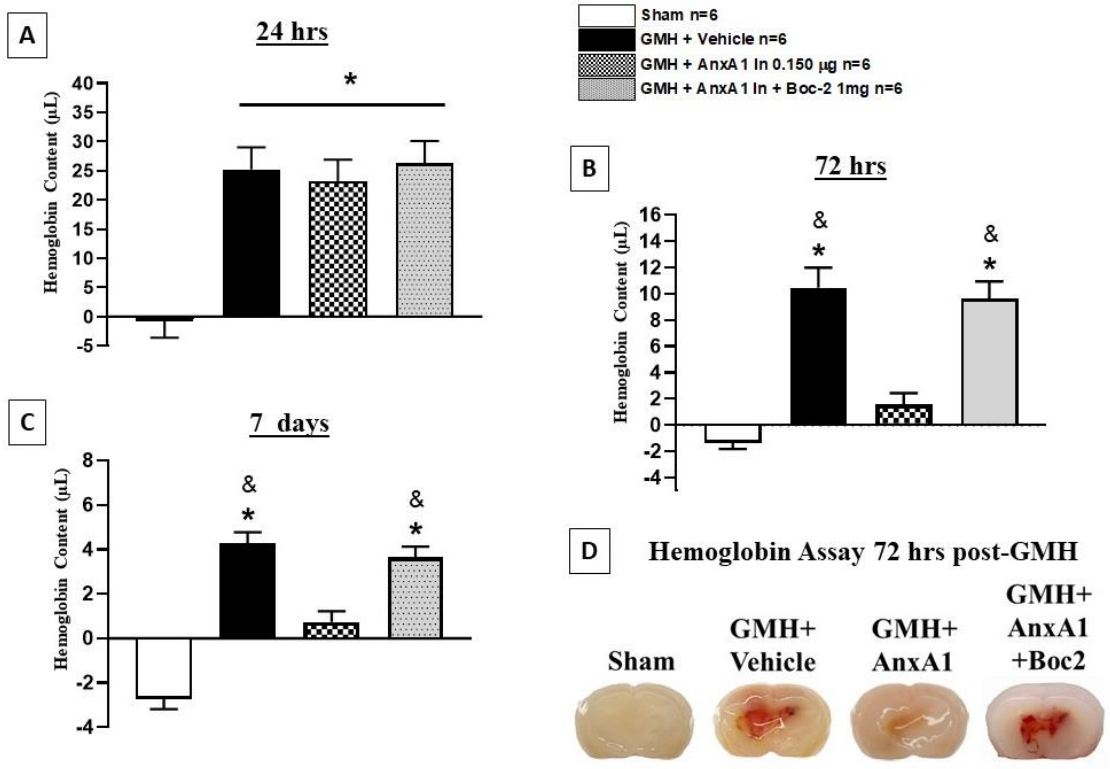

Figure 2.6. Hemoglobin Assay A) 24 hours, B) 72 Hours, and C) 7 days after GMH. D) representative picture of the progression of hematoma resolution at 72 hours. AnXA1 significantly reduced hematoma volume at 72 hrs (B and D) and 7 days (C) after GMH, yet boc-2 reversed these effects. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * P<0.05 vs. sham. & P<0.05 vs. AnxA1.

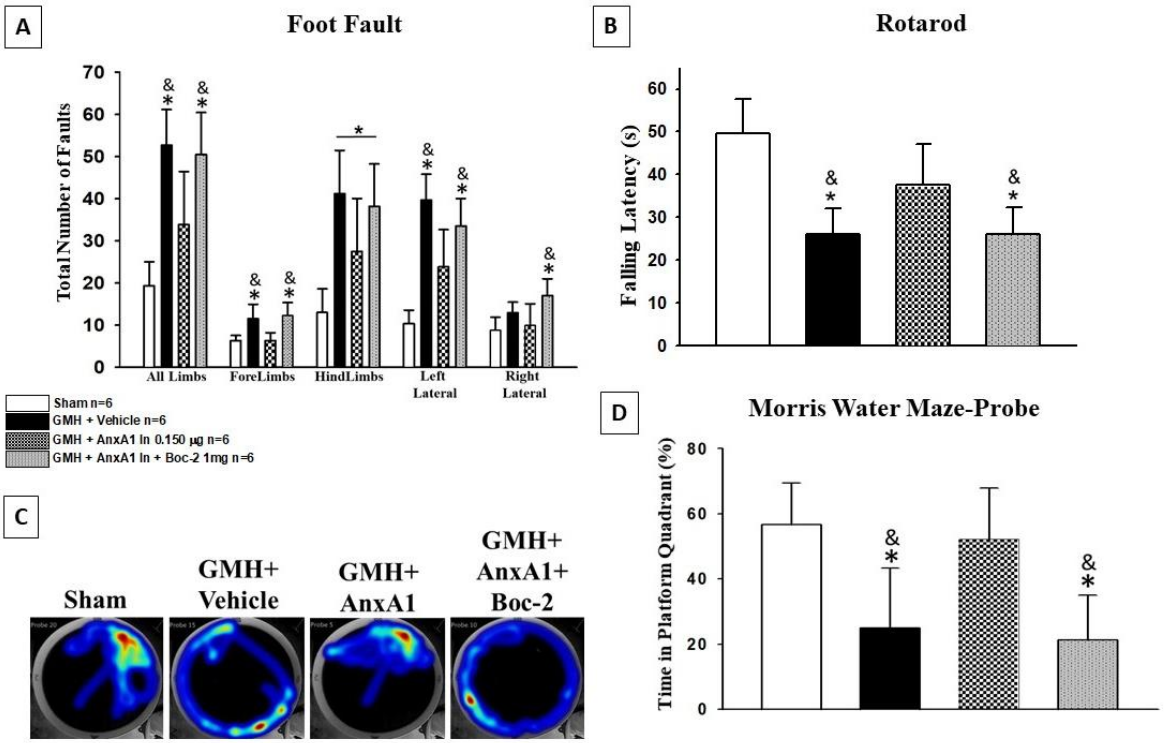

Figure 2.7: Annexin A1 improved locomotor, motor coordination, and memory at 4 weeks post-GMH, Boc-2 attenuated these effects. A) Annexin A1 Treatment improved locomotor coordination 4 weeks post-ictus, C) treated animals significantly improved motor coordination, and C-D) treated animal groups significantly improved in reference memory. FPR2 inhibitor reversed these effects. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * P<0.05 vs. sham. & P<0.05 vs. AnxA1.

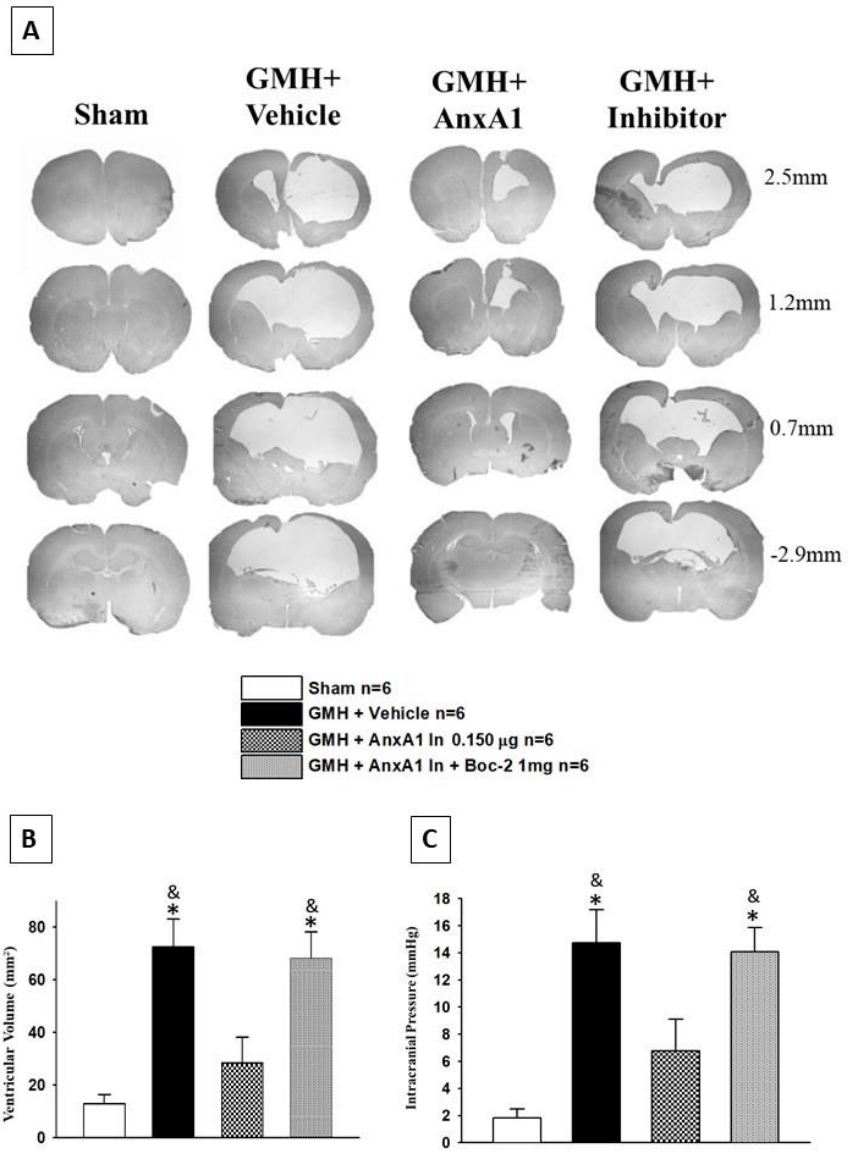

Figure 2.8. Annexin A1 treatment reduced ventricular dilation and intracranial pressure at 4 weeks after GMH. A and B) AnXA1 treated groups decreased ventricular dilation. Yet, FPR2 inhibitor Boc-2 reversed these effects. C) Annexin A1 significantly reduced CSF pressure when compared to both vehicle groups and Boc-2+AnxA1 group. n=8. Mean±SEM; one-way ANOVA followed by Tukey's test. * $P<0.05$ vs. sham. & $P<0.05$ vs. AnxA1. mmHG, millimeters of Mercury. Note: measurement on the right side of the representative pictures indicates the location of the brain section from bregma.

3.4 AnxA1 enhanced M2 microglia/macrophage cells 72 hours after GMH

CD206 (M2 marker) or TNF-Alpha was colocalized with CD11b to determine the amount of M1 or M2 microglia/macrophage cells at the site of perihematomal and were quantified as described in (Flores, Klebe et al. 2016). At 72 hours after GMH, Mannose receptor positive microglia/macrophage cells were significantly increased in treated animals when compared among all groups (Figure 2.9 A and B). Furthermore, AnxA1 treatment significantly decreased TNF-α positive microglia/macrophage cells when compared to the vehicle and FPR2 inhibitor group (Figure 2.10A and B). This data demonstrates that AnxA1 treatment significantly increased M2 populations and decreased M1 microglia populations.

To determine if the actions of FPR2 agonism were mediated through microglia cells, animals were given i.c.v injection of liposomal clodronate 2 days prior to GMH induction. WB was used at 24 hours to examine the expression of Iba1 in liposomal clodronate animals and hemoglobin assay was conducted at 72 hours after GMH. At 24 hrs after GMH, Iba1 expression was significantly decreased when compared to the sham (Figure 2.11A). At 24 hrs, immunohistochemistry demonstrated that expression was less readily expressed on microglia cells in GMH animals that received liposomal clodronate (Figure 2.11B). At 72 hours after GMH, hemoglobin assay established that AnxA1 treatment was unsuccessful in reducing the blood clot when compared to sham, and there was no significant difference when compared to the Vehicle control (Figure 2.11C). This data suggests that FPR2 agonist mediates its hematoma resolution actions through microglia cells.

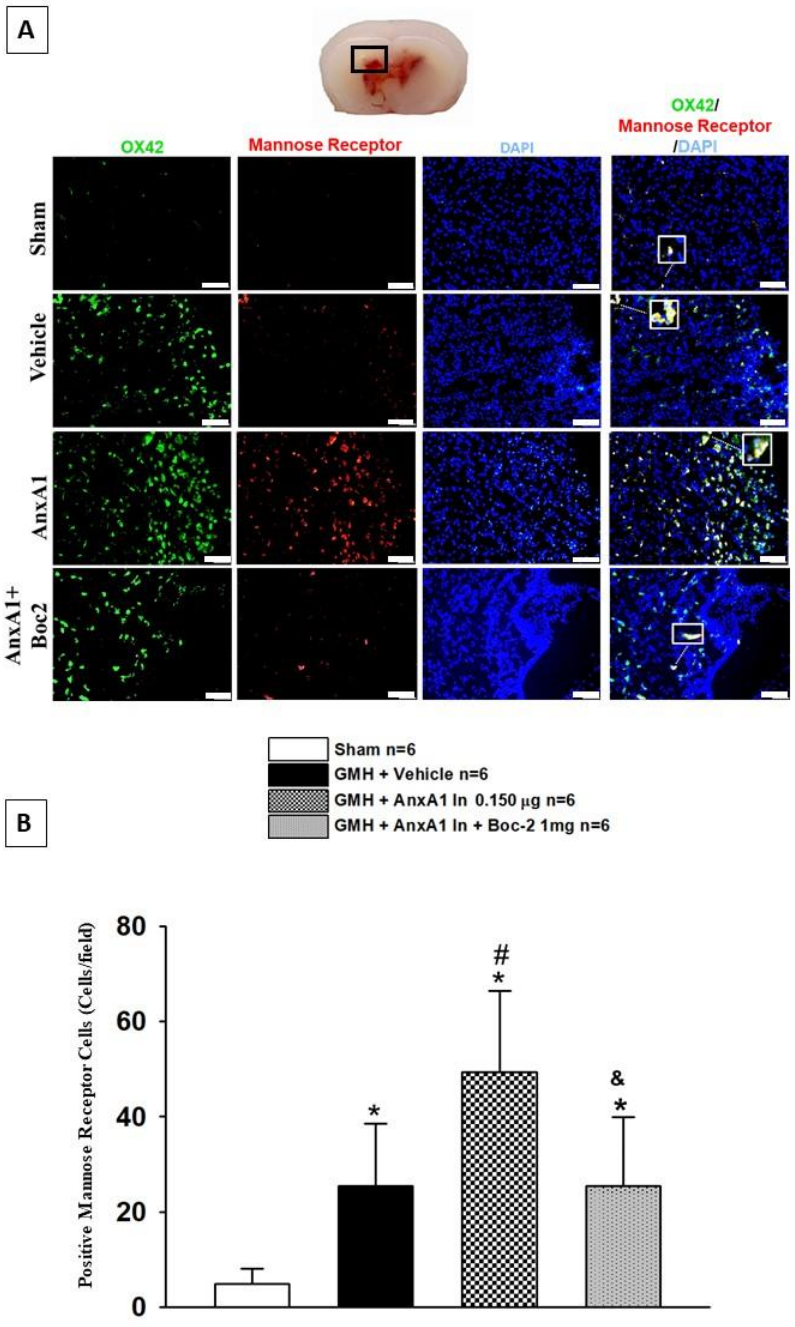

Figure 2.9. Annexin A1 treatment significantly increased M2 cells. Immunohistochemistry was performed to detect activated microglia (OX42;Green) and Mannose Receptor (CD206;Red) in all animal groups at 72 hours after GMH induction. Annexin A1 increased M2 microglia cells at the site of perihematomal, and Boc-2 reversed these effects (Fig. 8A). Additionally, M1 microglia expression was reduced in the treated group (Fig. 8B). Scale bar = 20µm. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * $P<0.05$ vs. sham. & $P<0.05$ vs. AnxA1.

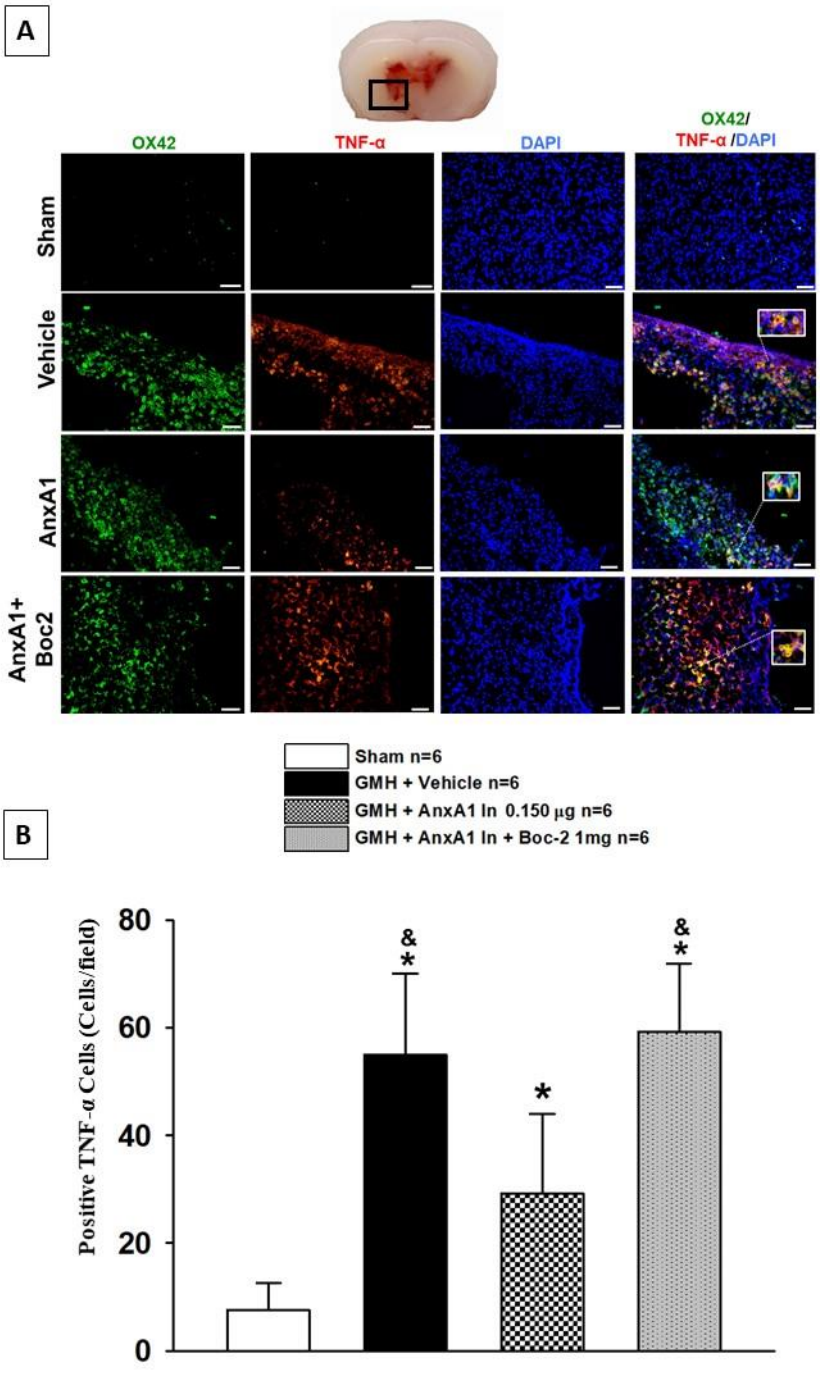

Figure 2.10. Annexin A1 treatment significantly decreased M1 cells after GMH. Immunohistochemistry was performed to detect activated microglia (OX42;Green) and M1 Marker (TNF-α;Red) in all animal groups at 72 hours after GMH induction. Annexin A1 significantly decreased M1 microglia (TNF-α;Red) expression and Boc-2 reversed these effects (Fig. 8A). Scale bar = 20µm. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * P<0.05 vs. sham. & P<0.05 vs. AnxA1.

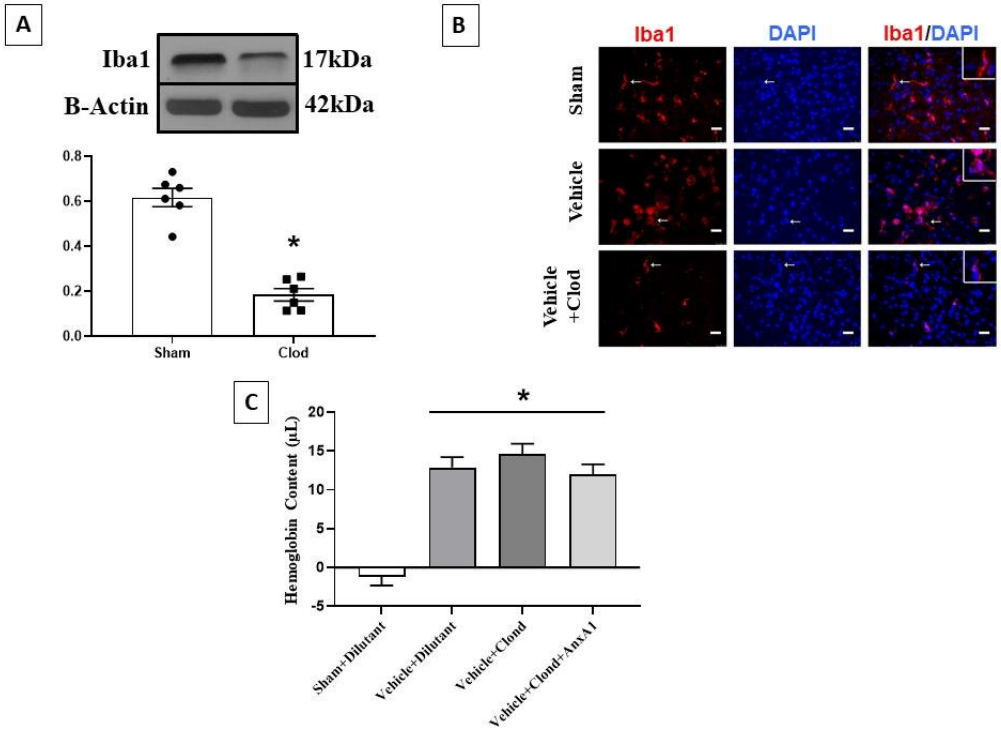

Figure 2.11. Inhibition of microglia cells ameliorates AnxA1 treatment effects on hematoma resolution. A) Western Blot at 24 hours confirmed the inhibition of microglia in the neonatal CNS. B) IHC demonstrated the decreased presence of microglia cells (red) 24 hours after GMH. C) Hemoglobin assay at 72 hours demonstrated AnxA1 treatment had no effect on hematoma content. n=6, Mean±SEM; t-test, one-way ANOVA followed by Tukey's test. * $P<0.05$ vs. sham.

3.5 FPR2 agonism, via AnxA1, activated p-ERK(1/2)/DUSP1/CD36 signaling pathway

FPR2 has been shown to activate p-ERK(1/2), which plays a role in the activation of DUSP1 (Ramon, Bancos et al. 2014) (Finelli, Murphy et al. 2013). FPR2 and DUSP1 have been shown to play a role in phagocytosis and activation of scavenger receptor CD36 (Cattaneo, Parisi et al. 2013, Ramon, Bancos et al. 2014) . Thus, we evaluated the effects of AnxA1 on FPR2, ERK (1/2), DUPS1, and CD36 protein expression at 72 hrs after GMH. AnxA1 treatment significantly increased the expression of FPR2, p-ERK (1/2), DUSP1, and CD36 when compared to the sham and the Vehicle Group (Figure 2.12 A, B, C, D, E respectively). Whereas FPR2 inhibitor attenuated the up regulation of these proteins (Figure 2.12).

To further investigate the signaling pathway mechanism, CRISPR targeting FPR2 was used to determine if the signaling pathway would be affected after AnxA1 treatment. Animals were randomly assigned as detailed in the experimental design of Experiment 5. FPR2 CRISPR significantly decreased the expression of FPR2, p-ERK (1/2), DUSP1, and CD36 when compared to the control group that received AnxA1 72 hours after GMH (Figure 2.13). Additionally, WB was conducted to determine if FPR2 CRISPR would inhibit the neuroprotective effects of AnxA1 on FPR2 agonism at 72 hours post-GMH. At 72 hours after GMH, FPR2 CRISPR significantly inhibited the AnxA1 induced hematoma resolution (Figure 2.13).

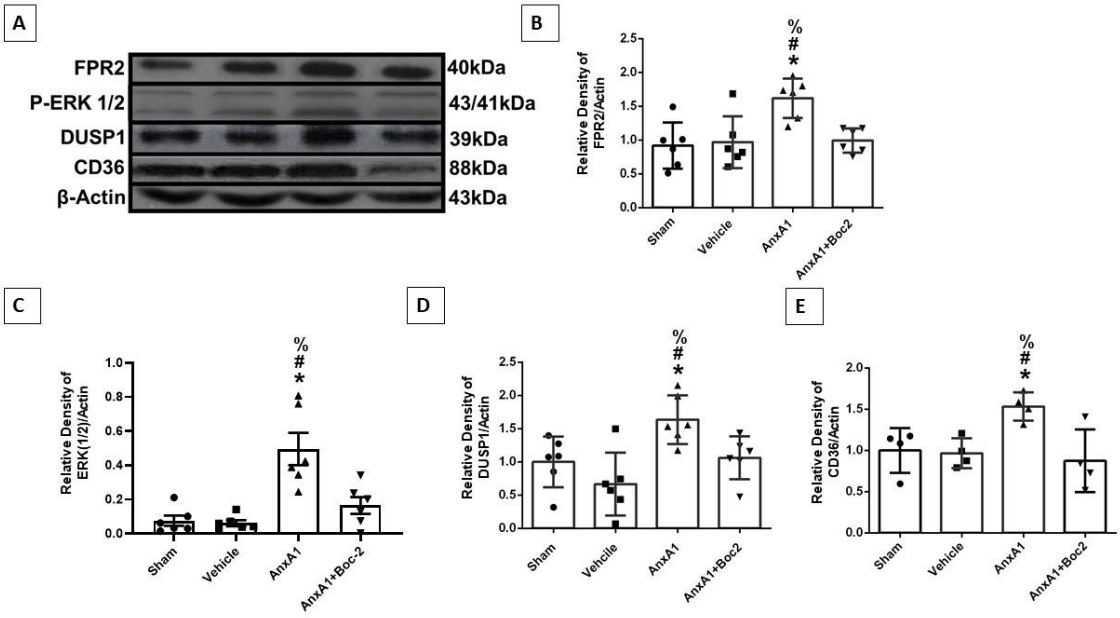

Figure 2.12. Annexin A1 treatment increased the expression of FPR2, p-ERK1/2, DUSP1, and CD36 72 hrs post-GMH. Expression of FPR2, p-ERK1/2, DUSP1, and CD36 were measured 72 hours post-ictus (Fig. 10A). FPR2 (B), p-ERK1/2 (C), DUSP1 (DC), and CD36 (E) expression was shown to be significantly induced in the treated group compared to vehicle. n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * P<0.05 vs. sham. & P<0.05 vs. AnxA1, % P<0.05 AnxA1+Boc-2.

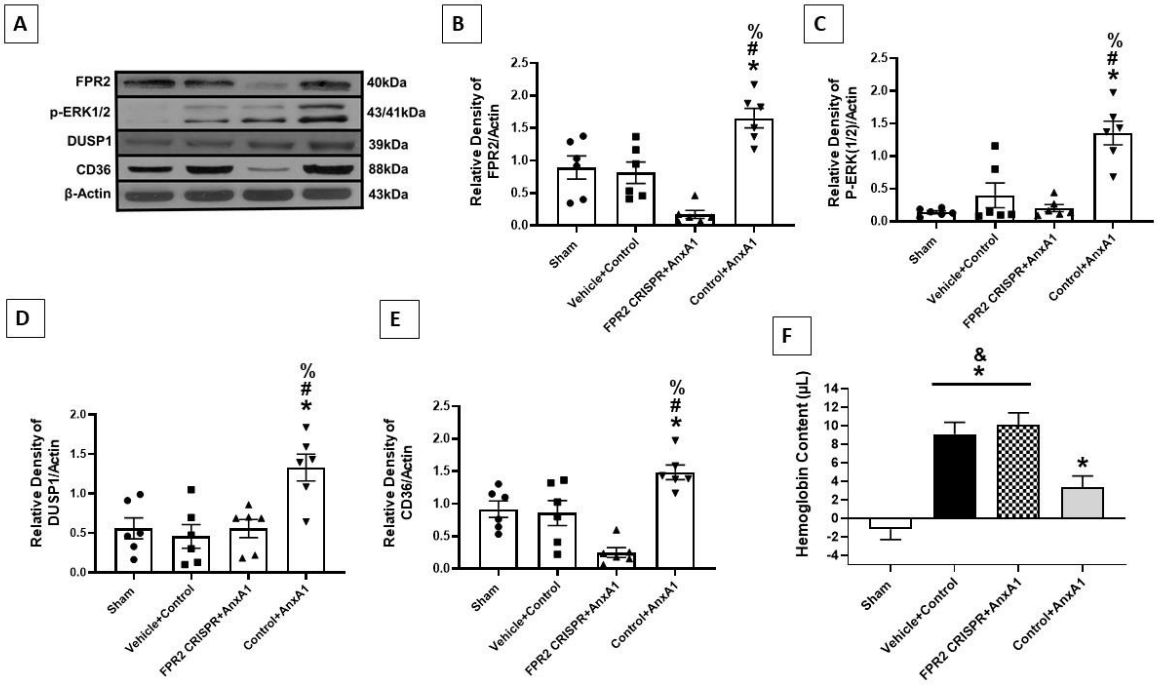

Figure 2.13. FPR2 CRISPR inhibited the upregulation of FPR2, p-ERK1/2, DUSP1, and CD36 after AnxA1 treatment at 72 hrs post-GMH. Expression of FPR2, p-ERK1/2, DUSP1, and CD36 were measured 72 hours post-ictus (A). FPR2 (B), p-ERK1/2 (C), DUSP1 (D), and CD36 (E) expression was shown to be significantly decreased in the FPR2 CRISPR with treatment when compared to the AnxA1 control group. Hemoglobin assay was done at 72 hours after GMH. FPR2 CRISPR inhibited the AnxA1 hematoma resolving effects when compared to the control treatment group (E). n=6. Mean±SEM; one-way ANOVA followed by Tukey's test. * P<0.05 vs. sham. & P<0.05 vs. AnxA1, % P<0.05 AnxA1+Boc-2.

4) Discussion

GMH is one of the leading causes of mortality and morbidity in premature infants; debilitating consequences of GMH include the formation of post-hemorrhagic hydrocephalus, cerebral palsy, and motor and cognitive deficits (Ballabh 2010, Heron, Sutton et al. 2010, Koschnitzky, Keep et al. 2018). Intracerebroventricular blood clots have been identified as causative factors of hydrocephalus formation, as the blood clots directly impairs the circulation and absorption of cerebrospinal fluid (CSF) (Whitelaw, Cherian et al. 2004, Stein, Luecke et al. 2010, Li, Ding et al. 2018). The enhancement of hematoma resolution neuroprotective in adult and neonatal hemorrhagic stroke (Zhao, Sun et al. 2015, Flores, Klebe et al. 2016, Liu, Flores et al. 2021). Our research group previously demonstrated that stimulation of signaling pathways that upregulate scavenger receptors CD36 and CD163 in microglia/macrophages lead to the rapid hematoma resolution after Germinal Matrix Hemorrhage (Flores, Klebe et al. 2016, Liu, Flores et al. 2021). In this study, we examined the effects of FPR2 stimulation, via AnxA1, on hematoma resolution as well as its effects on short- and long-term outcomes after germinal matrix hemorrhage. Additionally, we determined if FPR2 inhibition reversed the observed beneficial effects of FPR2 agonism. This is the first study to investigate a completely new mechanism of action for FPR2 in hemorrhagic stroke for the enhancement of hematoma resolution and first to evaluate the therapeutic potential for attenuating post-hemorrhagic hydrocephalus and neurological deficits in the short- and long-term.

N-formyl peptide receptors (FPR) belong to a family of G-protein-coupled receptors expressed on microglia in the central nervous system. Stimulation of the n-

formyl peptide receptor 2 (FPR2) by Annexin A1 (AnxA1) has been shown to be neuroprotective in models of stroke (McArthur, Cristante et al. 2010, Bena, Brancaleone et al. 2012). Although FPR2 has been previously studied in adult stroke, it is unclear if FPR2 is readily expressed in the neonatal CNS. Our data suggest that after GMH, FPR2 expression steadily increased after GMH and reached significance from sham at 72 hours and remained elevated for up to 7 days, indicating that FPR2 may play a role after GMH (Figure 2.2C). Immunohistochemistry confirmed that FPR2 was detected on microglia cells, indicating that FPR2 may play a beneficial role in the modulation of microglia cells (Figure2.3).

AnxA1 is a Ca2+-dependent phospholipid-binding protein and an endogenous activator of FPR2 (Parente and Solito 2004, Gavins 2010). Our research group has previously demonstrated that FPR2 stimulation via AnxA1 improved neurological outcomes after adult hemorrhagic stroke (Ding, Flores et al. 2020). The role of FPR2 agonism via AnxA1 in hematoma resolution remains unknown in neonatal stroke. A time-course was conducted to determine if endogenous AnxA1 and FPR2 shared similar expression levels after stroke, our data indicates that both proteins reached significance at 72 hours after GMH (Figure 2.2B). This data indicated that both proteins play an active role in the GMH pathophysiology, thus we conducted a dose-response study to assess the best therapeutic dose and route of recombinant Annexin A1 administration, with specific interest in hematoma resolution, neurobehavioral outcomes, and post-hemorrhagic hydrocephalus. Our data demonstrates that the high dose of AnxA1 (0.150 µg/per rat pup) administered intranasally was most beneficial in increasing hematoma resolution (Figure 2.3) and attenuating neurobehavioral deficits (Figure 2.3) at 72 hours after GMH

induction. Pharmacological inhibition of FPR2 was adapted from our previous study (Ding, Flores et al. 2020), where FPR2 antagonist Boc2 ameliorated the beneficial effects of AnxA1 on hematoma resolution (Figure. 2.6) and improvements in neurobehavioral function at 72 hours (Figure 2.5). Furthermore, in our long-term study we found that therapeutic regiments of intranasal AnxA1 resulted in improved cognitive and motor function at 4 weeks after GMH induction (Figure 2.7). In agreement with this long-term data, AnxA1 significantly reduced intracranial pressure (Figure 2.8C) and reduced ventricular dilation (Figure 2.8A and B) brought on by GMH at 4 weeks post-ictus. In contrast, Boc2 inhibition of FPR2 reversed the therapeutic effects of AnxA1 treatment in all long-term outcomes.

FPR2 activation has also been found to polarize microglia into the M2 phenotype (Li, Cai et al. 2011), primarily responsible for mediating wound healing and enhancing macrophage phagocytosis of blood clots in GMH (Flores, Klebe et al. 2016). These findings suggest that FPR2 may play a significant role in immunomodulation that provides protection against multiple maladies. M2 phenotype has been shown to be vital in the repair mechanisms after stroke and plays a key role in removing neurotoxic iron from the injury inflicted area, providing further neuroprotection (Flores, Klebe et al. 2016, Klebe, Flores et al. 2017). Yet M2's role in germinal matrix hemorrhage has been greatly understudied, further characterization of microglia subtypes and function needs to be conducted in the neonatal CNS. Our research group was the first to demonstrate that M2 polarization after GMH resulted in the enhancement of hematoma blood clot clearance and demonstrated that the activation of various signaling pathways that stimulated this microglia state had beneficial effects after GMH (Flores, Klebe et al.

2016, Liu, Flores et al. 2021). FPR2 stimulated, via AnxA1, significantly increased the number of M2 positive microglia cells at the site of peri-hematoma (Figure 2.9). Additionally, AnxA1 treatments significantly reduced the population of M1 positive microglia cells at the site of peri-hematoma (Figure 2.10). The introduction of FPR2 inhibitor Boc2 reversed the polarization of microglia cells. To establish that FPR2 therapeutic effects were executed through the stimulation of microglia cells, animals were given i.c.v injections of liposomal clodronate to globally inhibit microglia cells in the CNS. Western blot demonstrated a decreased expression of microglia marker Iba1 at 24 hours after GMH (Figure 2.11A). Furthermore, immunohistochemistry conducted at 24 hours after GMH indicated the inhibition of microglia cells in the CNS as there was low expression of microglia cells after GMH (Figure 2.11B). Lastly, Hemoglobin assay at 72 hours demonstrated that the inhibition of microglia cells resulted in the attenuation of AnxA1's therapeutic effect on the hematoma blood clot, where no significance was found in hematoma content between the Vehicle control, Vehicle+liposomal clodronate, and liposomal clodronate+AnxA1 groups (Figure 2.11b). This data indicates the link between hematoma resolution, microglia, and FPR2 agonism.

FPR2 signaling has been associated with the activation of ERK1/2, which in turn promotes the transcription of the DUSP1 gene (Arthur and Ley 2013, Finelli, Murphy et al. 2013). Current literature suggests that DUSP1 may play a role in CD36 signaling (Wancket, Meng et al. 2012). Interestingly, FPR2 stimulation has been shown to mediate CD36 scavenger and has also been reported to transform microglia into phagocytes (Cattaneo, Parisi et al. 2013, Ramon, Bancos et al. 2014). CD36 play an important role in mediating phagocytosis and transfection of CD36 on cells lacking phagocytic abilities

acquired this function (Ren, Silverstein et al. 1995). Thus, it is deducible that ERK/DUSP1/CD36 pathway is a potential mechanism by which FPR2 stimulation increases hematoma absorption. In this study we examined the underlying phagocytic mechanism of FPR2. We first co-treated the neonatal pups with FPR2 pharmacological antagonist Boc-2 and agonist AnxA1to determine if AnxA1 treatment elicits its neuroprotective effects through the agonism of the proposed FPR2 signaling pathway. At 72 hours, WB data showed that AnxA1 treatment significantly increased the expression of FPR2, p-ERK(1/2), DUSP1, and CD36 when compared to all other groups (Figure A-E). Whereas introduction of Boc-2 significantly reduced the expression of all proteins. This suggests that AnxA1 neuroprotective effects are modulated through FPR2 stimulation and all signaling protiens. To further confirm these results, FPR2 CRISPR was administered to neonatal pups 48 hours prior to GMH modeling. Our data showed that the introduction of FPR2 CRISPR significantly reduced the expression of the protein levels of FPR2, ERK(1/2), DUSP1, and CD36 when compared to the CRISPR control group that received AnxA1 treatment (Figure 2.13 A-E). Lastly, to determine if the knock-down of FPR2 via CRISPR inhibited the effects of FPR2 on hematoma resolution, hemoglobin assay was conducted at 72 hours. FPR2 CRISPR significantly ameliorated the resolution of hematoma (Figure 2.13F). This data illustrates that the neuroprotective effects of FPR2 agonism are mediated through the FPR2/ERK(1/2)/DUSP1/CD36 signaling pathway.

Although our data demonstrates the actions and signaling pathway of FPR2 agonism, there are limitations to this study. First, this study does not investigate the microglia subtypes involved in FPR2 agonism. More specifically, M2 microglia are

divided into three subtypes- M2a, M2b, M2c. Where both M2b and M2c participate in phagocytosis and removal of tissue debris, while M2a plays a role in cell regeneration (Roszer 2015, Lan, Han et al. 2017). Further characterization of M2 subtypes and function needs to be conducted. Second, this study briefly investigates the role of FPR2 in the formation of post-hemorrhagic hydrocephalus formation. Although intracranial pressures and ventricular volume were measured, FPR2 role in iron overload and CSF production was not, which are very important parameters of PHH. Third, only microglia cells were investigated in this study. FPR2 is located on various other monocytes as well as astrocytes. Further experiments must be conducted so that the function of FPR2 on other cell types can be established in GMH. Fourth, AnxA1-FPR2 might trigger multiple intracellular signaling pathways and the signaling network can complicate this study. Interestingly, C/EBP alpha transcription factor is also upregulated by ERK1/2 and has been known to activate CD36 transcription in the nucleus which induces CD36 receptor activation (Muto, Yachi et al. 2013). Recently, C/EBP alpha has shown to be upregulated in the CNS, more specifically in rat primary cultured microglia cells (Ramberg, Tracy et al. 2011).

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CHAPTER THREE

1) DISCUSSION AND CONCLUSION

Germinal matrix hemorrhage (GMH) is defined as the rupture of immature blood vessels within the subependymal (or periventricular) germinal matrix (Ballabh 2010). Occurring in approximately 3.5 per 1,000 births, GMH presents a leading cause of mortality and morbidity in premature infants (Heron, Sutton et al. 2010, Koschnitzky, Keep et al. 2018). Debilitating consequences of GMH include the formation of post-hemorrhagic hydrocephalus (Ballabh 2010, Heron, Sutton et al. 2010). The expansion of the cerebroventricular system leads to mechanical compression and consequent injury of the surrounding brain tissue, causing neurological deficits in patients that survive the initial bleed. Intracerebroventricular blood clots have been identified as causative factors of hydrocephalus formation. Blood clots directly impair the circulation and absorption of cerebrospinal fluid (CSF) (Whitelaw, Cherian et al. 2004, Stein, Luecke et al. 2010, Li, Ding et al. 2018). After hemorrhage, erythrocyte lysis releases hemoglobin and iron into the surrounding tissue. Iron is released from metabolized hemoglobin, consequently causing iron overload. Iron overload has been associated with CSF overproduction and consequent post-hemorrhagic hydrocephalus development (Klebe, Krafft et al. 2014). Current clinical management of hydrocephalus profoundly relies on the surgical insertion of shunts that drain excess CSF from the ventricles into the peritoneum, where it can be absorbed by the vasculature (Woernle, Winkler et al. 2013). Unfortunately, this procedure can cause post-surgical complications, and shunts often become obstructed and must be replaced over time (Woernle, Winkler et al. 2013). Therefore, a safe and non-

invasive treatment for the reduction of blood clots early in the pathophysiology to reduce post-hemorrhagic hydrocephalus would be essential in the management of GMH.

Activated microglia have shown to recruit hematogenous phagocytes to the site of injury, which engulf the blood clot and damaged surrounding tissues (Zhao, Sun et al. 2007, Flores, Klebe et al. 2016). At 24 hours, M2 markers were found at the ischemic core, suggesting that microglia cells are alternatively activated and promote tissue repair (Perego, Fumagalli et al. 2011). However, microglia M2 polarization post-GMH has been understudied, but our research group has shown that this phenotype plays a significant role in phagocytosis in GMH (Flores, Klebe et al. 2016). M2 microglia can be categorized into three subtypes- M2a, M2b, M2c. Both M2b and M2c have been shown to play a role in phagocytosis and removal of tissue debris, while M2a plays a role in cell regeneration (Roszer 2015, Lan, Han et al. 2017). Further characterization of M2 subtypes and function needs to be conducted. Recent studies in GMH have shown therapeutics that shift microglia/macrophages into the M2 phenotype, enhancing phagocytic blood clot clearance, attenuating short and long-term neurological deficits, and reduce PHH (Flores, Klebe et al. 2016, Zhang, Ding et al. 2018, Zhang, Xu et al. 2018). Furthermore, M2 microglia play a large role in regulating iron as the cell type can contain large intracellular labile iron pools, which effectively take up and spontaneously release iron at low concentrations away from the site of injury to avoid iron overload, which plays a major role in CSF production and hydrocephalus formation (Corna, Campana et al. 2010).

Currently, many of the studies conducted on phagocytic hematoma clearance are based on adult hemorrhagic stroke models. Previous work in intracerebral hemorrhage

(ICH) has shown that CD36 scavenger receptor plays a major role in the phagocytosis of blood clots and its upregulation was shown to be neuroprotective (Zhao, Sun et al. 2007, Zhao, Gonzales et al. 2015). CD36 modulation has been connected to various pathways such as peroxisome proliferator-activated receptor gamma (PPAR-γ) and nuclear factor-erythroid 2 p45-related factor. Such pathways have been shown to mediate microglia/macrophage polarization to the M2 phenotype, which is responsible for microglia phagocytic actions (Lan, Han et al. 2017). However, M2's role in germinal matrix hemorrhage has been greatly understudied, further characterization of microglia subtypes and function needs to be conducted in the neonatal CNS. Recently our research group demonstrated that PPAR-γ receptor activation upregulated CD36, which resulted in an increase in M2 positive cells and enhanced hematoma resolution. Currently, our proposal studied a novel pathway to induce phagocytic blood clot clearance via the FPR2 pathway. FPR2 has only been recently studied in the CNS and was shown to be neuroprotective through anti-inflammatory mechanisms in adult stroke models (Vital, Becker et al. 2016, Ding, Flores et al. 2019). Yet, FPR's role in the phagocytosis of hematoma has never been investigated. Here we investigated a complete mechanism which has not been fully evaluated. The majority of the investigated mechanisms of FPR2 have focused on the inhibition of prothrombotic activity and MAPK/P38 signaling pathway after stroke (Guo, Hu et al. 2016, Vital, Becker et al. 2016, Senchenkova, Ansari et al. 2019, Ding, Flores et al. 2020). Whereas, here we focused on FPR2 upregulation of ERK(1/2)/DUSP1/CD36 signaling pathway. Although FPR2 and DUSP1 have been associated with the upregulate CD36, the interactions of these proteins and signaling

pathway have not been connected nor evaluated (Wancket, Meng et al. 2012, Cattaneo, Parisi et al. 2013).

FPR2 agonist Annexin A1 has been shown to have therapeutic effects in preclinical models of stroke (Gavins, Dalli et al. 2007). It has been demonstrated that endogenous AnxA1 is commonly expressed on glial cells in the CNS of adult human and rodent brains, more specifically microglia/macrophages (McArthur, Cristante et al. 2010). Previous studies have found that endogenous AnxA1 was increased in conjugation with an influx of neutrophils at the site of injury (D'Amico, Di Filippo et al. 2000). Specifically, AnxA1 is released from neutrophil cytosolic granules to the cell surface, where it interacts with FPR's in a autocrine/paracrine fashion (Gavins 2010). Because AnxA1 stimulation of FPR2 may act on various other cell types, we globally inhibited microglia cells with liposomal clodronate to determine if the actions of FPR2 on hematoma resolutions was elicited by microglia cells. Various publications have demonstrated the protective anti-inflammatory characteristics of AnxA1 treatment in adult stroke models (Thygesen, Larsen et al. 2019, Ding, Flores et al. 2020). Furthermore, AnxA1 has been attributed to play a key role in the microglia phagocytosis of various molecules (McArthur, Cristante et al. 2010, Purvis, Solito et al. 2019). For these very reasons, AnxA1 was used to assess the neuroprotective effects of FPR2 on hematoma resolution. Thus, it is clinically relevant to use AnxA1 as a therapeutic treatment to upregulate the neuroprotective effects of FPR2 as we would be targeting an endogenous mechanism.

To investigate the actions FPR2 agonism on hematoma resolution after hemorrhagic stroke, a neonatal rat model of GMH was used. The primary findings of our

study are as follows: 1) endogenous AnxA1 and FPR2 was significantly upregulated at 72 hours post-GMH and remained elevated for up to 7 days and was found to be expressed on microglia cells. 2) FPR2 stimulation via AnxA1 significantly decreased hematoma content and improved neurobehavior in at 72 hours post-GMH, whereas FPR2 inhibition via Boc2 reversed these effects. 3) AnxA1 treatment significantly improved cognitive and motor coordination, reduced intracranial pressure, and reduced ventricular dilation after GMH at 4 weeks after GMH. These beneficial effects were reversed by Boc2. 4) AnxA1 treatment significantly increased M2 microglia cells and decreased M1 microglia cells at the site of perihematoma.5) Global inhibition of microglia cells attenuated the therapeutic effects of AnxA1 on the blood clot. 6) FPR2 agonism via AnxA1 significantly increased the expression of FPR2, p-ERK(1/2), DUSP1, and CD36 at 72 hours after GMH. 7) FPR2 CRISPR significantly inhibited the protein expression of all signaling proteins at 72 hours post-ictus.

2) Potential of FPR2 used as a Therapeutic Target

Currently, there are no clinical trials that target the blood clot after GMH, yet clinical trials in adults hemorrhagic stroke give us insight into the potential approaches of directly removing the blood clot. So far clinical trials in adult hemorrhagic stroke showed no beneficial effect in the direct removal of the blood clot. As a result, combinational treatments which involve the direct removal and the delivery of therapeutics such as anti-inflammatory agents that target secondary brain injury have been executed in adult hemorrhagic stroke trials. This suggests that an endogenous mechanism that targets the removal of the blood clot and promotes wound healing may be more beneficial then solely removing the blood clot (Sembill, Huttner et al. 2018). Our data from this

investigation elucidated a novel endogenous mechanism that directly resolves the primary causative factor of secondary brain injury after GMH. Here we provide a complete mechanism of action of FPR2 stimulation after GMH. FPR2 stimulation resulted in quick removal of the blood clot after GMH, which improved outcomes in behavior and brain morphology. Additionally, FPR2 agonism increased M2 microglia cells suggesting that the actions of FPR2 on hematoma resolution is mediated through microglia phagocytosis. Our findings provide pertinent information on viable therapeutic target to not only attenuate long-term PHH development after GMH, but also for a therapeutic approach for adult hemorrhagic stroke.

3) Summary/conclusion

Great progress is currently being made in elucidating mechanisms of hematoma resolution in germinal matrix hemorrhage. Here we identified a novel role for FPR2 in neonatal germinal matrix hemorrhage pathophysiology. We found that FPR2 played a significant role in enhancing hematoma resolution, thus improving overall outcomes in the short- and long-term. Additionally, FPR2 actions are mediated through the polarization of microglia into the M2 phenotype, which play a primary role in the phagocytosis of red blood cells. We then investigated a novel signaling pathway, where FPR2 agonism resulted in the upregulation of FPR2, p-ERK(1/2), DUSP1, and CD36. Lastly, pharmacological and gene knock-down of FPR2 resulted in the decreased expression of this signaling pathway. Although we are shedding light on a new mechanism of action, further studies need to be conducted to investigate the overall actions of FPR2 such as the role that this receptor has on microglia subtypes, cell types such as neutrophils, etc after GMH.

4) *Future Directions*

Our Current study is limited in that it primarily focused on hematoma resolution, microglia cells, short- and long-term outcomes, and one signaling pathway after FPR2 agonism. Although both male and female rodents were used for this study, further investigation needs to be conducted to investigate hormone differences between male and females and how they may affect the pathophysiology of GMH and treatment mechanisms. In our current study we treated only at the 1 hr time point after GMH, thus delayed treatment regimens need to be assessed to investigate the time-window of AnxA1 treatment. Additionally, other FPR2 agonist need to be assessed to compare if they stimulate the same mechanisms of action that AnxA1 acts on. Lastly, to investigate the role of FPR2 on other cell types such as astrocytes and neutrophils that also have the capacity to transform into phagocytes.

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APPENDIX A

PPARγ-INDUCED UPREGULATION OF CD36 ENHANCES HEMATOMA RESOLUTION AND ATTENUATES LONG-TERM NEUROLOGICAL DEFICITS AFTER GERMINAL MATRIX HEMORRHAGE IN NEONATAL RATS

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Preface:

The Primary Objective of the Dissertation was on FPR2's role on the upregulation of hematoma resolving mechanisms, more specifically on the FPR2/p-ERK(1/2)/DUSP1/CD36 signaling pathway. Current GMH manuscripts which have been published by our research group were the foundation of the direction of this proposal. Although this manuscript does not investigate the actions of FPR2 on hematoma resolution, it was the first to investigate CD36 and microglia polarization in the GMH pathophysiology.

Abstract

Germinal matrix hemorrhage remains the leading cause of morbidity and mortality in preterm infants in the United States with little progress made in its clinical management. Survivors are often afflicted with long-term neurological sequelae, including cerebral palsy, mental retardation, hydrocephalus, and psychiatric disorders. Blood clots disrupting normal cerebrospinal fluid circulation and absorption after germinal matrix hemorrhage are thought to be important contributors towards post-hemorrhagic hydrocephalus development. We evaluated if upregulating CD36 scavenger receptor expression in microglia and macrophages through PPARγ stimulation, which was effective in experimental adult cerebral hemorrhage models and is being evaluated clinically, will enhance hematoma resolution and ameliorate long-term brain sequelae using a neonatal rat germinal matrix hemorrhage model. PPARγ stimulation (15d-PGJ_2) increased short-term PPARγ and CD36 expression levels as well as enhanced hematoma resolution, which was reversed by a PPARγ antagonist (GW9662) and CD36 siRNA. PPARγ stimulation (15d-PGJ_2) also reduced long-term white matter loss and post-hemorrhagic ventricular dilation as well as improved neurofunctional outcomes, which were reversed by a PPARγ antagonist (GW9662). PPARγ-induced upregulation of CD36 in macrophages and microglia is, therefore, critical for enhancing hematoma resolution and ameliorating long-term brain sequelae.

1) Introduction

The ganglionic eminence consists of neuronal and glial precursor cells located at the head of the caudate nucleus below the lateral ventricles of the developing fetus, and the highly vascularized region within the subependymal tissue is the germinal matrix. Cerebral blood flow fluctuation associated with hemodynamic and respiratory instability in preterm infants in conjunction with the inherent fragility of the germinal matrix often leads to germinal matrix hemorrhage, a very common and major neurological complication of prematurity. Germinal Matrix Hemorrhage (GMH) occurs when immature blood vessels rupture within the subependymal (or periventricular) germinal region of the ganglionic eminence in the immature brain (Ballabh 2010). In the United States alone, GMH occurs in approximately 12,000 lives births per year, and the number of moderate-to-severe GMH cases has remained steady over the past two decades (Fanaroff, Stoll et al. 2007; Jain, Kruse et al. 2009; Osterman, Kochanek et al. 2015). Clinical studies indicate GMH afflicted infants often suffer from long-term neurological deficits, cerebral palsy, mental retardation, hydrocephalus, and psychiatric disorders (Kadri, Mawla et al. 2006; Ballabh 2014). Prenatal glucocorticoid treatment remains the best treatment for preventing GMH, yet minimal advancements have been made in GMH clinical management post-ictus (Shankaran, Bauer et al. 1995; Roberts and Dalziel 2006).

Hemodynamic and respiratory instability in preterm infants results in fluctuations of cerebral blood flow in the inherently frail germinal matrix vasculature, often resulting in spontaneous bleeding (Ballabh 2014). The consequent hematoma applies mechanical pressure to glia and neurons, resulting in cytotoxicity and necrosis, as well as evokes an inflammatory response, leading to secretion of destructive proteases and oxidative

species (Lekic, Klebe et al. 2015). In adult cerebral hemorrhage, clinical studies indicate hematoma volume is the best prognostic indicator; larger hematoma volumes have worsened outcomes (Keep, Xi et al. 2005; Xi, Keep et al. 2006). Experimental adult cerebral hemorrhage studies proved more rapid hematoma resolution is necessary for quickly ameliorating inflammation and improving neurological recovery (Zhao, Sun et al. 2007; Zhao, Grotta et al. 2009). Additionally, blood clots directly impair cerebrospinal fluid circulation and absorption after GMH, significantly contributing towards post-hemorrhagic hydrocephalus development (Cherian, Whitelaw et al. 2004; Aquilina, Chakkarapani et al. 2011). Therefore, we hypothesize enhancing hematoma resolution will improve GMH outcomes.

Microglia are resident macrophages of the central nervous system and are critical drivers of the neuro-inflammatory response after GMH and other hemorrhagic brain injuries (Aronowski and Zhao 2011; Tang, Chen et al. 2015). Activated microglia recruit hematogenous phagocytes to the injured site, which engulf the hematoma as well as damaged or dead tissue (Cox, Crossley et al. 1995; Aronowski and Hall 2005). The role microglia play in hemorrhagic brain injury pathogenesis is different in neonates than adults (Woo, Wang et al. 2012). Unlike the adult brain where microglia cells and macrophages contribute to brain injury after stroke through the production of inflammatory cytokines (Vexler and Yenari 2009), neonatal brains demonstrate the opposite as the depletion of these cells enhances injury by removing endogenous protective mechanisms (Faustino, Wang et al. 2011).

Scavenger receptor CD36, a trans-membrane glycoprotein, is involved in several biological functions, such as foam cell formation, immune cell chemotaxis, and

phagocytosis of apoptotic cells (Woo, Wang et al. 2012). CD36 receptor is reportedly located on the cell surface of several cell types, including monocytes, endothelial cells, and microglia. CD36 plays an important role in phagocytosis, and upregulating its expression beneficially enhances hematoma resolution (Zhao, Sun et al. 2007). Transfection of non-phagocytic cells with a CD36-expressing gene converted those cells into phagocytes (Ren, Silverstein et al. 1995). CD36 genetic deletion worsened injury after acute focal stroke in neonatal mice, partially by decreasing removal of apoptotic cells (Woo, Wang et al. 2012).

Peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear hormone receptor superfamily, plays a major role in the upregulating CD36 expression (Zhao, Sun et al. 2007; Zhao, Grotta et al. 2009). PPARγ stimulation exerts anti-inflammatory effects in several central nervous system injuries and disorders (Pereira, Hurtado et al. 2005; Landreth, Jiang et al. 2008). Many studies demonstrated PPARγ is neuroprotective in various experimental stroke models (Pereira, Hurtado et al. 2005; Zhao, Sun et al. 2007; Woo, Wang et al. 2012; Shao and Liu 2015). In adult intracerebral hemorrhage (ICH) experimental models, PPARγ activation was directly associated with upregulation of CD36 expression, leading to enhanced phagocytosis-mediated clearance of the hematoma as well as dead or damaged cells by microglia and macrophages. PPARγ stimulation reduced expression of pro-inflammatory mediators, ameliorated secondary brain injury, and improved functional recovery after ICH (Zhao, Sun et al. 2007). Currently, PPARγ stimulation for enhancing hematoma resolution and ameliorating secondary brain injury is being clinically tested in ICH patients (Gonzales,

Shah et al. 2013). Yet, no clinical trials are evaluating PPARγ stimulation in GMH patients.

In this study, we assess if PPARγ stimulation enhances CD36-mediated hematoma resolution in a neonatal rat germinal matrix hemorrhage model. We hypothesize PPARγ stimulation, using 15d-PGJ_2, will augment microglia/macrophage phagocytosis of blood clots, reducing post-hemorrhagic hydrocephalus, inflammation, behavioral dysfunction, and neuronal loss, which will be reversed by CD36 knockdown or PPARγ antagonist administration.

2) Materials and methods

2.1 Animals and Surgeries

All experimental procedures were conducted in accordance with the National Institutes of Health guidelines for the treatment of animals and were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Two hundred and sixty P7 Sprague-Dawley neonatal pups (Harlan, Indianapolis, IN) weighing 12-15 g (brain development is comparable to 30–32-week gestation humans) were used in this study. Germinal matrix hemorrhage was achieved by stereotactic-guided injection of bacterial collagenase, as previously described (Lekic, Manaenko et al. 2012). Pups were anesthetized with 3% isoflurane (delivered through medical grade oxygen and mixed air) while being stabilized onto a stereotaxic frame. Isopropyl alcohol followed by betadine was applied to the incision site. Incision was made on the longitudinal plane to expose the skull and reveal bregma. The stereotactic coordinates from bregma were as follows: 1.6 mm (rostral), 1.6 mm (right lateral), and 2.8 mm (depth) from the dura. A burr hole (1 mm) was drilled, into which a 27-gauge needle was inserted at a rate of 1 mm/min. 0.3

units of clostridial collagenase VII-S (Sigma Aldrich, MO) in 1 µL was infused over a period of 3 minutes with a Hamilton syringe guided by a microinfusion pump (Harvard Apparatus, Holliston, MA). After completing infusion, the needle is left into position for an additional 10 minutes after injection to prevent "back-leakage" and then removed at a rate of 1 mm/min. Once the Hamilton is removed, the burr hole is sealed with bone wax and the incision site sutured. Animals are then given buprenorphine and allowed to recover on a 37°C heated blanket. When fully recovered, pups are then placed back with the mother. Surgery time per animal is approximately 30 minutes. Sham animals were subject to needle insertion without collagenase infusion. The same procedures were performed for intraventricular injection of siRNA, except the stereotactic coordinates from bregma were as follows: 1.0 mm (rostral), 1.0 mm (left lateral), and 1.8 mm (depth) of the dura.

2.2 Animal Treatments and Experimental Groups

P7 rat pups were randomly divided into the following groups: sham-operated (n=38), Vehicle (n=38), GMH + 15d-PGJ_2 (n=38), GMH + 15d-PGJ_2 + GW9662 (n=38), GMH + CD36 siRNA (n=12), GMH + 15d-PGJ_2 + CD36 (n=12), GMH + scrambled siRNA (n=12). The PPARγ agonist (15d-PGJ_2, 0.1mg/kg; Sigma Aldrich), antagonist (GW9662, 4mg/kg; Sigma Aldrich) + agonist, and saline (for sham and vehicle groups) were administered intraperitoneally to experimental animals at 1 hour post-GMH and then once daily for 7 days. CD36 siRNA (1.2 ng, Cell Signaling & Santa Cruz), and scrambled siRNA (1.2 ng, Santa Cruz) were administered via intraventricular injection to experimental animals 24 hours prior to GMH induction. Buprenorphine (0.01 mg/kg) was administered subcutaneously to all groups after completing surgery.

2.3 Animal Perfusion and Tissue Extraction

Animals were euthanized using isoflurane (≥5%) followed by trans-cardiac perfusion with ice-cold phosphate buffered saline (PBS) for hemoglobin assay and Western blot samples or with ice-cold PBS followed by 10% formalin for histology samples. Forebrains for hemoglobin assay and Western blot were snap-frozen with liquid-nitrogen, then stored in -80°C freezer before protein extractions or spectrophotometric quantification. Histological brain samples were post-fixed in 10% Fomaldehyde for at least 3 days and then 30% sucrose for at least 3 days, all stored in 4°C fridge. Forebrains were next embedded in Optimal Cutting Temperature compound and stored in -20°C freezer.

2.4 Hemoglobin Assay

Spectrophotometric measurements were used to assess hemorrhagic volume using well-established protocols (Choudhri, Hoh et al. 1997; Tang, Liu et al. 2004; Lekic, Manaenko et al. 2011). Frozen extracted forebrains were placed into individual glass tubes containing 3 mL of PBS. The tissue was homogenized for 60 seconds (Tissue Miser Homogenizer; Fisher Scientific, Pittsburgh, PA) followed by ultrasonication for 1 minute to lyse erythrocyte membranes. The products were then centrifuged for 30 minutes and the supernatant was separated from the pellets. A 4:1 ratio of Drabkin's reagent (Sigma-Aldrich) and supernatant were combined, which were left to react for 15 minutes. Absorbance, using a spectrophotometer (540 nm; Genesis 10uv; Thermo Fisher Scientific, Waltham, MA), was calculated into a hemorrhagic volume (μL) on the basis of a standard curve as routinely performed (Lekic, Manaenko et al. 2011).

2.5 Intracranial Pressure (ICP) Measurements

At 28 days post-ictus, animals were anesthetized and mounted onto a stereotaxic frame, where the head was inclined downward at a 30-degree angle. A midline skin incision was made to expose the atlanto-occipital membrane. The cisterna magna was punctured with a 26G Hamilton needle, which was connected to a pressure transducer of a Digi-Med LPA 400-low pressure Anayzer (Micro-Med-Louisville, Kentucky, USA) as described (Lackner, Vahmjanin et al. 2013)

2.6 Western Blotting

Protein concentrations for immunoblot (Lekic, Manaenko et al. 2011) were determined by DC protein assay (Bio-Rad, Hercules, CA). 30 µg protein per sample were loaded into wells of 4-20% gels, ran for 30 minutes at 50V then 90 minutes at 125V, then transferred onto nitrocellulose membranes at 0.3A for 120 minutes (Bio-Rad). Membranes were incubated for 2 hours in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween20. Then the following primary antibodies were incubated overnight: anti-PPARy (1:1000; Santa Cruz, Dallas, Texas), CD36 (1:500, Santa Cruz, Dallas, Texas), and mannose receptor (CD206) (1:500, ABcam, Cambridge, Massachusetts). Secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) were then applied to the membranes and incubated for 2 hours and then processed with the ECL plus Kit (GE Healthcare and Life Science, Piscataway, NJ). β-actin was used as an internal control against the anti-body (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). ImageJ software (4.0, Media Cybernetics, Silver Spring, MD) was used to analyze the relative density of the resultant protein immunoblot images as described (Tang, Liu et al. 2004).

2.7 Histological Volumetric Analysis

10 μm thick coronal brain sections were cut every 600 μm using a cryostat (Leica Microsystems LM3050S) and were placed onto poly-L-lysine-coated slides. Brain slices were Nissl stained morphometrically analyzed using computer-assisted (ImageJ 4.0, Media Cybernetics, Silver Spring, MD) hand delineation of the ventricle system (lateral, third, cerebral aqueduct, and fourth), hemisphere (cortex, subcortex), caudate, thalamus, hippocampus, and corpus callosum (white matter) (Lekic, Manaenko et al. 2011). These structures were delineated using optical dissector principles from prior stereological studies (Reisert, Wildemann et al. 1984; Oorschot 1996; Tang, Lopez et al. 2001; Bermejo, Jimenez et al. 2003; Avendano, Machin et al. 2005; Ekinci, Acer et al. 2008; Klebe, Krafft et al. 2014). Volumes were calculated using the following equation: [(Average [(Area of coronal section) × Interval × Number of sections) (MacLellan, Silasi et al. 2008).

2.8 Immunohistochemistry

10 μm thick slices were first stained with OX-42 (1:1000, ABcam) and mannose receptor (1:1000, ABcam) overnight at 4 °C, followed by incubation with appropriate fluorescence conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). The peri-hemorrhagic area was imaged by a Fluorescent Olympus-BX51 microscope and analyzed using MagnaFire SP 2.1B software (Olympus, Melville, NY). At least six sections per animal group over a microscopic field of 20 × (for microglia) were averaged and expressed as cells/field, as described (Wang and Dore 2007).

2.9 Neurobehavioral Analysis

Neurobehavioral function was evaluated in a blinded manner using a battery of tests, described below, to detect sensorimotor and cognitive deficits 28 days after GMH

as previously described (Hartman, Lekic et al. 2009; Klebe, Krafft et al. 2014): Foot Fault Test: Rats were placed on a wire grid (20x40 cm) kept above the floor level and allowed to walk on the grid for 2 minutes. Number of foot faults will be recorded when a complete paw falls through the openings in the grid. Rotarod Test: Rats were placed on a rotarod (Columbus Instruments, Columbus, OH), which consists of a rotating horizontal cylinder (7 cm diameter) divided into 9.5-cm-wide lanes. Rats walked forward when the cylinder is rotating to avoid falling down. Rats were tested at a starting 5 RPM or 10 RPM with acceleration at 2 RPM per 5 seconds. A photobeam circuit detected the latency to fall off the cylinder. Water Maze Test: Rats were released in a metal pool (110 cm diameter) filled with water and containing spatial cues on the walls, and they were allowed to swim to find a submerged platform (11 cm diameter). Each animal performed 10 trials per day for 4 days, 5 blocks of 2 consecutive trials, with a 10-min interval between successive blocks. Over the next 3 days, the platform was submerged 1 cm below the water and surface and the rats had to find and remember the platform location. Additionally, at the end of each day, the platform was removed and animals were allowed to swim to find the platform quadrant. An overhead camera with a computerized tracking system (Noldus Ethovision; Noldus, Tacoma, WA) recorded the swim path and measured the swim distance, swim speed, and time spent in probe quadrant.

2.10 Statistical Analysis

A power analysis using a type I error rate of 0.05 and a power of 0.8 on a 2-sided test was used to estimate sample size. Data are expressed in Mean ± Standard Deviation. One-way ANOVA on ranks using the Student-Newman-Keuls post-hoc test was used to analyze

behavioral, histological, western blots, and immunohistochemistry. A P-value <0.05 was considered statistically significant.

3) Results

3.1 PPARγ Stimulation Ameliorated Long-term Neurological Deficits

The vehicle group performed significantly worse compared to sham in the Morris Water Maze evaluation, yet PPARγ stimulation improved spatial learning and memory compared to vehicle and was not significantly different from sham. Treatment effect was reversed by the PPARγ antagonist (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.1.A-B). Treatment also significantly improved sensorimotor function in the foot fault test compared to other GMH groups (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.1.C). Yet no significant difference was achieved in the treated group compared to vehicle and antagonist groups in the rotarod sensorimotor evaluation (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.1.D).

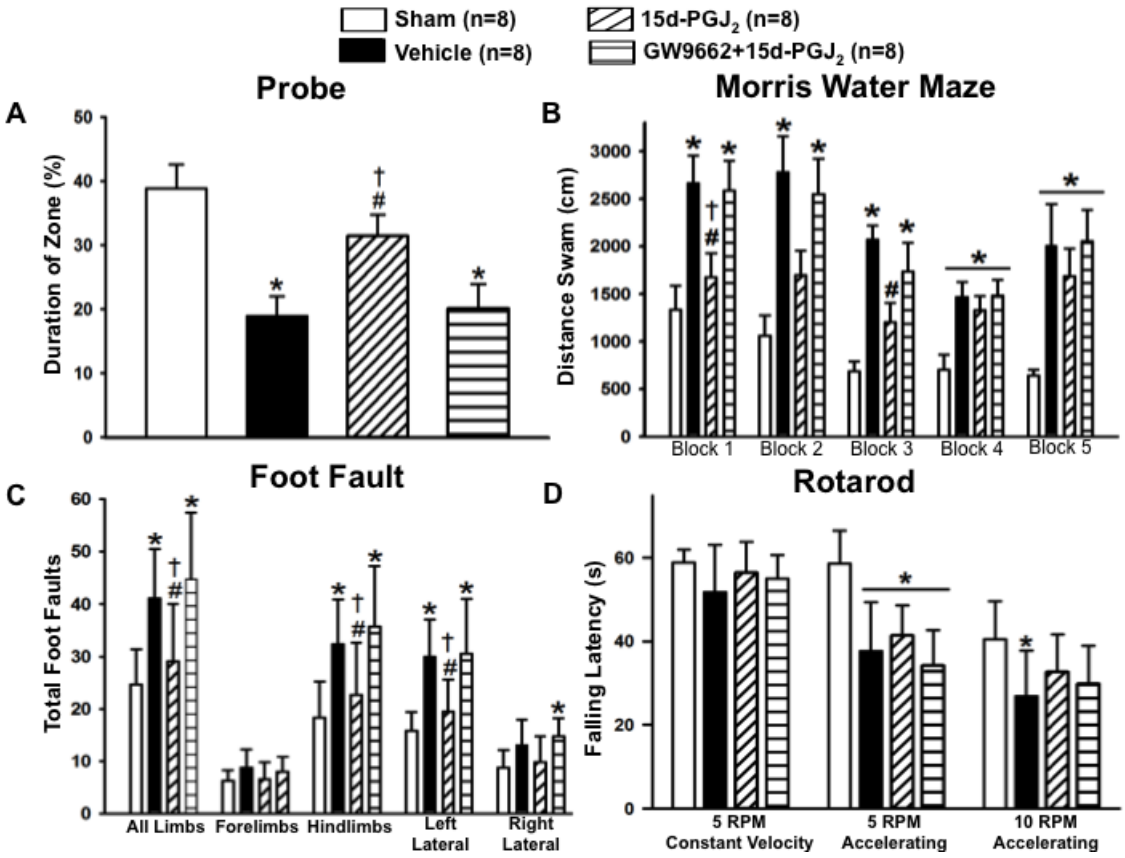

Figure A.1: Long-term neurocognitive and sensorimotor outcomes after 15d-PGJ$_2$ treatment and PPARγ inhibition with 15d-PGJ$_2$ treatment at 3-4 weeks after GMH. Neurofunctional assessment of (A and B) Morris water maze, (C) foot fault, and (D) rotarod at 21 to 28 days after germinal matrix hemorrhage. Values are expressed as mean±SD. *P<0.05 compared with sham, #P<0.05 compared with vehicle, and †P<0.05 compared with inhibitor and agonist. N=8 per group; and RPM, rounds per minute.

3.2 PPARγ Stimulation Improved Long-term Brain Morphology

Since increased intracranial pressure is associated with hydrocephalus, ICP was measured in rats at 4 weeks post-ictus. ICP was significantly decreased in treated groups when compared to vehicle and PPARγ antagonist groups (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.2.B). Calculated cortical thickness is presented as a ratio to the mean of sham and was significantly deceased in the vehicle and PPARγ antagonist group, but the PPARγ stimulated group had significantly less cortical loss (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.3.A). Ventricular volume was significantly increased in vehicle and PPARγ antagonist groups, but 15d-PGJ_2 treatment reduced post-hemorrhagic ventricular dilation (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.3.B). White matter loss is presented as a percentage of white matter present to mean of sham. White matter loss was reduced by PPARγ stimulation, but vehicle and PPARγ antagonist groups had significant white matter loss (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.3.C). Basal ganglia loss is presented as a percentage of basal ganglia present to mean of sham. The 15d-PGJ_2 treated group had significantly decreased basal ganglia loss compared to the vehicle group. Surprisingly, basal ganglia loss in the GW9662 group was not significantly different from PPARγ agonist or vehicle groups (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.3.D).

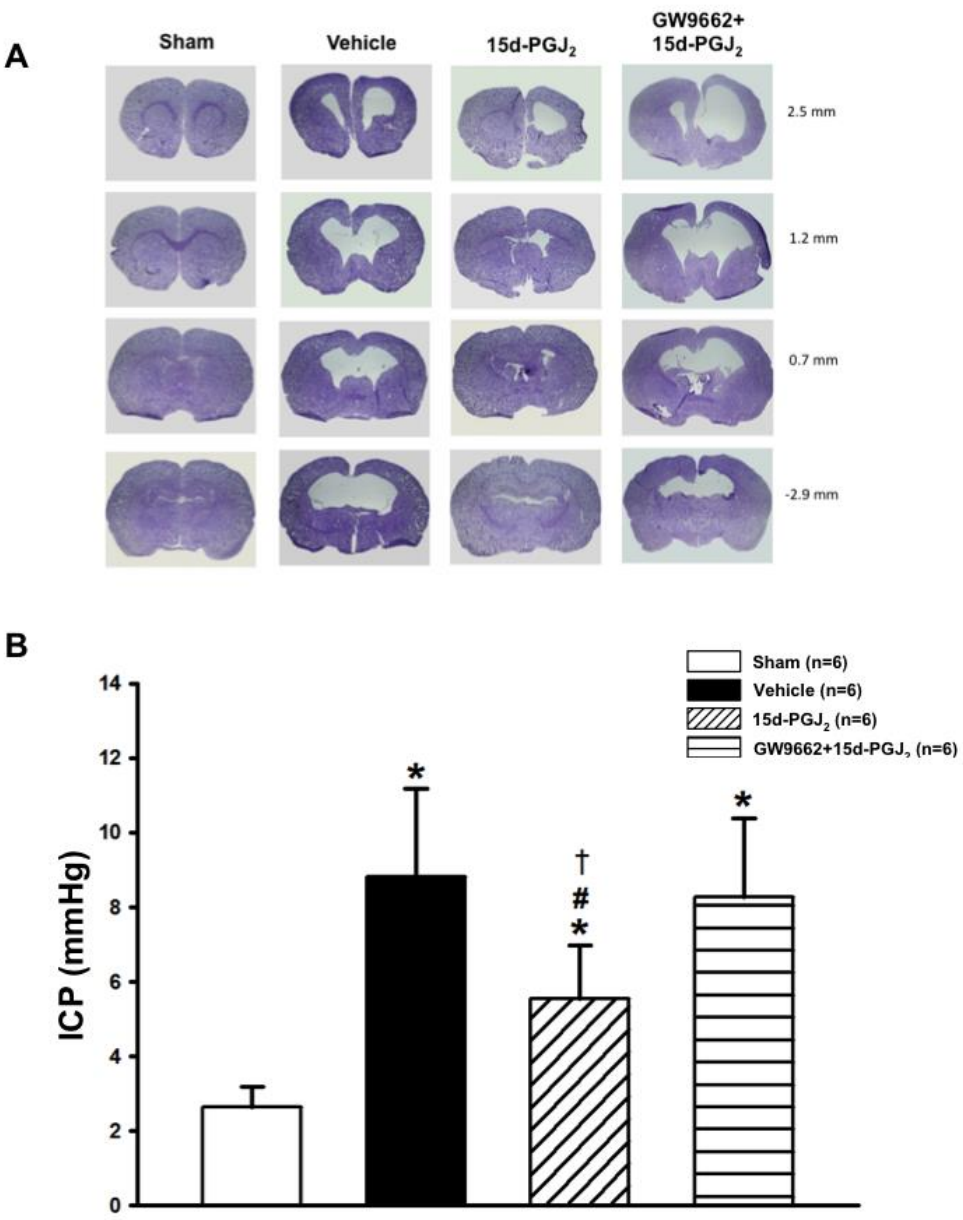

Figure A.2: Effects of 15d-PGJ$_2$ treatment and PPARγ inhibition with 15d-PGJ$_2$ treatment on brain morphology and intracranial pressure 4 weeks after GMH. 28 days after germinal matrix hemorrhage. Representative microphotographs of (A) Nissl-stained brain sections were taken and (B) Intracranial pressure (ICP) in mm Hg**.** Note: measurement on the right side of the representative pictures indicates the location of the brain section from bregma. Values are expressed as mean±SD. *P<0.05 compared with sham, #P<0.05 compared with vehicle, and †P<0.05 compared with inhibitor and agonist. N=6 per group; and mmHG, millimeters of Mercury.

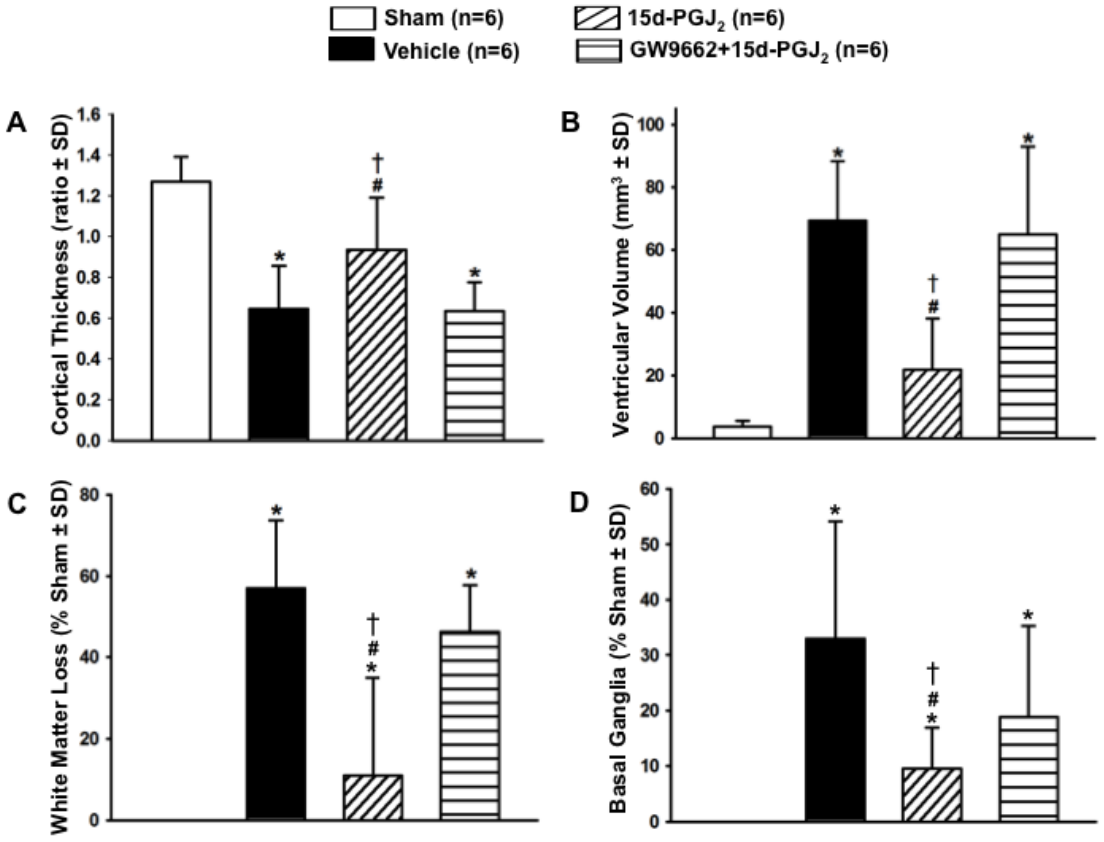

Figure A.3: Quantification of brain morphological outcomes from 15d-PGJ$_2$ treatment and PPARγ inhibition with 15d-PGJ$_2$ treatment at 4 weeks post-GMH. Quantification of (A) cortical thickness, (B) ventricular volume, (C) white matter loss, and (D) basal ganglia loss at 28 days after germinal matrix hemorrhage. Values are expressed as mean±SD. *P<0.05 compared with sham, #P<0.05 compared with vehicle, and †P<0.05 compared with inhibitor and agonist. N=6 per group.

3.3 PPARγ Stimulation Enhanced Hematoma Resolution, Increased Activated Microglia, and Induced M2 Polarization

A hemoglobin assay time-course was conducted at 24 hours, 72 hours, and 7 days to determine PPARγ's role in hematoma resolution. At 24 hours, all groups had significantly greater hemoglobin content in the brain compared to sham (*P<0.05 versus Sham; Figure 2.4.A). At 72 hours, all groups had significantly greater hemoglobin content in the brain compared to sham, but 15d-PGJ_2 treatment had significantly less hemoglobin content compared to vehicle, which was reversed by GW9662 treatment (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.4.B). At 7 days, only the vehicle and PPARγ antagonist groups had significantly greater hemoglobin content compared to sham, but the PPARγ agonist group had significantly less hemoglobin content compared to vehicle and PPARγ antagonist groups (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.4.C). Since significantly greater hematoma resolution was observed in the PPARγ stimulated group at 72 hours, representative photographs of immuno-stained activated microglia/macrophages (OX-42) in the peri-hematoma region are presented at this time point (Figure 2.5.A). The 15d-PGJ_2 treated group had relatively increased number of microglia/macrophages compared to sham, vehicle, and PPARγ antagonist group ((*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.5.B). Additionally representative photographs of the co-localization of activated microglia/macrophages (OX-42) and mannose receptor (CD206) in the peri-hematoma region are presented at 72 hours, showing an increase in OX-42/mannose

receptor expression in 15d-PGJ_2 treated group in comparison to all other groups (Figure 2.5.A).

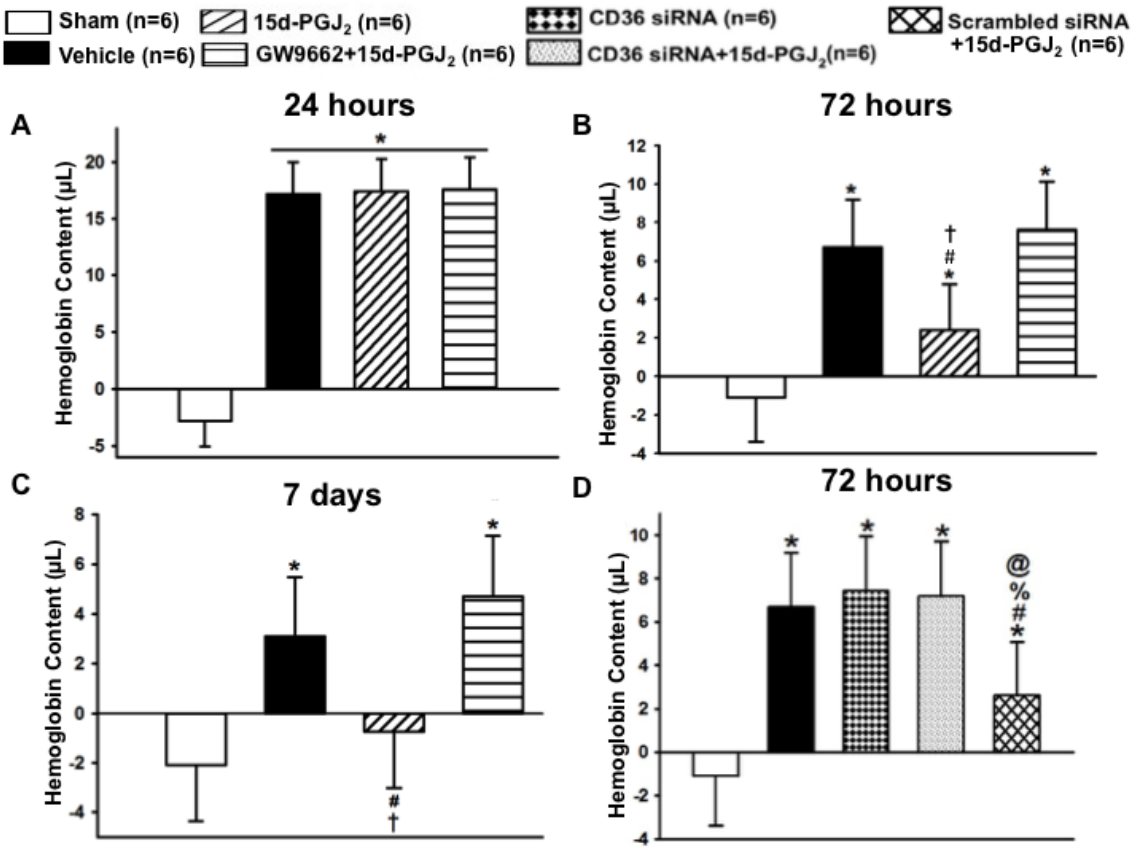

Figure A.4: Short-term hematoma resolution after GMH from 15d-PGJ$_2$ treatment, PPARγ inhibition with 15d-PGJ$_2$ treatment, CD36 knockdown, and CD36 knockdown with 15d-PGJ$_2$ treatment. Hemoglobin assay at (A) 24 hours, (B) 72 hours, and (C) 7 days. At 72 hours (D) Hemoglobin assay was conducted on siRNA groups. Values are expressed as mean±SD. *P<0.05 compared with sham, #P<0.05 compared with vehicle, and †P<0.05 compared with inhibitor and agonist, %P<0.05 compared CD 36 siRNA, and @P<0.05 compared to scrambled siRNA. N=6 per group.

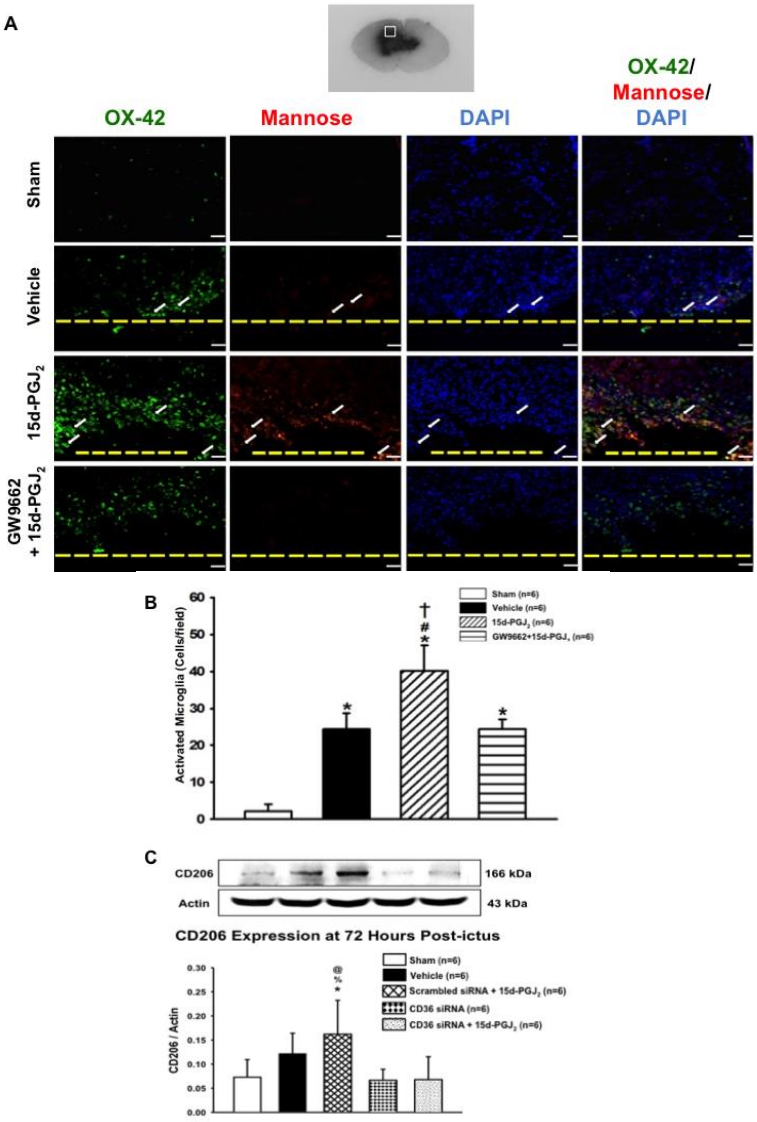

Figure A.5: Microglia / Macrophage activation and differentiation into M2 subtypes at 3 days post-GMH following 15d-PGJ$_2$ treatment, CD36 knockdown, and CD36 knockdown with 15d-PGJ$_2$ treatment. Immunohistochemistry representative pictures were taken showing the co-localization of (A) activated microglia (OX-42; scale bar: 20 μm) with Mannose Receptor and DAPI staining and (B) quantification of activated microglia was conducted at 72 hours. Western blots were conducted at 72 hours for (C) CD206 Expression. Note: peri-hematoma region is below the yellow broken line (A) and the representative GMH brain indicates where IHC images were taken. Values are expressed as mean±SD. *P<0.05 compared with sham, #P<0.05 compared with vehicle, †P<0.05 compared with inhibitor and agonist, %P<0.05 compared CD36 siRNA, and @P<0.05 compared to scrambled siRNA. N=6 per group; and ICH, Immunohistochemistry. N=6 per group.

3.4 PPARγ Stimulation Increased CD36 and PPARγ Expression at 72 Hours

A time-course study was conducted to determine endogenous PPARγ and CD36 expression levels in GMH at 0, 3, 6, 12 hours and 1, 3, 5, and 7 days after GMH. Endogenous PPARγ expression was significantly increased at 3, 6, 12 hours and 1 day compared to 0 hour (*P<0.05 versus 0 hours; Figure 2.6.A). Correspondingly, endogenous CD36 expression was significantly increased at 3, 6, 12 hours, and 1 day compared to 0 hour (*P<0.05 versus 0 hours; Figure 2.6.B), and tended to remain elevated by 7 days. PPARγ and CD36 expression levels were determined at 72 hours for all experimental groups. PPARγ expression was significantly increased in the 15d-PGJ_2 group compared to all other groups (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.6.C). Much like PPARγ, CD36 expression was significantly increased in the 15d-PGJ_2 group compared to all other groups (*P<0.05 versus Sham; #P<0.05 versus Vehicle; †P<0.05 versus GW9662 + 15d-PGJ_2; Figure 2.6.D).

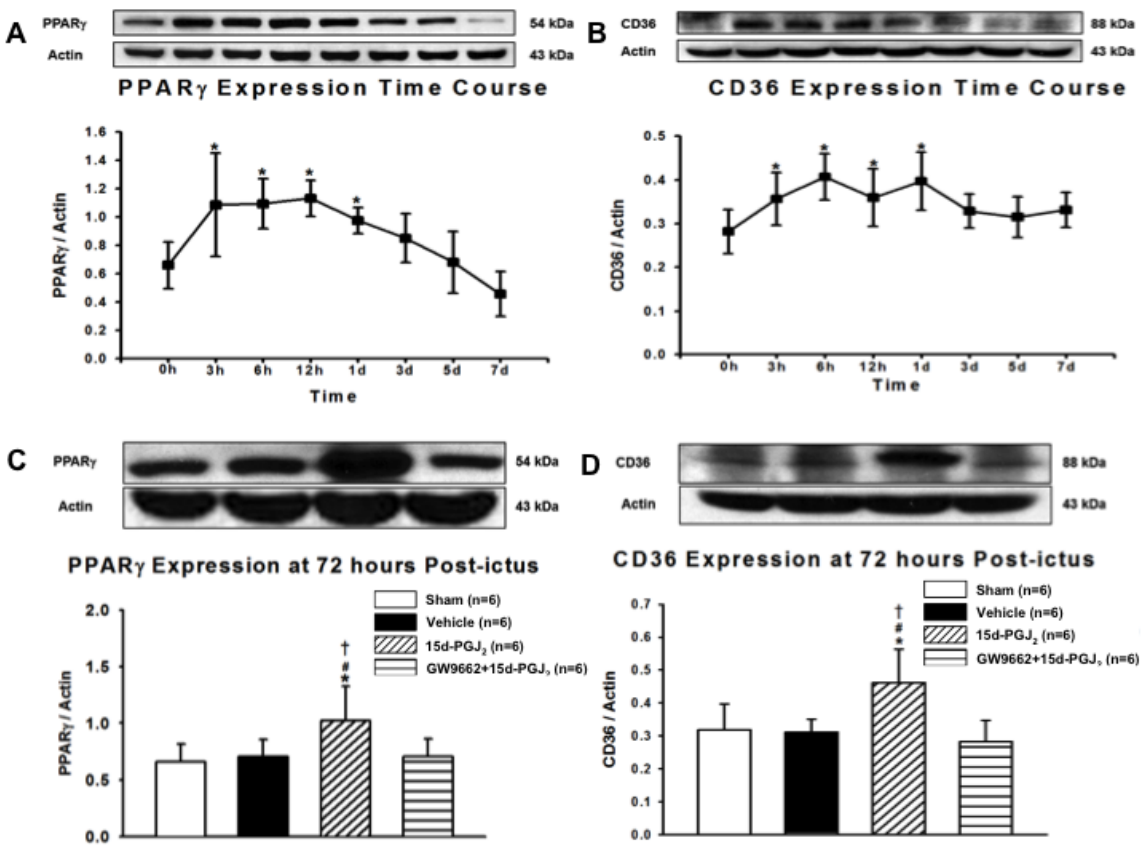

Figure A.6: Short-term time course of CD36 and PPARγ expression after GMH and the effects of 15d-PGJ$_2$ treatment, PPARγ inhibition with 15d-PGJ$_2$ treatment, CD36 knockdown, and CD36 knockdown with 15d-PGJ$_2$ treatment on CD36 and PPARγ expression levels. Western blot was conducted for time-course studies of (A) PPARγ and (B) CD36 at 0, 3, 6 , 12 hours, 1, 3, 5, 7 days after GMH. Western blot was then conducted at 72 hours for (C) PPAR-Y and (D) CD36. Values are expressed as mean±SD. *P<0.05 compared with sham, #P<0.05 compared with vehicle, and †P<0.05 compared with inhibitor and agonist. N=6 per group; and h and d, hour(s) and day(s).

3.5 CD36 Knockdown Reversed PPARγ Agonist-enhanced Hematoma Resolution and M2 Expression at 72 Hours.

At 72 hours, CD36 knockdown reversed PPARγ agonist-enhanced hematoma resolution, which was not reversed in the scrambled siRNA group (*P<0.05 versus Sham, #P<0.05 versus Vehicle, %P<0.05 versus CD-36 siRNA + 15d-PGJ_2, @P<0.05 versus scrambled siRNA+15d-PGJ_2, Figure 2.4.D). Additionally, CD36 knockdown reversed PPARγ mannose receptor expression, which was not reversed in the scrambled siRNA group (*P<0.05 versus Sham, #P<0.05 versus Vehicle, %P<0.05 versus CD-36 siRNA + 15d-PGJ_2, @P<0.05 versus scrambled siRNA+15d-PGJ_2, Figure 2.5.C).

4) Discussion

Neonatal brain hemorrhage is a common affliction of premature infants. The resultant hematoma is thought to play a major role in causing post-hemorrhagic hydrocephalus development because blood clots disrupt cerebrospinal fluid circulation and absorption in the ventricles (Crews, Wyss-Coray et al. 2004). Rapid hematoma resolution was neuroprotective in adult hemorrhagic stroke models (Zhao, Sun et al. 2007; Zhao, Sun et al. 2015). In particular, PPARγ stimulation upregulates CD36 expression in microglia/macrophages, leading to increased phagocytosis of blood products and more rapid hematoma resolution after adult intracerebral hemorrhage (Zhao, Sun et al. 2007). In this study, we examined the effects of stimulating PPARγ with 15d-PGJ_2 on hematoma resolution as well as long-term brain morphological and neurofunctional outcomes after collagenase-induced GMH in neonatal rats. In addition, we determined if PPARγ and CD36 inhibition reversed observed therapeutic effects from PPARγ stimulation by 15d-PDJ_2. This study is the first to assess this treatment approach in neonates for enhancing hematoma resolution as well as the first to evaluate its therapeutic potential for ameliorating long-term white matter loss, post-hemorrhagic hydrocephalus development, and neurological deficits.

We evaluated the efficacy of 15d-PGJ_2 treatment, a PPARγ agonist, administered 1 hour post-ictus as a potential therapeutic modality for GMH-induced brain injury. Although treatment did not significantly increase hematoma resolution at 24 hours, it did significantly enhance hematoma resolution at 72 hours and 7 days after GMH (Figure 2.4.A-C). PPARγ stimulation demonstrated more rapid hematoma clearance after GMH in neonates, starting at 72 hours, than after ICH in adults, which took 7 days (Zhao, Sun

et al. 2007). 15d-PGJ_2 co-admiration with GW9662, a PPARγ antagonist, reversed 15d-PGJ_2 treatment effects on enhanced hematoma resolution at 72 hours and 7 days after GMH. To determine if endogenous expression of PPARγ and CD36 changes after GMH, we performed a Western blot time course using 0, 3, 6, 12 hour, 1, 3, 5, and 7 day endpoints following GMH induction. Endogenous PPARγ expression increased at 3, 6, 12 hour, and 1 day after GMH induction, then returned to baseline by 3 days (Figure 2.6.A). Endogenous CD36 expression significantly increased at 3, 6, 12 hours, and 1 day after GMH induction, and tended to remain elevated through 7 days (Figure 2.6.B). Because endogenous PPARγ and CD36 expression returned near baseline at 72 hours and because 15d-PGJ_2 treatment enhanced hematoma resolution at 72 hours, we determined treatment effects on PPARγ and CD36 expression levels by Western blot at 72 hours. As expected, 15d-PGJ_2 treatment significantly increased PPARγ expression compared to sham and vehicle, and 15d-PGJ_2 co-administration with GW9662 reversed this effect (Figure 2.6.C). Similarly, 15d-PGJ_2 treatment significantly increased CD36 expression compared to sham and vehicle, and 15d-PGJ_2 co-administration with GW9662 reversed this effect (Figure 2.6.D).

CD36 receptor is an important scavenger receptor located on several cell types, including monocytes, endothelial cells, and microglia/macrophages. CD36 plays an important role in microglia/macrophage phagocytosis, and upregulating its expression beneficially enhances hematoma resolution (Zhao, Sun et al. 2007). More activated microglia were observed in the peri-hematoma region of treated GMH animals than all other groups, providing evidence of PPARγ's treatment effects are dependent upon these critical immune cells (Figure 2.5.A-B). All GMH groups seemed to have increased DAPI

stained cells post-ictus compared to sham. The germinal matrix has many growing and dividing neuronal and glial precursor cells. Prior GMH studies have documented increased proliferative cytokines and pathways, such as TGF-β and mTOR, after injury, and we speculate proliferative signaling triggers more profound gliosis after neonatal brain hemorrhage (Lekic, Klebe et al. 2015). While brain cell death is definitely occurring, we speculate proliferative signaling in conjunction with increased leukocyte infiltration contributes towards these observed results in neonatal brains. Although activated microglia/macrophages contribute to brain injury in adults, some evidence suggests microglia/macrophages have important defense mechanisms against injury in neonates, which could be explained by the vital role microglia play in neonatal brain development (Faustino, Wang et al. 2011; Harry and Kraft 2012). Furthermore, activated microglia/macrophages have two differentiated states, a pro-inflammatory classically activated state (M1), and an immune dampening and tissue regenerative alternatively activated state (M2) (Klebe, McBride et al. 2015). CD36 stimulation can contribute towards microglia/macrophage activation as well as M2 polarization (Kouadir, Yang et al. 2012; Rios, Koga et al. 2013; Chavez-Sanchez, Garza-Reyes et al. 2014). PPARγ stimulation also polarizes microglia/macrophages towards the M2 state (Yoon, Jeon et al. 2008; Pisanu, Lecca et al. 2014; Penas, Mirkin et al. 2015). To corroborate these findings in GMH, immunohistochemical co-localization of activated microglia and mannose receptor (CD206), an M2 marker, demonstrated increased co-expression in 15d-PGJ_2 treated GMH animals compared to other groups, providing more evidence PPARγ induces M2 polarization (Figure 2.5.A). Similarly, CD206 Western blots demonstrated increased CD206 expression levels in treated GMH animals compared to sham, GMH

animals with CD36 siRNA alone, and GMH animals with CD36 siRNA and 15d-PGJ_2 treatment. Interestingly, vehicle treated GMH animals showed a small tendency towards having increased CD206 expression levels, which also did not achieve a statistically significant difference compared to 15d-PGJ_2 treated GMH animals. After hemorrhage, M2 microglia/macrophages are expected to increase over time as the peri-hematoma milieu transitions into an immune dampened tissue repair phase in which M2 microglia/macrophages play a pivotal role (Klebe, McBride et al. 2015). CD36 knockdown eliminated the tendency observed in the vehicle group, achieving a significantly reduced expression compared to 15d-PGJ_2 treatment. CD36 knockdown also reversed 15d-PGJ_2 induced upregulation of CD206 expression (Figure 2.5.C). CD36, thus, is important for M2 polarization, particularly after PPARγ stimulation. To further confirm microglial/macrophage CD36 plays a pivotal role in PPARγ-induced blood clot clearance, siRNA was used to knockdown CD36 expression. CD36 knockdown reversed 15d-PGJ_2 treatment effects on enhanced hematoma resolution at 72 hours, which was not reversed by scrambled siRNA (Figure 2.4.D).

In our long-term evaluations, vehicle treated GMH animals had significant cortical, white matter, and basal ganglia loss as well as post-hemorrhagic ventricular dilation, but 15d-PGJ_2 treatment ameliorated these brain morphological maladies, which were reversed by PPARγ antagonist, GW9662, co-administration (Figure 2.3.A-D). Surprisingly, GW9662 co-administration did not completely reverse 15d-PGJ_2's effects on reducing basal ganglia loss, although a tendency was observed. Our ICP measurements agreed with the brain morphological assessment, where ICP levels were significantly lower in treated groups when compared to vehicle and PPARγ antagonist

group **(**Figure 2.2.B). Additionally, vehicle treated GMH animals performed poorly in the Morris Water Maze, Foot Fault, and Rotarod tests, but 15d-PGJ_2 treatment significantly improved spatial memory and motor function, which were reversed by GW9662 co-administration (Figure 2.1.A-D). The foot fault test evaluates locomotor function, the rotarod test evaluates sensorimotor coordination and balance, and Morris Water Maze evaluates spatial learning and memory (Schaar, Brenneman et al. 2010). Although 15d-PGJ2 reduced the number of foot faults, it did not significantly improve performance in the rotarod test. GMH and consequent post-hemorrhagic ventricular dilation may cause cerebellar injury that affects motor coordination (Volpe 2009; Brouwer, de Vries et al. 2015; Fumagalli, Bassi et al. 2015). More thorough investigations are needed to elucidate the pathophysiology between GMH and cerebellar injury. Although motor coordination is an important factor in both the rotarod and foot fault tests, the element of balancing on an accelerating cylinder platform leads us to speculate cerebellar injury may more profoundly affect performances in the rotarod evaluations. 15d-PGJ2 treatment may not be effective enough to ameliorate potential cerebellar injury from GMH and consequent post-hemorrhagic hydrocephalus, and, if this is the case, modulating the immune response after GMH may not be sufficient to promote complete functional recovery. Additionally, the rotarod test may not be sensitive enough for detecting improved sensorimotor outcomes in treatment groups, which is why we performed multiple neurofunctional evaluations. Nonetheless, these results provide sufficient evidence that enhanced hematoma resolution corresponds with improved long-term brain morphological and neurocognitive outcomes after GMH. Most importantly, more rapid blood clot clearance resulted in significantly decreased post-hemorrhagic ventricular

dilation, providing evidence that blood products play an important role in post-hemorrhagic hydrocephalus development.

The CD36 scavenger receptor is important in microglia/macrophage-mediated phagocytosis of cellular debris and blood products. Our results suggest PPARγ stimulation by 15d-PGJ_2 increases microglia/macrophage phagocytic function by upregulating CD36 scavenger receptor expression in our experimental GMH model, leading to enhanced hematoma resolution. We are first to report the positive long-term effects on brain morphological and neurofunctional outcomes from more efficient hematoma resolution after GMH. Blood products disrupt cerebrospinal circulation and absorption in the cerebroventricular system, often resulting in post-hemorrhagic hydrocephalus. Herein, we provide evidence that more rapid blood clot clearance reduces long-term post-hemorrhagic ventricular dilation after GMH. Removing the hematoma without damaging surrounding tissues is ideal and clinically relevant. PPARγ stimulation could be a promising therapeutic approach for GMH patients, especially since PPARγ stimulation by Pioglitazone is already being evaluated in clinical trials for adult cerebral hemorrhage.

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