UNIVERSITY OF CALIFORNIA SAN DIEGO

Lysophosphatidic acid receptor 1 signaling initiates neonatal post-hemorrhagic hydrocephalus through ciliated ependymal cell loss

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2018

The Dissertation of Nicole Lummis is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

18:1, carbon number (18) and unsaturation state (1) of a phospholipid tail

BrP, α-bromomethylene phosphonate

Aβ, beta-amyloid

AcTub, acetylated tubulin

AC, adenylyl cyclase

AD, Alzheimer's disease

ATX, autotaxin

cAMP, cyclic adenosine monophosphate

Cas3, cleaved caspase 3

CNS, central nervous system

CSF, cerebrospinal fluid

CTGF, connective tissue growth factor

E13.5, embryonic day 13.5

EC, endothelial cell

Edg, endothelial differentiation gene

ERK, extracellular signal-regulated kinase

FAK, focal adhesion kinase

G protein, guanine nucleotide-binding protein

$G_\alpha/G_\beta/G_\gamma$, heterotrimeric G protein subunits

G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$, G_α subtypes

GABA, γ-aminobutyric acid

GBM, glioblastoma multiforme

GFAP, glial fibrillary acidic protein

GPCR, G protein-coupled receptor

GSK3, glycogen synthase kinase 3

H&E, hematoxylin and eosin histology dyes

HIF, hypoxia inducible factor

Iba1, ionized calcium-binding adapter molecule 1

ICP, intracranial pressure

IL, interleukin

INM, interkinetic nuclear migration

IPF, idiopathic pulmonary fibrosis

IZ, intermediate zone

Kd, dissociation constant

LCAT, lecithin cholesterol acyltransferase

LP, lysophospholipid

LPA, lysophosphatidic acid

LPA_{1-6}, lysophosphatidic acid receptors 1-6, protein nomenclature

LPAR, lysophosphatidic acid receptor

LPAR1-6, lysophosphatidic acid receptors 1-6, human gene nomenclature

Lpar1-6, lysophosphatidic acid receptors 1-6, murine gene nomenclature

LPC, lysophosphoditylcholine

LPE, lysophosphatidylethanolamine

LPP, lipid phosphate phosphatase

LPS, lyosphosphatidylserine or lipopolysaccharide

MAG, monoacylglycerol

MBP, myelin basic protein

MEK, mitogen activated protein kinase kinase

MEMF, mouse embryonic meningeal fibroblast

MZ, marginal zone

NF-κB, nuclear factor kappa-light-chain-enhancer of acrivated B cells

NGF, nerve growth factor

NPC, neuroprogenitor cell

OMPT, oleoyl-methoxy phosphothionate

PHH, post-hemorrhagic hydrocephalus

P8, postnatal day 8

P8+7, 7 days following P8 procedure

PC, phosphatidylcholine

PDGF, platelet derived growth factor

PE, phosphatidylethanolamine

PHH, post-hemorrhagic hydrocephalus

PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase

PLA1, Phospholipase A1

PLA2, Phospholipase A2

PPI, prepulse inhibition

PS, phosphatidylserine

PSNL, partial sciatic nerve ligation

Rac, Ras-related C3 botulinum toxin substrate

ROCK, rho-associated kinase

S1P, sphingosine 1-phosphate

SC, schwann cell

SEM, scanning electron microscopy

SVZ, subventricular zone

TEM, transmission electron microscopy

TGFβ, transforming growth factor β

TRPV1, transient receptor potential cation channel subfamily V member 1

UVB, ultraviolet radiation B

VEGF, vascular endothelial growth factor

VM, ventriculomegaly

VZ, ventricular zone

VZG1, ventricular zone gene 1

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ABSTRACTS

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FIELDS OF STUDY

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Cell Regulation (oncology) with Dr. Daniel Anderson at Genentech

Toxicology with Dr. Paul Jean at Dow Corning

Chemical Research & Development with Melinda Howell at Dow Corning

ABSTRACT OF THE DISSERTATION

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Nicole Lummis

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Lysophosphatidic acid (LPA) is a signaling phospholipid that binds to G protein-coupled receptors designated LPA_1-LPA_6. It has several activities throughout the body, including modulating cellular survival and migration. LPA circulates in the blood bound to carriers such as albumin, and LPA concentrations are increased during cases of hemorrhage or trauma. In the

nervous system, LPA helps modulate development and differentiation, but high concentrations of the lipid are often involved in pathology. Post-hemorrhagic hydrocephalus is one such disorder. Hydrocephalus results from an accumulation of cerebrospinal fluid (CSF) in the brain, which expands the fluid-filled ventricles, causing permanent brain damage. This disorder is often chronic and is treated by invasive neurosurgical procedures which drain excess CSF. Here we provide evidence that LPA, introduced to the CSF in the blood, may be a key mediator of post-hemorrhagic hydrocephalus. Animals were injected with LPA at postnatal day 8 and hydrocephalus was observed by measuring increased ventriculomegaly and intracranial pressure 7 days later. Histology and immunohistochemistry demonstrated that the ciliated ependymal cells which generate CSF flow were heavily disrupted 3-6 hours post-LPA exposure. However, development of hydrocephalus and degeneration of the ependymal monolayer was prevented by knockout of LPA_1, and to a lesser extent by LPA_3 knockout. Pretreatment with the LPA_1 antagonist AM095 also protected LPA-injected animals from developing hydrocephalus, signifying that this is a receptor-mediated phenomenon that could be prevented by timely application of a pharmacological inhibitor. As there are currently no efficacious pharmacological treatments for hydrocephalus patients or methods used to prevent hydrocephalus post-hemorrhage, the identification of LPA_1-expressing ependymal cells provides a novel target for therapeutic development.

Chapter 1:

INTRODUCTION

Hydrocephalus is a common neurological disorder that affects 1 in 1000 people worldwide (Chi et al., 2005; Dewan et al., 2018). It is typically a chronic condition associated with persistent migraines, cognitive deficits, epilepsy, and motor dysfunction (Ding et al., 2001; Lacy et al., 2008; Laurence and Coates, 1962; Vinchon et al., 2012). Clinically, hydrocephalus is characterized by an accumulation of cerebrospinal fluid (CSF) pressure in the brain and subsequent enlargement of the fluid-filled ventricles, termed ventriculomegaly (Bergsneider et al., 2006). Despite the chronic nature of this disorder, current treatments are limited to palliative neurosurgical interventions that physically drain excess CSF (Cherian et al., 2004; Shooman et al., 2009; Walid and Robinson, 2012). Research into alternative treatment and prevention options is necessary to improve these patients' quality of life.

While hydrocephalus can have congenital origins, after birth hydrocephalus usually develops following severe hemorrhage or brain trauma (Robinson, 2012). During a hemorrhagic event, many foreign molecules are introduced to the CSF, including lysophosphatidic acid (LPA). LPA is a signaling lipid that binds to 6 known receptors, named LPA_1-LPA_6 (Yung et al., 2014). As G protein-coupled receptors, LPARs mediate diverse cellular functions, including survival, cytoskeletal changes, and proliferation (Stoddard and Chun, 2015). LPA receptors are expressed differentially throughout the body and serve many functions in health and disease. In the nervous system, LPA_1-LPA_6 are spatiotemporally regulated, demonstrating altered cell type expression in the developing and mature brain (Yung et al., 2015) (see Chapter 3, Figure 2).

Of particular relevance, bloodborne LPA levels are significantly elevated in cases of hemorrhage or trauma (Sano et al., 2002). More than 50% of bloodborne LPA is produced from lysophosphotidylcholine (LPC) by the enzyme autotaxin (ATX) (Aoki et al., 2002; Sonoda et al., 2002). Once converted, LPA circulates bound to carrier proteins such as albumin. ATX is highly expressed by the choroid plexus, an organ present throughout the ventricular system that produces CSF, meaning that LPC introduced to the CSF during intracerebral hemorrhage may be efficiently converted to LPA (Allen Brain Atlas, 2015; Segal, 2000; Speake et al., 2001) . Therefore, severe hemorrhage introduces large concentrations of LPA into the ventricular system through direct (bloodborne LPA) or indirect (enzymatic LPC conversion) means, wreaking havoc on LPA receptor-expressing cells and altering healthy CSF dynamics.

Post-hemorrhagic hydrocephalus has a high prevalence in very low birth weight (VLBW) premature infants due to the extreme fragility of the germinal matrix vasculature (Ballabh, 2010). In this population, intraventricular hemorrhage often occurs after birth, allowing LPA and other signaling molecules to invade the brain and ultimately cause disability or disease. While our lab has successfully established an LPA_1-dependent model of hydrocephalus in the E13.5 fetal mouse (Yung et al., 2011), this model does not replicate the conditions surrounding hydrocephalus in the VLBW population, instead representing bleeding in the highly immature late 1st trimester brain. Therefore, this dissertation focuses on the characterization of a neonatal model beginning later in neural development. This model allows us to differentiate LPA signaling involvement between the distinct clinical populations of fetal and premature PHH patients and target therapies toward young infants at high risk for PHH.

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Chapter 2:

Promising Pharmacological Directions in the World of Lysophosphatidic Acid Signaling

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Abstract

Lysophosphatidic acid (LPA) is a signaling lipid that binds to six known lysophosphatidic acid receptors (LPARs), named LPA_1-LPA_6. These receptors initiate signaling cascades relevant to development, maintenance, and healing processes throughout the body. The diversity and specificity of LPA signaling, especially in relation to cancer and autoimmune disorders, makes LPA receptor modulation an attractive target for drug development. Several LPAR-specific analogues and small molecules have been synthesized and are efficacious in attenuating pathology in disease models. To date, at least three compounds have passed phase I and phase II clinical trials for idiopathic pulmonary fibrosis and systemic sclerosis. This review focuses on the promising therapeutic directions emerging in LPA signaling toward ameliorating several diseases, including cancer, fibrosis, arthritis, hydrocephalus, and traumatic injury.

Introduction

Lysophosphatidic acid (LPA) is a bioactive lipid that is concentrated in serum and is essential for a variety of cellular and developmental processes (reviewed in (Choi *et al.*, 2010)). While LPA does play a structural role in cell membranes, extracellular LPA is a highly selective and specific activator of a class of G protein-coupled receptors (GPCRs) called LPA receptors (LPARs) (reviewed in (Yung *et al.*, 2014)). There are currently six recognized LPARs, named LPA_{1-6}, with clear homologs between human (*LPAR1-6*) and mouse (*Lpar1-6*) genes (reviewed in (Chun *et al.*, 2010)). All six receptors are expressed throughout the body during development and adulthood in unique spatiotemporal patterns. These receptors are involved in a variety of necessary functions, including cell survival, proliferation, migration, differentiation, vascular regulation, and cytokine release (reviewed in (Yung *et al.*, 2014)).

LPA can be produced in several ways through the activity of intracellular or extracellular enzymes. The two most prominent pathways involve the conversion of lysophosphatidyl choline (LPC) to LPA by autotaxin (ATX/*Enpp2*) (Tokumura *et al.*, 2002; Umezu-Goto *et al.*, 2002) and conversion of phosphatidic acid to LPA by phospholipase A1 or A2 (PLA1/PLA2) (Fourcade *et al.*, 1995; Sonoda *et al.*, 2002). Intriguingly, ATX is highly expressed in blood, brain, kidney, the lymphatic system, and tissue surrounding injury (Bachner *et al.*, 1999; Kanda *et al.*, 2008; Savaskan *et al.*, 2007), suggesting important LPA-mediated mechanisms in these areas. Additionally, LPA is secreted by activated platelets and mature adipocytes (Eichholtz *et al.*, 1993; Sano *et al.*, 2002; Valet *et al.*, 1998). Because of its important roles throughout the body, aberrant LPA signaling has also been implicated in several diseases. This review focuses on the agents that have been developed to modulate LPA signaling and tested in disease models.

Lysophosphatidic Acid Receptor Signaling

Interest in LPA as a signaling molecule dates back to the late 1970s when effects on intracellular calcium release, platelet aggregation, and blood pressure were reported (Gerrard *et al.*, 1979; Tokumura *et al.*, 1978). While the involvement of G proteins was postulated (Moolenaar and van Corven, 1990), the mechanism of LPA signaling was not elucidated until 1996 when the first LPA receptor was cloned (Hecht *et al.*, 1996). Since the discovery of LPA_1 (originally Vzg-1 or Edg-2), five other LPARs have been validated. LPA_2 and LPA_3 were elucidated through homology searches by comparing amino acid sequences to that of LPA_1 (An *et al.*, 1998; Bandoh *et al.*, 1999). Through efforts aimed at finding ligands for orphan receptors, LPA_4 and LPA_5 were discovered (Kotarsky *et al.*, 2006; Lee *et al.*, 2006; Noguchi *et al.*, 2003). Most recently, LPA_6, a GPCR that is most closely related to LPA_4, was added to the ranks of LPA receptors (Pasternack *et al.*, 2008; Yanagida *et al.*, 2009).

LPAR signaling occurs through a variety of intracellular cascades (reviewed in (Mirendil *et al.*, 2013)). The binding of LPA or an LPA analog to its 7-transmembrane GPCR allows the G_α subunit to exchange used GDP for GTP. This results in G_α dissociating from G_β and G_γ, allowing the G_α and $G_{\beta\gamma}$ complexes to signal through downstream effectors. Several G_α subunits have been implicated in LPAR signaling, including $G_{\alpha 12/13}$, $G_{\alpha q/11}$, $G_{\alpha s}$, and $G_{\alpha i/O}$. Downstream effectors include activation of several pathways. The $G_{\alpha 12/13}$-mediated Rho/ROCK and Rho/SRF pathways have been implicated in cell motility, invasion, and cytoskeletal changes (Jeong *et al.*, 2012; Kim and Adelstein, 2011; Sotiropoulos *et al.*, 1999). The $G_{\alpha q/11}$ pathway activates phospholipase C (PLC), which induces IP3, and subsequently initiates Ca^{2+} and diacyl glycerol signaling (Sando and Chertihin, 1996). This cascade can result in vasodilation and a variety of transcriptional changes, including protein kinase C-induced cell growth, immune recruitment,

and changes in learning and memory (Cummings *et al.*, 2004; Lu *et al.*, 1999; Ruisanchez *et al.*, 2014; Seewald *et al.*, 1999). Induction of the $G_{\alpha s}$ pathway leads to adenylyl cyclase (AC) activation and the production of cAMP, preventing cell migration (Jongsma *et al.*, 2011). Activation of $G_{\alpha i/O}$ is the most versatile, as downstream effectors include PLC, Ras/MAPK-induced morphological changes (Kranenburg and Moolenaar, 2001), PI3K/Rac-mediated migration (Jimenez *et al.*, 2000), modulation of PI3K/Akt survival mechanisms (Kang *et al.*, 2004; Ye *et al.*, 2002), and inhibition of AC.

Each LPAR has multiple important regulatory functions throughout the body (reviewed in (Yung *et al.*, 2014)). Many of these have been elucidated through the use of knockout animals, pharmacological LPAR agonists or antagonists, and gene association studies. The first discovered LPAR, LPA_1, appears to be responsible for several developmental, physiological, and pathological processes. These include cell survival, proliferation, adhesion, migration, immune function, and myelination (reviewed in (Fukushima *et al.*, 2001)). LPA_2 signaling has also been implicated in cell survival, migration, immune function, and myelination (reviewed in (Ishii *et al.*, 2004)), often appearing to contribute to complementary LPA_1 mechanisms (Contos *et al.*, 2002). LPA_3, while expressed in many different tissues, is most heavily characterized as being involved in reproduction; it mediates fertility, embryo spacing, and embryo implantation (Ye *et al.*, 2005). LPA_4 influences cell aggregation, cell adhesion, vascular development, and osteogenesis regulation (reviewed in (Mirendil *et al.*, 2013)). Additionally, LPA_4-mediated adhesion appears to counteract LPA_1/LPA_2-stimulated migration processes (Lee *et al.*, 2008). LPA_5 also negatively regulates cell motility and is involved in chemokine release (Jongsma *et al.*, 2011; Lundequist and Boyce, 2011). Although LPA_6 is the most recently discovered LPAR, several genome screening studies have been published linking mutations in LPA_6 to genetic hair

loss and autosomal recessive hypotrichosis, or "wooly hair" syndrome (Azeem *et al.*, 2008; Pasternack *et al.*, 2008; Petukhova *et al.*, 2008). LPA_6 is also under investigation for further functionality. The effects of LPAR signaling are outlined in **Figure 1**.

Pharmacological Advances Modulating LPA Signaling

As LPAR signaling has been strongly implicated in many disease states, great interest has been expressed in developing specific LPAR inhibitors. Currently, no LPA or LPAR-targeting drugs have been FDA approved, though several are in development or undergoing clinical trials (Yung *et al.*, 2014) (**Table 1**). Furthermore, the ability to develop safe and efficacious drugs targeting lysophospholipid signaling has already been proven; fingolimod (FTY720), an analog of sphingosine 1-phosphate (S1P) and inhibitor of S1P receptors, has been FDA-approved for the treatment of multiple sclerosis (Brinkmann *et al.*, 2002; Calabresi *et al.*, 2014; Chun and Hartung, 2010).

LPA signaling has long been implicated in immune reactions (reviewed in (Lin and Boyce, 2006)). To this end, several therapeutic advances have been made concerning autoimmune disorders. In fact, an $LPA_{1/3}$ inhibitor, SAR100842, has completed phase II clinical trials to protect against systemic sclerosis (Sanofi, 2014), an autoimmune disorder characterized by accumulated collagen in connective tissue, leading to scarring of the skin and vasculature (Lafyatis, 2014). LPA_1 inhibitors are also of great interest in fibrosis, with BMS-986202 (previously AM152) having successfully completed phase I and BMS-986020 beginning phase II clinical trials for idiopathic pulmonary fibrosis (IPF) (2011; BMS, 2011, 2014). The LPA_1 inhibitor AM966 and the $LPA_{1/3}$ antagonist VPC12249 have also shown efficacy in murine IPF studies (Okusa *et al.*, 2003; Swaney *et al.*, 2010). Concurrently, an LPA_3 agonist, oleoyl-

methoxy phosphothionate (OMPT), enhanced IPF injury and reduced the therapeutic effects of VPC12249, suggesting that LPA_3 signaling may also be relevant in fibrotic disease. The pan-LPAR antagonist HLZ-56 and LPA_1 inhibitor AM095 attenuated kidney and dermal fibrosis in mouse models by preventing Smad2 phosphorylation, which reduced TGFβ signaling and subsequent CTGF release (Castelino *et al.*, 2011; Geng *et al.*, 2012; Swaney *et al.*, 2011), a mechanism that may be central to LPAR inhibitor effectiveness in other fibrotic disorders.

Much of the enthusiasm for LPAR therapies is directed at cancer, as LPAR signaling has been shown in numerous studies to promote motility and invasion of several cancer types, including breast, ovarian, colon, and brain tumors (Hama *et al.*, 2004; Hayashi *et al.*, 2012; Hoelzinger *et al.*, 2008; Mills *et al.*, 2002). *In vitro* studies utilizing the pan LPAR/ATX antagonist α-bromomethylene phosphonate LPA (BrP-LPA) and $LPA_{1/3}$ antagonists Ki16425, Ki16198, and Debio 0719 have been shown to decrease tumor aggressiveness and increase radiosensitivity through varied mechanisms, including inhibited Rho/ROCK and MEK/ERK signaling, prevention of FAK/paxillin localization to focal adhesions, and reduced matrix metalloproteinase accumulation (Hama *et al.*, 2004; Komachi *et al.*, 2012; Liao *et al.*, 2013; Marshall *et al.*, 2012; Schleicher *et al.*, 2011; Su *et al.*, 2013; Zhang *et al.*, 2009). While many studies focus on the migratory effects of LPA_1 signaling, use of the LPA_2 inhibitor "compound 35" attenuated Erk phosphorylation and reduced proliferation of colorectal cancer cells (Beck *et al.*, 2008). LPA itself has been proposed as a screening molecule for ovarian cancer, as increased levels of LPA have been repeatedly observed in the blood of patients with malignant ovarian tumors and may have prognostic value in lung cancer patients as well (Bai *et al.*, 2014; NCI, 2014; Sedlakova *et al.*, 2011). Although no LPAR-targeting cancer drugs have reached

clinical trial stages thus far, pharmaceutical inquiry is progressing rapidly and the initiation of cancer-focused clinical trials is projected to follow.

In addition to cancer and fibrosis, LPAR inhibitors have been utilized as potential therapeutics in other areas of study. For instance, Ki16425 and BrP-LPA have been shown to decrease the clinical score of murine arthritis (Nikitopoulou *et al.*, 2013; Orosa *et al.*, 2014). The development of an LPA-induced neonatal model of post-hemorrhagic hydrocephalus was also abrogated utilizing Ki16425 (Yung *et al.*, 2011). While LPA signaling is reported to be involved in wound-healing processes (Lee *et al.*, 2000), it may exacerbate severe trauma. In fact, anti-LPA antibodies that diminish LPAR binding and activation have shown some efficacy in modulating murine brain lesion severity and recovery (Crack *et al.*, 2014; Goldshmit *et al.*, 2012), although the actual mechanism of these immunological agents and remains to be determined. Additionally, Bristol-Myers Squibb has patented LPAR inhibitors for spinal cord injury and neuropathic pain indications (Nogueira and Vales, 2013), since there is a substantial body of evidence implicating LPA_1 and LPA_5 signaling in the initiation and maintenance of neuropathic pain (reviewed in (Ueda *et al.*, 2013)).

The most common output for screening drug efficacy against an LPAR is determining the status of Ca^{2+} influx within the tested cell types. Generally, LPAR agonists will increase intracellular Ca^{2+} mobilization while LPAR antagonists will inhibit Ca^{2+} release. Using this method, several studies have been published on the synthesis and relative efficacy of potential therapeutics against LPA_{1-3}, LPA_{1-5}, and more recently LPA_{1-6} (reviewed in (Im, 2010)). While this article only discusses pharmacological modulators with functional, disease-related readouts, a more comprehensive list of LPAR agonists and antagonists can be found in a previous review (Yung *et al.*, 2014).

Compounds Targeting ATX Inhibition

In addition to direct pharmacological modulation of LPARs, several research groups have targeted the upstream enzyme ATX for discovery of potential therapeutics (**Table 1**). ATX inhibitors prevent the enzymatic conversion of LPC to LPA. As ATX expression can account for at least half of plasma LPA levels (Tanaka *et al.*, 2006; van Meeteren *et al.*, 2006), these drugs ultimately attenuate LPA signaling. Although this pathway lies upstream of LPAR signaling, targeting ATX allows for structure-based drug design (Fells *et al.*, 2013; Kawaguchi *et al.*, 2013; Norman *et al.*, 2013), a process that is limited in LPAR drug discovery because of the lack of receptor crystal structures; work in progress should rectify this deficiency.

In particular, oncology researchers are interested in developing these agents. Several ATX inhibitors have been synthesized and tested in tumor migration, metastasis, survival, and radiosensitivity studies. These inhibitors include the small molecules ONO-8430506 (Benesch *et al.*, 2014) and PF-8380 (Bhave *et al.*, 2013; St-Coeur *et al.*, 2013), lipid analogs 4PBPA (Gupte *et al.*, 2011) and pan-ATX/LPAR antagonist BrP-LPA (Schleicher *et al.*, 2011; Xu and Prestwich, 2010), and gintonin – a plant-derived LPA/ginseng glycolipoprotein complex that results in feedback inhibition of ATX through LPAR signaling (Hwang *et al.*, 2013). These compounds ultimately reduced survival and invasive behaviors of *in vitro* cancer cells and tumor xenografts. As ATX and LPARs are often upregulated in cancer (reviewed in (Gotoh *et al.*, 2012)), the success of these compounds in research may spur therapeutic development.

ATX antagonism is also being investigated as a solution to inflammatory disease. PF-8380 has been shown to drastically reduce plasma LPA concentrations during inflammation (Gierse *et al.*, 2010), suggesting that targeting ATX may be useful to reduce chronic

inflammation. As mentioned above, BrP-LPA has been utilized to ameliorate arthritis in mice (Nikitopoulou *et al.*, 2013). Furthermore, GWJ-A-23 showed efficacy in attenuating allergen-induced asthmatic attacks and bleomycin-induced IPF (Oikonomou *et al.*, 2012; Park *et al.*, 2013). The effects of reduced LPA signaling stretch even further, as the potent ATX inhibitor S32826 has been utilized to decrease intraocular pressure in a rabbit model of glaucoma (Iyer *et al.*, 2012).

Conclusion

Over the past four decades, interest in the signaling lipid LPA has grown from understanding its synthesis to encompassing several key processes in development and disease. To this end, several compounds have been fine-tuned by researchers and pharmaceutical companies to inhibit LPARs and ATX in order to mitigate the destructive pathologies related to cancer, autoimmune diseases, and other afflictions. The LPA_1-targeting inhibitors SAR100842, BMS-986202, and BMS-986020 have passed phase I or phase II clinical trials with the potential of advancing further toward FDA approval. The increasing availability of chemical tool compounds will enhance our understanding of LPAR signaling mechanisms in disease towards the development of new disease-modifying therapeutics.

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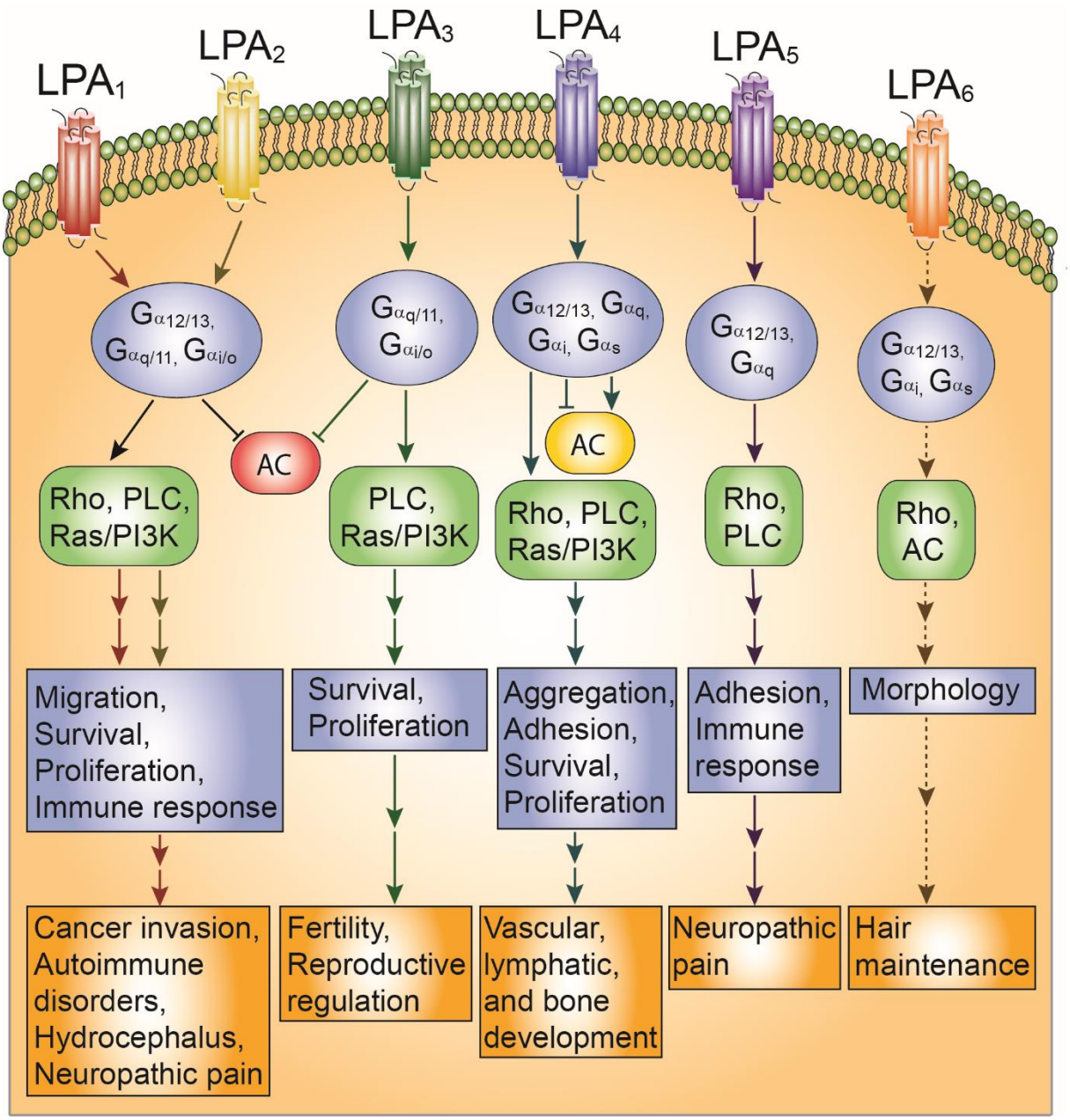


Figure 2.1: LPAR signaling details and functional outcomes.

LPAR signaling details are highlighted for each receptor, based on canonical GPCR pathways that have been validated. Dashed lines indicate preliminary data that require further confirmation. Activated downstream effectors are shown in green, inhibited effectors in red, and effectors that are differentially activated or inhibited in yellow. The cellular effects of activating each LPAR are listed beneath the G_{α} cascades, followed by ultimate phenotypical outcomes as highlighted in this review. Antagonism or functional knockout of each LPAR has been proven to inhibit these disorder phenotypes.

Table 2.1: Summary of compounds that target LPA signaling.

The name, target, structure and development stage for each LPA signaling antagonist discussed in the article are outlined, along with their therapeutic indications.

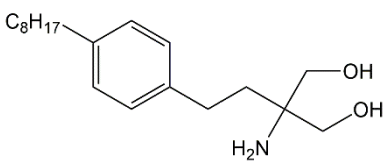
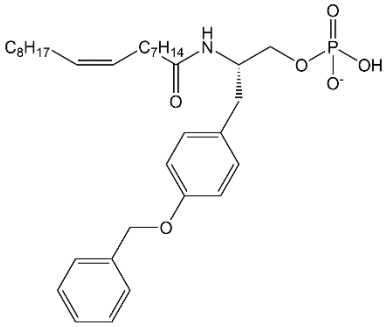
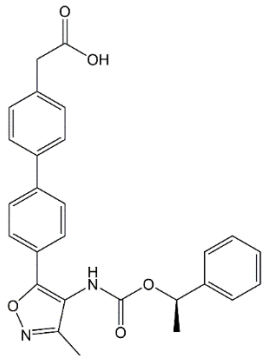
Drug	Target	Structure	Phase	Indication	Reference
FTY720	$S1P_1$, $S1P_{3-5}$		FDA approved	Multiple sclerosis	(Brinkmann *et al.*, 2002; Chun and Hartung, 2010)
BMS-986202/ AM152	LPA_1	See patent WO/2012/162592 A1 for more information	Phase I complete	Idiopathic pulmonary fibrosis	(BMS, 2011; Bradford, 2012)
BMS-986020	LPA_1	See patent WO/2012/162592 A1 for more information	Phase II complete	Idiopathic pulmonary fibrosis	(BMS, 2014; Bradford, 2012)
VPC 12249	LPA_1		Preclinical	Idiopathic pulmonary fibrosis	(Okusa *et al.*, 2003)
AM095	LPA_1		Preclinical	Dermal fibrosis, kidney fibrosis	(Castelino *et al.*, 2011; Swaney *et al.*, 2011)

Table 2.1: Summary of compounds that target LPA signaling, continued

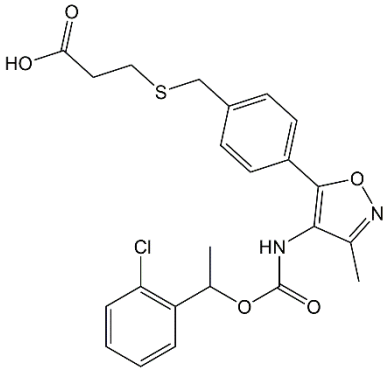
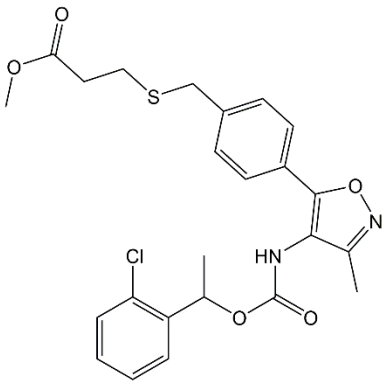
Drug	Target	Structure	Phase	Indication	Reference
BMS patent	LPA_1	See patent WO/2013/070879 A1 for more information	Preclinical	Spinal injury, neuropathic pain	(Nogueira and Vales, 2013)
SAR 100842	LPA_1, LPA_3	See patent WO/2012/162592 A1 for more information	Phase II complete	Systemic sclerosis	(Bradford, 2012; Sanofi, 2014)
Ki16425	LPA_1, LPA_3		Preclinical	Cancer, rheumatoid arthritis, hydro-cephalus	(Hama *et al.*, 2004; Liao *et al.*, 2013; Orosa *et al.*, 2014; Su *et al.*, 2013; Yung *et al.*, 2011)
Debio 0719	LPA_1, LPA_3	R-stereoisomer of Ki16425	Preclinical	Cancer	(Marshall *et al.*, 2012)
Ki16198	LPA_{1-3}		Preclinical	Cancer	(Komachi *et al.*, 2012)

Table 2.1: Summary of compounds that target LPA signaling, continued

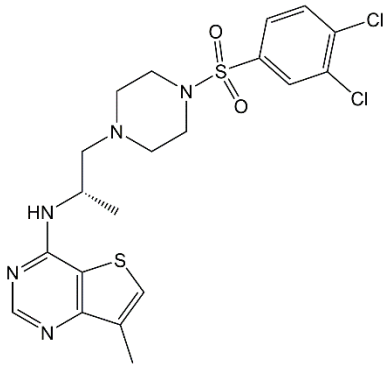
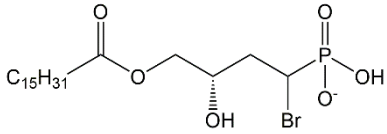
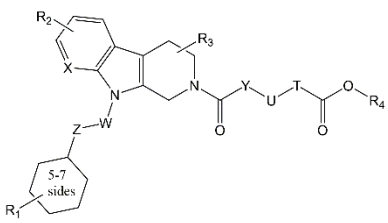
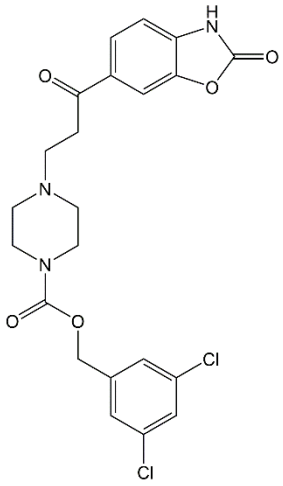
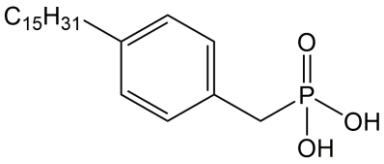
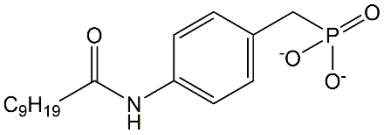
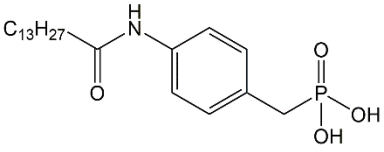
Drug	Target	Structure	Phase	Indication	Reference
Cmpd. 35	LPA_2		Preclinical	Cancer	(Beck *et al.*, 2008)
Anti-LPA	All LPAR signaling	Antibody	Preclinical	Traumatic brain injury	(Crack *et al.*, 2014; Goldshmit *et al.*, 2012)
HLZ-56	All LPARs	Unavailable	Preclinical	Kidney fibrosis	(Geng *et al.*, 2012)
BrP-LPA	ATX, all LPARs		Preclinical	Cancer, rheumatoid arthritis	(Nikitopoulou *et al.*, 2013; Schleicher *et al.*, 2011; Xu and Prestwich, 2010; Zhang *et al.*, 2009)
ONO-8430506	ATX	 Backbone only, see patent WO/2012/005227 A1	Preclinical	Cancer	(Benesch *et al.*, 2014; Morimoto, 2012)

Table 2.1: Summary of compounds that target LPA signaling, continued

Drug	Target	Structure	Phase	Indication	Reference
PF-8380	ATX		Preclinical	Cancer, inflamm-ation	(Bhave *et al.*, 2013; Gierse *et al.*, 2010; St-Coeur *et al.*, 2013)
4PBPA	ATX		Preclinical	Cancer	(Gupte *et al.*, 2011)
Gintonin	ATX	Glycolipoprotein, structure not available	Preclinical	Cancer	(Hwang *et al.*, 2013)
GWJ-A-23	ATX		Preclinical	Asthma, idiopathic pulmonary fibrosis	(Oikonomou *et al.*, 2012; Park *et al.*, 2013)
S32826	ATX		Preclinical	Glaucoma	(Iyer *et al.*, 2012)

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Chapter 3:

Lysophosphatidic acid (LPA) signaling in the nervous system

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Summary

The brain is composed of many lipids with varied forms that serve as essential structural and signaling molecules. Lysophosphatidic acid (LPA) is an important bioactive lipid species that is part of the lysophospholipid (LP) family. LPA is primarily derived from membrane phospholipids and signals through six cognate G protein-coupled receptors (GPCRs), LPA_{1-6}. These receptors are expressed on most cell types within central and peripheral nervous tissues and have been functionally linked to many neural processes and pathways. This review covers a current understanding of LPA signaling in the nervous system, with particular focus on the relevance of LPA to both physiological and diseased states.

Introduction

The human brain is composed of approximately 60-70% lipids by dry weight (O'Brien and Sampson, 1965; Svennerholm et al., 1994). These lipids can be divided into two major pools, structural and signaling, that include well-known families such as cholesterol, fatty acids, eicosanoids, endocannabinoids, and prostaglandins (**Fig. 1**). Structural lipids classically comprise cell membranes and may be passively organized by protein factors (reviewed in (Rossy et al., 2014)), whereas signaling lipids function in a predominantly extracellular fashion through receptors to activate downstream pathways (Bieberich, 2012). Lysophospholipids (LPs) are an important family of lipid signaling molecules and lysophosphatidic acid (LPA) is a major member of this family. LPA is found in numerous tissues and fluids, notably within the developing and adult nervous system, with widely varying concentrations that can be influenced by many factors. Despite early speculations on non-receptor mechanisms, LPA effects have been shown to act through cognate, cell surface GPCRs termed LPA receptors (LPARs, reviewed in (Noguchi et al., 2009; Choi et al., 2010; Yung et al., 2014)).

Since the discovery of the first LP receptor for LPA in the developing brain (Hecht et al., 1996), five other LPARs have been characterized and are all expressed in the nervous system. Many roles for LPA signaling have been elucidated, including effects on neuroprogenitor cell (NPC) function (Hecht et al., 1996; Fukushima et al., 2000; Fukushima et al., 2002b; Yung et al., 2011), myelination (Weiner et al., 1998; Anliker et al., 2013), synaptic transmission (Trimbuch et al., 2009), and brain immune responses (Moller et al., 2001; Schilling et al., 2004), which influence neural development, function, and behavior (**Fig. 1**). Additionally, LPA signaling influences endothelial cell function, in vasculogenesis and angiogenesis, and likely also influences the neurovasculature (English et al., 1999; Teo et al., 2009; Yukiura et al., 2011; Chen

et al., 2013). LPAR discovery also led to the identification of another LP receptor subclass, sphingosine 1-phosphate (S1P) receptors, that bind this distinct LP (Kihara et al., 2014). S1P and its five receptors have important nervous system activities (Chun, 1999; Chun et al., 1999; McGiffert et al., 2002; Meng and Lee, 2009; Meng et al., 2011; Choi and Chun, 2013; van Echten-Deckert et al., 2014), but they will not be covered here.

The ecosystem of neural LPA signaling has been considerably expanded with the characterization of both LPA precursors and LPA synthesis/degradative enzymes (Xu et al., 2000; Brindley et al., 2002; Koike et al., 2006; van Meeteren et al., 2006). In view of the broad neurobiological influences of LPA signaling, its dysregulation may lead to diverse neuropathologies, including brain malformations, neuropsychiatric disorders, neuropathic pain, and brain cancers (Bandoh et al., 2000; Houben and Moolenaar, 2011; Yung et al., 2011; Ueda et al., 2013). Wound healing can also be regulated by LPA signaling (Lee et al., 2000; Lee et al., 2013b), and regenerative processes following nervous system injury can also be LPA dependent. LPs and their relevance to non-nervous system tissues have been reviewed elsewhere (Choi et al., 2010; Mirendil et al., 2013).

LPA Metabolism and Distribution

LPA is a metabolite in the biosynthesis of membrane phospholipids and is ubiquitously present in all cells, tissues, and organ systems examined thus far. LPA exists in multiple chemical forms, with variations in acyl carbon chain saturation, chain length, and position on the glycerophosphate backbone. The generic term LPA (mono-acyl-*sn*-glycerol-3-phosphate) often refers to 18:1 oleoyl-LPA (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate) because of its widespread laboratory use. However, at least 15 distinct chemical forms have been described to date in

various biological systems, including the nervous system (Scherer et al., 2009; Aaltonen et al., 2010; Morishige et al., 2010; Triebl et al., 2014).

LPA is prominently found in blood fractions, where it ranges from 0.1 μM in plasma to more than 10 μM in serum. These concentrations are significantly higher than the estimated nanomolar K_ds of LPA_{1-6} (Aoki et al., 2002; Watanabe et al., 2007a; Hosogaya et al., 2008; Yanagida et al., 2009). Platelet release of precursor LPs and subsequent conversion generates the majority of LPA in serum, while plasma LPA can be derived from conversion of basal precursor LPs (Aoki et al., 2002; van Meeteren et al., 2006). Serum and plasma LPC can be bound to the carrier protein albumin (Ojala et al., 2006) and there is evidence that LPA may also be transported in this fashion (Eichholtz et al., 1993; Desmaret et al., 2005). Within the central nervous system (CNS), LPA is found in the embryonic brain, spinal cord, and cerebrospinal fluid (CSF) at low nanomolar to micromolar levels (Gerrard et al., 1979; Tokumura et al., 1986; Liliom et al., 1998; Sano et al., 2002; Tanaka et al., 2004; Ma et al., 2010; Yung et al., 2011; Tokumura et al., 2012; Yung et al., 2014). LPA is generated through several different enzymatic pathways (**Fig. 1**). One route of LPA formation is the enzymatic conversion of precursor phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), or phosphatidylethanolamine (PE) into the derivative chemical species lysophosphatidylcholine (LPC), lysophosphatidylserine (LPS), or lysophosphatidylethanolamine (LPE). In plasma or serum, this involves the sequential actions of lecithin cholesterol acyltransferase (LCAT) and phospholipase A1 (PLA_1). In platelets, this occurs via phosphatidylserine-specific PLA_1 (PS-PLA_1) or secretory phospholipase A2 ($sPLA_2$). In either location, these LPs can then be converted to LPA by autotaxin (ATX), a lysophospholipase D and major LPA-producing enzyme (Tanaka et al., 2006; van Meeteren et al., 2006; Albers et al., 2010). It is expressed

and/or present in various neural-related tissues, including the neural tube, choroid plexus, meninges, and blood vessels (Bachner et al., 1999; Savaskan et al., 2007), as well as CSF and blood (Sato et al., 2005; Nakamura et al., 2007; Nakamura et al., 2009).

Constitutive removal of ATX results in neural and vascular defects with organismal death by embryonic day E9.5 (Tanaka et al., 2006; van Meeteren et al., 2006). *Enpp2*$^{+/-}$ (the ATX gene name) mice have an approximately 50% reduction of LPA levels in plasma, but not serum (van Meeteren et al., 2006; Fotopoulou et al., 2010). In addition, Sox-2 mediated *Enpp2*-$^{-/-}$ conditional deletion from epiblasts resulted in failure of neural tube closure, reduced mitosis, and increased cell death in the neural tube (Fotopoulou et al., 2010). ATX structure, splice variants, and functional domains are being actively examined (reviewed in (Perrakis and Moolenaar, 2014)).

Through a second major pathway, phosphatidic acid (PA) is generated from phospholipids by phospholipase D or from diacylglycerol through diacylglycerol kinase. Subsequently, PA is converted into LPA by either PLA_1 or PLA_2 (reviewed in (Aoki et al., 2008)). In all of these metabolic pathways, the generated chemical forms of LPA directly reflect its precursor phospholipid structure (e.g., 18:1 LPC produces 18:1 LPA) (Sugiura et al., 1999; Aoki, 2004). Other pathways for generating LPA involve the acylation of glycerol-3-phosphate by glycerophosphate acyltransferase (GPAT) or the phosphorylation of monoacylglycerol (MAG) by monoacylglycerol kinase (MAG-kinase) (Bektas et al., 2005). Further enzymatic pathways to produce LPA also exist (reviewed in (Pages et al., 2001; Aoki et al., 2008)).

These biosynthetic pathways generate both intracellular and extracellular LPA (reviewed in (Pages et al., 2001)). Extracellular LPA produces signaling through its receptors (reviewed in (Choi et al., 2010; Lin et al., 2010; Mutoh et al., 2012; Mirendil et al., 2013; Yung et al., 2014)

and noted below). Intracellular LPA serves as an intermediate for the *de novo* biosynthesis of complex glycerolipids, including mono-, di-, and triglycerides, as well as other phospholipids (reviewed in (Pages et al., 2001)). Additionally, these lipid pools can affect membrane vesicular curvature (McMahon and Gallop, 2005).

LPA can be degraded by liberation of the phosphate group to produce MAG. This process is mediated by several classes of enzymes, including three lipid phosphate phosphatases (LPPs) (Brindley and Pilquil, 2009; Tomsig et al., 2009), LPA acyltransferase (LPAAT), and various phospholipases (e.g., PLA_1 or PLA_2) (Pages et al., 2001; Saba, 2004; Kok et al., 2012). LPA can also be converted to PA by LPAAT, hydrolyzed by LPP(1-3), or converted by phospholipases into glycerol-3-phosphate (Pages et al., 2001; Brauer et al., 2003). Inhibition of LPP(1-3)-mediated degradation can elevate LPA levels in cerebral cortical tissue (Aaltonen et al., 2012).

The tissue and fluid distributions of LPA and its related metabolites are relevant to both basic and medical applications (reviewed in (Yung et al., 2014)). Total LPA can be measured via radioenzymatic, fluorometric, colorimetric, or immunoenzymatic assays (Hosogaya et al., 2008; Jesionowska et al., 2014). Individual chemical forms can be parsed by strategies that involve chromatography coupled to mass spectrometry (Smyth et al., 2008; Aaltonen et al., 2010; Lee et al., 2013a; Triebl et al., 2014), including visualization of the spatial distribution of lipids (Patti et al., 2010). These and future techniques should prove useful in analytical and diagnostic settings (Watanabe et al., 2007a; Watanabe et al., 2007b; Okudaira et al., 2010; Yung et al., 2011; Dohi et al., 2012; Ikeda et al., 2013).

LPARs

There are currently six LPARs: protein names **LPA_1–LPA_6** and gene names ***LPAR1-LPAR6*** (human) and ***Lpar1-Lpar6*** (non-human) (reviewed in (Chun et al., 2010; Davenport et al., 2013; Kihara et al., 2014; Yung et al., 2014)). These 7-transmembrane GPCRs activate heterotrimeric G proteins defined by their G_α subunits ($G_{12/13}$, $G_{q/11}$, $G_{i/o}$, and G_s) to initiate a variety of signaling cascades. The spatiotemporal neural distributions of LPA, related metabolites, and LPAR expression drive diverse physiological and pathophysiological processes within the nervous system, as discussed below (**Fig. 2**). During development, the cell types that comprise the brain arise from distinct lineages, undergo cellular processes including proliferation, apoptosis, morphological changes, migration, and differentiation, develop specialized functions, and integrate into a functional network (Franco and Muller, 2013; Greig et al., 2013). LPARs and involved enzymes are expressed on these cell types in varying spatiotemporal patterns from fetal through mature life (**Fig. 2**).

Fetal brain development

During cerebral cortical development, neuroepithelial cells proliferate to give rise to the ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), cortical plate, and marginal zone (MZ) (Bystron et al., 2008) (**Fig. 2**). NPCs that reside in the VZ undergo interkinetic nuclear migration (INM) and proliferate to generate distinct progenitor pools, differentiate to generate nascent neurons, and migrate outward to locate within cell layers and establish functional connections (Noctor et al., 2001; Noctor et al., 2004). Astrocytes and oligodendrocytes are generated during late embryonic and early postnatal periods, and together with neurons, comprise basic cellular elements of the cortex, complemented by other cell types

such as endothelial cells, pericytes, ependymal cells, microglia, meninges, and cells of the choroid plexus **(Fig. 2)**.

Enrichment of *Lpar1* in the VZ and meninges of the developing cortex suggests roles for LPA signaling in cortical development, as reflected in its original name, ventricular zone gene-1 (VZG1) (Hecht et al., 1996; Fukushima et al., 1998; Weiner et al., 1998). The generation and characterization of LPAR-null mice have been crucial to elucidating the receptor dependency of diverse LPA-associated neural phenotypes. Constitutive deletion of *Lpar1* results in 50% perinatal lethality associated with olfactory and other nervous system defects (Contos et al., 2000; Harrison et al., 2003). *Ex vivo* whole cerebral cortical cultures treated with LPA form thicker cortices through decreased cell death within the VZ (rather than through increasing cell proliferation) and have an increased post-mitotic neuronal population (Kingsbury et al., 2003). The concomitant removal of *Lpar1* and *Lpar2* prevents these effects, demonstrating receptor dependency. Meninges at E13, as assessed by mouse embryonic meningeal fibroblasts (MEMFs), respond to the addition of LPA by the formation of actin-based stress fibers (Contos et al., 2002). MEMFs appear to have relatively high levels of *Lpar1*, low levels of *Lpar2*, and undetectable expression of *Lpar3* (Contos et al., 2002). Removal of both *Lpar1* and *Lpar2* prevents formation of LPA-induced MEMF stress fibers, which was not blocked in single *Lpar1* or *Lpar2* knockouts.

LPA has multiple effects on NPCs. The earliest calcium conductance changes appear to be mediated by LPA signaling and occur before development of ionic responsivity induced by neurotransmitters such as GABA and glutamate (Dubin et al., 1999; Dubin et al., 2010). Interestingly, fluctuations in electrical activity based on calcium signaling are known to impact NPC proliferation, neuronal differentiation, chemotaxis, dendritic morphology, axon growth and

guidance, and neurotransmitter phenotype (reviewed in (Rosenberg and Spitzer, 2011)), all of which can be influenced by LPA signaling.

INM is the to-and-fro movement of nuclei from apical to basal positions in the VZ during cell cycle progression. *Ex vivo* and *in vivo* cortical exposure to LPA disturbs normal INM such that an increased percentage of these mitotic cells are found in the basal position, resulting in mitotic displacement (Kingsbury et al., 2003; Yung et al., 2011). Mitotic displacement also occurs in an *Lpar1*-dependent manner under hypoxic culture conditions (Herr et al., 2011). Future studies could refine the understanding of LPA signaling on specific progenitor populations in this context, such as basal progenitors (Haubensak et al., 2004), intermediate progenitors (Englund et al., 2005), or outer subventricular zone radial glia-like cells (oRGs) (Hansen et al., 2010; Wang et al., 2011).

A hallmark of cortical development is the migration of newly generated neurons to their final superficial location within the cortical plate. This migration is aided by leading processes that help sense the surrounding environment for molecular signaling cues, such as growth factors, chemoattractants, and chemorepellents. *In vitro* studies utilizing the B103 cell line, wild type E12 embryonic nestin-positive cortical progenitor cells, and E12 cortical explant cultures identified protruding lamellipodia and growth cones that rapidly retracted upon LPA exposure in a Rho-dependent manner, leaving fine F-actin retraction fibers, supporting LPA's role as an inhibitory cue during cell migration or process outgrowth (Fukushima et al., 1998; Fukushima et al., 2000; Campbell and Holt, 2001, 2003).

During corticogenesis, programmed cell death can affect significant populations of developing cells (Blaschke et al., 1996; Kuida et al., 1996; Blaschke et al., 1998; Yung et al., 2009; Peterson et al., 2012). Culturing *ex vivo* cortices in LPA reduces cell death in an *Lpar1*

and *Lpar2* dependent manner (Kingsbury et al., 2003). LPA signaling activates cell survival pathways, including thymoma viral proto-oncogene (Akt) (Weiner and Chun, 1999) and β-catenin (Weiner et al., 2001). Moreover, β-catenin and T cell factor signaling can be activated by LPA and can contribute to the suppression of apoptosis in H197 cells (an embryonic hippocampal progenitor cell line) (Sun et al., 2013). LPA signaling has also been reported to influence neurosphere proliferation in culture (Svetlov et al., 2004). NPCs express multiple LPAR subtypes (**Fig. 2**) and LPA signaling can influence aspects of their development and function.

Neurons

In mice, cortical neurogenesis extends from E10-E18, during which NPCs divide to produce other progenitors and young postmitotic neurons. LPA promotes cortical NPCs to commit to the neuronal lineage via LPA_1 and the $G_{i/o}$ pathway (Kingsbury et al., 2003; Fukushima et al., 2007). Additionally, LPA can alter the actin cytoskeleton and promote microtubule rearrangement within neurons (Fukushima et al., 2002a; Fukushima and Morita, 2006). Therefore, it is not surprising that the morphology and motility of young postmitotic neurons is also influenced by LPA exposure (Fukushima et al., 2002b). The retraction of neurites, which is an important neuronal response to chemical gradients, can be mediated by LPA via the ROCK pathway (Smalheiser and Ali, 1994; Tigyi et al., 1996; Magazin et al., 1998; Satoh et al., 2011). More recently, "transient receptor potential channel, subfamily M, member 2 (TRPM2)" was reported to mediate LPA-induced neurite retraction in the developing brain (Jang et al., 2014). Finally, neurite branching, an important process for neuronal network formation, was induced through the introduction of LPA_3 and the addition of LPA into hippocampal cell

cultures (Furuta et al., 2012). This physiological response was initiated through G_q and the Rho family GTPase 2 (Rnd2) (Furuta et al., 2012).

Maturing neurons establish polarity through the specification and development of neurites into axons and dendrites. In hippocampal neuronal cultures, the bases of axons were predominantly found distal to an exogenous LPA source (Yamane et al., 2010). Interestingly, localization of the Golgi apparatus and centromeres, which are associated with the establishment of neuronal polarity before axonogenesis (de Anda et al., 2005), were positioned distal to the LPA source, suggesting that LPA signaling can influence the site of axonal sprouting (Yamane et al., 2010). *Lpar6*, the most recently characterized LPAR family member, is enriched in the neural plate of Xenopus neurulae. Deletion of *Lpar6* results in forebrain defects with concomitant reduction of telencephalic markers, as well as defects extending into the hindbrain (Geach et al., 2014). The roles of *Lpar6* in the development and function of the mammalian nervous system remain to be characterized, although human mutations of this receptor gene have most notably been associated with forms of hair loss (Pasternack et al., 2008; Pasternack et al., 2009; Shimomura et al., 2009; Kurban et al., 2013).

Astrocytes

In vivo, astrocytes appear to express few LPA receptor subtypes aside from low levels of *Lpar1* (Allard et al., 1998; Tabuchi et al., 2000; Weiner et al., 2001; Cervera et al., 2002), whereas cultured astrocytes express *Lpar1-5* (Sorensen et al., 2003; Shano et al., 2008). Receptor subtype expression depends on age, species (Rao et al., 2003), and cell activation state. The expression of newly classified *Lpar6* in astrocytes remains to be examined.

During development, LPA has numerous effects on cultured astrocytes, including intracellular calcium mobilization (Tabuchi et al., 2000), generation of reactive oxygen species, and actin cytoskeletal rearrangement (Guasch et al., 2003; Spohr et al., 2008). Most studies have shown LPA-mediated DNA synthesis and proliferation in astrocytes (Ramakers and Moolenaar, 1998; Sorensen et al., 2003; Shano et al., 2008), though some conflicting reports exist. This discrepancy may be a function of the employed LPA concentration (Fuentes et al., 1999; Shano et al., 2008), astrocyte origin, species, or cell selection in culture (Pebay et al., 1999; Furukawa et al., 2007). Additionally, LPA can induce astrocytes to express immediate early genes through pertussis-toxin sensitive G proteins, as well as cytokine genes, including nerve growth factor (NGF), interleukin (IL)-1β, IL-3, and IL-6 (Tabuchi et al., 2000).

In culture, cross-talk between neurons and astrocytes through LPA signaling has been reported. For example, LPA-activated astrocytes can induce neuronal differentiation (Spohr et al., 2008) as well as axonal outgrowth of neurons via the extracellular matrix protein and epidermal growth factor signaling pathway (Spohr et al., 2011). The production of NGF can also be enhanced by astrocytic exposure to LPA and other LPs (Furukawa et al., 2007). Astrocyte proliferation and astrogliosis appear to be mediated through LPA signaling, likely through *Lpar1-3* (Sorensen et al., 2003). Finally, stimulation of astrocytes, which are normally insensitive to LPA, by lipopolysaccharide (LPS) or IL-1β induced LPA-responsive astrocyte migration (Sato et al., 2011). It is likely that other LPA-induced paracrine signals exist amongst cell types within the nervous system.

Oligodendrocytes

Oligodendrocytes express *Lpar1* postnatally in a spatiotemporal manner that correlates with maturation and myelination (Allard et al., 1998; Weiner et al., 1998), peaking between postnatal P15-P21 (Weiner et al., 1998). *In vitro*, LPA induces calcium mobilization, extracellular-regulated-kinase 1/2 (ERK1/2) phosphorylation, process retraction, and cell rounding in mature oligodendrocytes, but not in oligodendrocyte precursors (Moller et al., 1999). Early studies reported that *Lpar1* in oligodendrocytes does not appear to promote myelination (Stankoff et al., 2002). However, a subsequent study using a combination of the ATX knockout with LPA exposure supports a role for LPA signaling on the transition from process outgrowth to membrane sheath formation as oligodendrocytes switch from premyelinating to myelinating forms (Nogaroli et al., 2009). This is consistent with increasing ATX expression during initial myelination (Nogaroli et al., 2009). The maintenance of *Lpar1* in oligodendrocytes does not appear to require an axonal signal from neurons, suggesting that it is regulated by an intrinsic oligodendrocyte program (Stankoff et al., 2002). Finally, LPA stimulates process formation and increases the number of differentiating, but not mature, oligodendrocytes (Nogaroli et al., 2009).

Schwann cells

Schwann cells (SCs), which are peripheral nervous system counterparts to oligodendrocytes, are derived from the neural crest and are also responsive to LPA signaling. At least three types of SCs exist. Myelinating SCs form the myelin sheath around neuronal axons and express *Lpar1* and possibly *Lpar2*. Perisynaptic SCs (also called terminal SCs) are present at the neuromuscular junction, express both *Lpar1* and *Lpar3* (Weiner et al., 2001; Kobashi et al., 2006), and have known functions in synaptic transmission, synaptogenesis, and nerve

regeneration (Armati and Mathey, 2013). The expression of LPARs in non-myelinating SCs, which ensheath non-myelinated fibers, is currently unknown. LPA_1 signaling reduces SC death through G_i, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and Akt *in vitro* (Weiner and Chun, 1999), which is supported *in vivo* by observations that *Lpar1*-null mice have increased apoptosis of SCs in sciatic nerves (Contos et al., 2000).

In addition to promoting SC survival, LPA also induces dynamic alterations to the actin cytoskeleton, with corresponding morphological and cell adhesion changes (Weiner and Chun, 1999; Weiner et al., 2001). *In vitro*, the effects of LPA on SCs include promoting wreath formation, activating N-cadherin dependent cell aggregation, and enhancing focal adhesions. These responses are dramatically reduced in *Lpar1*-null SCs (Weiner et al., 2001). P0 protein, a glycoprotein that is a major structural component of the myelin sheath, can be increased in SCs by LPA_2 signaling, which may contribute to SC differentiation (Li et al., 2003). Recently, it was shown that LPA via LPA_1 signaling coupled to $G\alpha_i$ and Rac1, can promote embryonic SC migration, myelination, and cell-to-axon segregation. By contrast, *Lpar1*-null mice showed delayed SC-to-axon segregation, abnormal polyaxonal myelination, and thinner myelin sheaths (Anliker et al., 2013).

In adult SCs isolated from axotomized sciatic nerve, *Lpar1* and *Lpar2* are coincidentally upregulated during post-axotomy SC proliferation, suggesting that LPA signaling promotes SC division in regenerating peripheral nerves (Weiner et al., 2001; Frohnert et al., 2003). In contrast, LPA also induces demyelination of SCs in dorsal root *ex vivo* culture as well as in mice that have been intrathecally injected with LPA (Inoue et al., 2004; Fujita et al., 2007).

Microglia

Microglia, derived from hematopoietic stem cells, are the resident macrophages of the CNS, showing multiple types and activation states including ramified (resting or quiescent) or activated. Activated microglia can scavenge, present antigens, phagocytose, and release inflammatory mediators during injury and neurodegeneration (Hu et al., 2014). Microglia from mouse or rat mainly express *Lpar1* and/or *Lpar3* (Moller et al., 2001; Tham et al., 2003), while human microglial cell lines express *Lpar1-3* (Moller et al., 2001; Bernhart et al., 2010). Receptor activation by LPA can induce intracellular calcium mobilization and potassium channel activation, as well as cell proliferation, cell morphology changes, upregulated chemokinesis, and membrane ruffling (Schilling et al., 2004; Fujita et al., 2008; Muessel et al., 2013). GPCR reciprocal modulation by both LPA and stromal cell-derived factor-1 (SDF-1) on their respective receptors produces microglial morphological changes through inward-rectifier potassium channel 2.1 (Kir2.1) modulation, although the specific LPAR subtype remains to be defined. As with other cell types in the brain, LPAR profiles and responses likely vary with microglia maturation, activation state, and species source.

Choroid plexus

The choroid plexus is the major site of CSF production in the nervous system and is present in the lateral, third, and fourth ventricles. The choroid plexus consists of cuboidal epithelial cells surrounding a central area with capillaries and is contiguous with the ependymal layer. Unlike the ependyma, the choroid plexus is structurally distinct with tight gap junctions that connect the apical surface of the epithelial cells (Dziegielewska et al., 2001). There is a report of *Lpar5* expression in the fourth ventricular choroid plexus of the developing mouse

embryo, while none of the other LPARs were reported in choroid plexus in other ventricles (Ohuchi et al., 2008). *LPAR6* is found in human lateral ventricular choroid plexus, and reduced expression is seen in major depressive disorder (Turner et al., 2014). Importantly, LPA-related metabolic enzymes are highly expressed in the choroid plexus. For example, ATX shows perhaps the highest expression levels compared to all tissues in the choroid plexus during embryonic and adult life (Bachner et al., 1999; Koike et al., 2006; Savaskan et al., 2007). The presence of ATX in the choroid plexus, and the general proximity of the choroid plexus to neurogenic structures, may catalyze blood-derived LPC into bioactive LPA that can affect many aspects of neurodevelopment. Future studies should lead to new insights into the choroid plexus-CSF-brain axis.

Brain Vasculature

Development of the nervous system occurs in tandem with establishment of the vascular network, which encompasses vasculogenesis, angiogenesis, vessel maturation and stability, as well as blood brain barrier (BBB) formation (Greenberg and Jin, 2005; Eichmann and Thomas, 2013). Numerous factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and Notch also have roles in CNS development (Eichmann and Thomas, 2013; Ruhrberg and Bautch, 2013). In parallel, LPARs and LPA signaling can also influence vascular biology in health and disease (Teo et al., 2009). Notably, LPA and VEGF signaling are intertwined, since LPA can induce VEGF expression via NF-κB signaling and VEGF can induce ATX and *Lpar1* expression in vascular endothelial cells (Lin et al., 2008; Ptaszynska et al., 2010; Dutta et al., 2011).

Studies linking LPA to vascular development demonstrated that ATX-null mice die by E9.5 with vascular defects in the yolk sac and embryo (Tanaka et al., 2006; van Meeteren et al., 2006). These defects are similar to those found in $G\alpha_{13}$-null mice (Offermanns et al., 1997); LPA receptor subtypes that are known upstream activators of $G\alpha_{12/13}$ (Mirendil et al., 2013; Yung et al., 2014), which link LPAR signaling to some of these vascular processes. Indeed, specific LPAR-null mice have vascular defects, including *Lpar1*-null and *Lpar1* and *Lpar2* double null mice that have frontal cerebral hematomas (Contos et al., 2000; Contos et al., 2002), and more recently, *Lpar4*-null mice that have dilated blood and lymphatic vessels related to impaired pericyte recruitment (Sumida et al., 2010). Additionally, pericytes appear to mediate blood vessel stabilization by increasing the rate of LPA degradation, based upon an *in vitro* model of angiogenesis (Motiejunaite et al., 2014).

Cultured endothelial cells (ECs) have been shown to express *LPAR1-6* (Lin et al., 2007; Schleicher et al., 2011; Ren et al., 2013). LPA promotes the survival and proliferation of ECs from a variety of sources (English et al., 1999; Lee et al., 2000), including brain microvascular bEND.3 cells (Schleicher et al., 2011). LPA can also stimulate EC migration (English et al., 1999; Lee et al., 2000; Wu et al., 2005) and influence vascular tone. An early report, using a piglet model of intracranial hematoma demonstrated that LPA exposure produces dose-dependent vasoconstriction, which is reminiscent of that produced by hemorrhage (Tigyi et al., 1995). More recently, LPA has been found to mediate vasodilation via LPA_1, phospholipase C, and endothelial nitric oxide synthase (Ruisanchez et al., 2014). LPA overexposure has been reported to increase BBB permeability (On et al., 2013; Yu et al., 2014), as may occur during pathological conditions like stroke.

Stressors and Neuropsychiatric Disorders

CNS Injury

Beyond the roles of LPA in the development and function of the nervous system, LPAR signaling is also important during CNS stress or damage. During injury, LPA concentrations in brain and CSF are significantly elevated by production and/or release from multiple sources, including activated platelets and pro-inflammatory cells (Eichholtz et al., 1993; Tigyi et al., 1995; Goldshmit et al., 2010). Concentrations can reach thousands of times the apparent Kd of LPA receptors (Yung et al., 2011) to produce maximal activation of LPA receptor signaling. Traumatic injury has been reported to induce *LPAR2* expression – which is typically low or undetectable in adult brain – in human ependymal cells as well as mouse cortical and spinal cord astrocytes (Goldshmit et al., 2010; Frugier et al., 2011). *Lpar1* is also increased in reactive murine spinal cord astrocytes, while *Lpar3* expression is reportedly increased in cortical and spinal cord neurons (Goldshmit et al., 2010). Additionally, ATX levels in the human cerebral cortex are decreased after fatal closed head injury (Frugier et al., 2011). Whether this is due to negative feedback from injury-mediated LPA release (van Meeteren et al., 2005) or other factors remains to be determined. Conversely, ATX levels are dramatically increased in white matter adjacent to injury lesions in the rat cortex, demonstrating a localized upregulation during injury (Savaskan et al., 2007) and suggesting that overactive LPA signaling may contribute to inflammatory consequences. Notably, LPA levels altered by antibodies have been reported to improve traumatic brain injury outcomes in animal models (Goldshmit et al., 2012; Crack et al., 2014).

Hypoxia

Ischemia, or restriction of blood supply to tissues, is a major cause of hypoxia, even in the absence of direct trauma or infection. Hypoxic states result in altered signaling pathways, decreased synaptic transmission, inflammation, and neural death (Corcoran and O'Connor, 2013). LPA_1 signaling has been tied to hypoxia through distinct mechanisms. For instance, hypoxia enhances LPA-induced hypoxia inducible factor-1 alpha (HIF-1α) expression in cancer cells and VEGF expression in the vasculature (Kim et al., 2006; Lee et al., 2006; Lee et al., 2013c). Embryonic HIF-1α expression is also dependent on the presence of ATX and is rescued by LPA exposure in ATX-null animals (Fotopoulou et al., 2010). In the developing brain, the effects of short-term hypoxia can be devastating, altering development and often resulting in neurological disability or psychiatric disease (Herrera-Marschitz et al., 2014). A study utilizing cultured mouse cortical hemispheres in hypoxic conditions found that even short term exposure to a low oxygen environment caused cellular abnormalities, including mislocalized mitotic NPCs and neurite retraction through overactivation of LPA_1 and subsequent downregulation of G protein-coupled receptor kinase 2 (GIRK2) (Herr et al., 2011). These effects were previously seen upon stimulation of LPA_1 signaling in NPCs (Kingsbury et al., 2003) and could be reduced or prevented by an $LPA_{1/3}$ inhibitor, use of *Lpar1*-null mice (Herr et al., 2011), or use of *Lpar1* and *Lpar2* double null mice (Kingsbury et al., 2003). Similar protection against mitotic displacement produced by hypoxia was seen with inhibition of Rac1, Rho-associated kinase (ROCK), and HIF-1α, indicating that these signaling molecules may be involved in this *Lpar1*-dependent pathway (Fotopoulou et al., 2010).

In postnatal brains, hypoxia can be caused by stroke or trauma (Busl and Greer, 2010). Increased LPA signaling promotes retinal cell survival under hypoxic conditions by upregulation

of *Lpar1* and *Lpar2* expression in ganglion cells and the inner retinal layers (Savitz et al., 2006). However, retinopathy models of prematurity in rat neonates, produced by alternating cycles of hypoxia and hyperoxia, showed conflicting results (Yang et al., 2009): while *Lpar1* was upregulated in retinal tissue, LPA exposure or *Lpar1* overexpression decreased cell viability and LPA_1 inhibition or short hairpin RNA knockdown was protective to cell survival. LPAR signaling is clearly involved during hypoxic/ischemic insult, though continued investigation will be necessary to resolve whether LPA acts as a protective or harmful effector.

Hydrocephalus

Several disorders have been strongly correlated with hemorrhagic events during early development, which could result in enhanced LPA signaling through blood exposure and cellular stress. Fetal hydrocephalus is one such disease. Hydrocephalus is characterized by increased CSF pressure associated with ventriculomegaly, motor deficiencies, and cognitive deficits, and has been linked to overactivation of LPA signaling in an embryonic mouse model of disease (Yung et al., 2011) (Fig. 3A). Hydrocephalus was recapitulated by injecting blood components such as plasma or serum into the lateral ventricles of mouse embryos. Injecting LPA alone resulted in 100% incidence of ventriculomegaly accompanied by other characteristics of developmental hydrocephalus, including neurorosette formation, displaced NPCs, cortical disruptions, and 3rd ventricular and aqueductal occlusions that have all been reported clinically (Fukumizu et al., 1995; Dominguez-Pinos et al., 2005). All of these phenotypes were reduced or prevented in an *Lpar1* and *Lpar2* double-null mutant or by use of pharmacological $LPA_{1/3}$ antagonism. Current treatments for hydrocephalus are neurosurgical and palliative, however this

disorder might be amenable to medical interventions involving LPAR signaling modulators, which are being actively pursued.

Neuropsychiatric models

Lpar1-null mice display a variety of negative behavioral signs and cognitive deficits, illustrating the importance of LPA_1 signaling in normal cognition. When *Lpar1*-null mice are born, they display problems in olfaction related to suckling as well as pronounced craniofacial dysmorphism (Contos et al., 2000), a trait commonly seen in autism (Ploeger et al., 2010). ma*Lpar1*-null mice, a spontaneous variant that arose during colony expansion of the *Lpar1*-null line in Málaga, Spain, exhibit several negative behavioral signs, including generalized anhedonia (Santin et al., 2009; Blanco et al., 2012; Castilla-Ortega et al., 2013), hypersensitivity to stress (Castilla-Ortega et al., 2010; Castilla-Ortega et al., 2011; Garcia-Fernandez et al., 2012), and significantly increased anxiety (Santin et al., 2009). *Lpar1*-null mice also display deficits in prepulse inhibition (PPI) of the startle reflex (Harrison et al., 2003), a cognitive attention-related test that is significantly impaired in schizophrenia patients, as well as learning and memory deficiencies particularly related to spatial memory retention (Santin et al., 2009), consolidation (Castilla-Ortega et al., 2011), and working memory (Castilla-Ortega et al., 2010).

LPA signaling has additionally been linked to many of the underlying molecular and neurotransmitter pathways involved in both genetic and environmental risk factors for neuropsychiatric disorders. Glycogen synthase kinase-3 (GSK3) – a signaling network node associated with risk factors such as disrupted in schizophrenia 1 (Disc1), neuregulin 1 (Nrg1), thymoma viral proto-oncogene 1 (Akt1), and reelin – is strongly regulated by *Lpar1* (Yang et al., 2005; Lovestone et al., 2007; Mao et al., 2009; Sun et al., 2011). Additionally, glutamatergic

signaling alterations are implicated in behavioral deficits associated with schizophrenia, autism, and other related neuropsychiatric disorders (Quiroz et al., 2004; Lin et al., 2012a; Hadley et al., 2014), and LPA is known to initiate glutamate uptake (Shano et al., 2008). Modification of LPA_1 expression has also been linked to altered miniature excitatory postsynaptic potential (mEPSP) kinetics and frequency, inhibitory postsynaptic potential (IPSP) amplitude, and entorhinal cortex gamma oscillations (Cunningham et al., 2006; Segal, 2006; Trimbuch et al., 2009). *Lpar1*-null mice also display alterations in serotonin (5-HT) neurotransmitter levels (Harrison et al., 2003), dysregulation of glutamatergic synapses particularly through regulation of the glutamate receptors GluR3, GluR1, and the NMDA receptor NR2A/B (Harrison et al., 2003; Roberts et al., 2005; Musazzi et al., 2010), increases in hippocampal CaMKII activity (Musazzi et al., 2010), and decreases in parvalbumin-positive neurons (Cunningham et al., 2006; Pedraza et al., 2013). These data present a compelling picture of LPA_1-initiated glutamatergic and GABAergic signaling dysregulation resulting in negative and cognitive behavioral deficits relevant to schizophrenia, depression, bipolar disorder, and anxiety disorders (**Fig. 3B**).

Many environmental risk factors for neuropsychiatric diseases, such as bleeding, infection, and hypoxia, are insults that overactivate LPA signaling (Eichholtz et al., 1993; Cummings et al., 2004; Li et al., 2010; Herr et al., 2011). In an adult model of increased LPA exposure, where investigators directly infused LPA into the cerebral ventricles, treated mice displayed significantly increased anxiety, anhedonia through decreased novelty exploration, and depression-associated immobility in the forced swim test (Castilla-Ortega et al., 2014). These negative behavioral deficits are remarkably similar to those seen in *Lpar1*-null mice, suggesting that precise regulation of LPA signaling is critical for regulation of certain behaviors. Indeed, since exposure to high concentrations of LPA during mid-development also induces

hydrocephalus, this suggests that either increased or decreased LPAR signaling could lead to a number of neurological disorders that might be considered "hypomorphs" of the severe changes seen in hydrocephalus. It is possible that the timing, severity, and/or duration of aberrant LPAR signaling contributes to specific pathologies.

The activity of PLA_2, one of the main phospholipases responsible for LPA production, can be significantly increased in first-episode schizophrenia patients and is strongly associated with symptom severity (Smesny et al., 2005; Ong et al., 2010; Smesny et al., 2010). In addition, rats with knocked-down PLA_2 expression show reductions in PPI, a common cognitive deficit seen in schizophrenia patients (Lee et al., 2009). The removal of a lipid enzyme homologue associated with LPA degradation and uptake, plasticity-related gene 1 (PRG-1 or LPPR4: lipid phosphate phosphatase-related protein type 4, reviewed in (Strauss and Brauer, 2013)), results in epilepsy in knockout mice (Trimbuch et al., 2009). Importantly, these seizures are prevented when LPA_2 signaling is also removed, suggesting a receptor-dependent pathology. LPA signaling is also dysregulated in bipolar I patients, where Ca^{2+} influx in lymphocytes from patients show a hypersensitive response to LPA exposure (Quiroz et al., 2004; Perova et al., 2008).

Alzheimer's disease

One of the central pathologies in Alzheimer's disease (AD) is an abundance of senile plaques and tangles composed of beta-amyloid (Aβ) and tau aggregates. Decreased ATX expression found in the frontal cortex of Alzheimer's patients (Umemura et al., 2006) suggests that altered LPAR signaling might contribute to the pathology of the disease. LPA enhances site-specific pathogenic tau phosphorylation, leading to GSK3-mediated growth cone collapse in

neurons (Sayas et al., 1999). LPA also increases β-secretase activity, leading to elevated Aβ production (Shi et al., 2013). Gintonin, a bioactive fraction isolated from ginseng that has been reported to improve cognition in AD, is composed of protein-bound LPA that activates LPA_{1-5} ($LPA_2 > LPA_5 > LPA_1 > LPA_3 > LPA_4$; LPA_6 was not assessed) (Hwang et al., 2012b). Gintonin promotes soluble secretory AβPPα release and decreased Aβ1-42 release (Hwang et al., 2012a). Treatment of Aβ transgenic mouse AD models with gintonin has been reported to attenuate amyloid plaque deposition and prevent long-term memory impairment, resulting in cognitive dysfunction rescue (Hwang et al., 2012a). It will be important to validate these reports, including determination of whether there are specific LPARs involved in Aβ plaque formation and cognitive deficits in AD.

Nerve injury and pain

LPA signaling is also involved in nerve injury and pain responses through LPARs and related metabolites (reviewed in (Ueda et al., 2013)) (**Fig. 3C**). Chronic pain affects up to 65% of the adult population and can broadly be classified as neuropathic or nociceptive in nature (Yawn et al., 2009). Several models used to study neuropathic pain include partial sciatic nerve ligation (PSNL), intrathecal LPA injection, UVB irradiation, and ischemia-induced pain. Neuropathic pain, with symptomatic allodynia and hyperalgesia, is commonly caused by trauma or inflammation of the nervous system, resulting in nerve damage, peripheral neuropathy, and Schwann cell demyelination (Campbell and Meyer, 2006). LPA exposure during tissue injury, as modeled by intrathecal LPA injection, initiates neuropathic pain in wild-type mice (Inoue et al., 2004; Ueda et al., 2013). Interestingly, the *de novo* synthesis of LPA through PLA_2 and ATX appears to mediate the initial phases of neuropathic pain (Ma et al., 2010). Furthermore, in

ex vivo studies, LPA induces demyelination and decreases myelin basic protein expression in isolated dorsal root ganglion fibers (Fujita et al., 2007). Intrathecally administered lysolecithin, an agent used to produce demyelination (Hall, 1972), appears to induce neuropathic pain via ATX-mediated conversion into LPA (Inoue et al., 2008b). This result is supported by studies on *Enpp2*$^{+/-}$ mice, which have a 50% decrease in LPA plasma concentration and also show a 50% recovery from PSNL-induced neuropathic pain (Inoue et al., 2008a). LPA-mediated nociception may be dependent on specific LPA forms, since pain responses were strongly elicited by 18:1 LPA but not by 16:0 LPA or 18:0 LPA (Ma et al., 2013). Cyclic phosphatidic acid, a structural analog of LPA, has also been reported to inhibit ATX and attenuate PSNL-induced allodynia and hyperalgesia (Kakiuchi et al., 2011).

Genetic removal of *Lpar1* blocks LPA-induced sequelae during PSNL injury, demonstrating both lipid and receptor specificity (Inoue et al., 2004). Allodynia and demyelination are also reduced using Rho pathway inhibitors, implicating LPA_1-mediated Rho activation (Inoue et al., 2004). Furthermore, LPA_1 activation can induce a nociceptive response, characterized by tissue, but not nerve damage, which mediates the release of substance P, a neuropeptide implicated in inflammation and pain (Renback et al., 1999). In addition, LPA produced through a feed-forward mechanism can signal through LPA_3 in the spinal cord dorsal horn and dorsal roots to initiate nerve injury-induced neuropathic pain (Ma et al., 2009). Intraperitoneal administration of Ki16425, an $LPA_{1/3}$ antagonist, can block LPA-induced nociception (Ma et al., 2013). PSNL-induced neuropathic pain can also be blocked in *Lpar5*-null mice, resulting in decreased phosphorylated cAMP response element-binding protein (pCREB) expression in spinal cord dorsal horn neurons and distinct involvement of cAMP, independent of LPA_1 signaling (Lin et al., 2012b).

Numerous lipids, including LPA, can indirectly activate or sensitize nociceptors by interacting with TRP or sodium channel families, or by recruiting immune cells to the site of inflammation. During UVB radiation-induced inflammatory hyperalgesia, distinct species of LPA and other lipids are elevated in the skin, but not in dorsal root ganglia or dorsal horn (Sisignano et al., 2013). Sensitization of afferent Aβ and Aγ, but not C fibers, are thought to be responsible for LPA-induced mechanical allodynia. Moreover, increased or newly expressed TRPV1 receptors in Aβ and Aγ fibers are thought to be involved in the maintenance of LPA-induced allodynia (Ohsawa et al., 2013).

Brain Cancer

LPA signaling has significant roles in cancer, particularly in the promotion of metastasis, tumor growth, neovascularization, and inhibition of apoptosis (Tsujiuchi et al., 2013; Yung et al., 2014) (**Fig. 3D**). Aberrant LPAR expression and signaling in CNS glioblastomas and neuroblastomas have been reported (Hoelzinger et al., 2008; Hayashi et al., 2012; Willier et al., 2013). For instance, *LPAR1* and *ENPP2* are overexpressed in highly malignant glioblastoma multiforme (GBM) (Kishi et al., 2006). Metabolism of LPC by ATX may provide a chemotactic source of LPA, since *ENPP2* knockdown in GBM reduces LPC-directed migration, inhibits invasion, and enhances tumor radiosensitivity (Kishi et al., 2006; Hoelzinger et al., 2008; Schleicher et al., 2011; Bhave et al., 2013). In neuroblastomas, induced expression of LPA_2 or LPA_3 in the B103 neuroblastoma cell line promotes increased baseline motility and invasion (Hayashi et al., 2012), while LPA_1 signaling stimulates increased cell motility in both neoplastic and non-neoplastic cells (Van Leeuwen et al., 2003; Hama et al., 2004). In contrast, LPA_4

expression in B103 cells has been reported to attenuate pro-migratory LPA_1 and LPA_2 responses (Lee et al., 2008).

Dysregulated Rho and Rac signaling appears to correlate with neural tumor proliferation and invasiveness (Khalil and El-Sibai, 2012), which can be altered by LPAR mechanisms. LPA_1 and LPA_2 signaling induces stress fiber formation, cytoskeletal rearrangement, and cell migration in GBM cells via downstream Rho/ROCK activation (Manning et al., 2000; Seasholtz et al., 2004). ROCK inhibition in LPAR-expressing tumor cells decreases motility and invasion (Salhia et al., 2005; Hayashi et al., 2012). This pathway is balanced and antagonized by Rac1-mediated adhesion and PI3K signaling (Van Leeuwen et al., 2003; Seasholtz et al., 2004; Salhia et al., 2005). Other significant pathways may include Ras/MAPK signaling, since MAPK phosphorylation was observed in LPA-mediated glioblastoma cell migration (Mattingly et al., 2001; Annabi et al., 2009). Additionally, downregulation of LPA-induced MAPK in drug resistant neuroblastoma cells sensitized them to therapy. Furthermore, constitutively active LPA_1-expressing B103 cells showed higher matrix metalloproteinase-2 expression and activation, suggesting that LPA signaling may stimulate invasion by processes other than basic motility (Kato et al., 2012).

Conclusion

Over the past two decades, the roles of LPA signaling have been progressively defined in the normal, developing, and adult nervous system, commencing with the identification of specific, cell-surface receptors. LPA signaling influences numerous developmental processes, including NPC proliferation, neural and glial development, proper cell migration, and cell survival. Environmental stressors such as hypoxia, inflammation, and hemorrhage that increase

LPA signaling have links to multiple neuropathologies, including hydrocephalus, traumatic brain injury, neuropathic pain, and neurodevelopmental and neuropsychiatric disorders. In addition, pathological conditions such as brain cancers and neurodegenerative disorders like AD have also been linked to LPA signaling. Genuine therapeutics targeting LPARs are anticipated in the future, especially in view of the compound known as fingolimod, an FDA-approved medicine for treating multiple sclerosis, which antagonizes LP (S1P) receptors (Chun and Brinkmann, 2011; Cohen and Chun, 2011; Groves et al., 2013), along with the entry of new chemical entities targeting LPARs into clinical trials for other indications ((Swaney et al., 2010; Castelino et al., 2011) and reviewed in (Lappano and Maggiolini, 2011; Rancoule et al., 2011)). The prospects are increasingly bright for uncovering new biology and medicines that impact the nervous system through LPA signaling.

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Chapter 3, in full, is a reprint of the material as it appears in Neuron. Yung YC*, **Stoddard NC***, Mirendil H, and Chun J, "Lysophosphatidic Acid signaling in the nervous system," *Neuron*. 85:669–82. PMC4400838, 2015. *co-first authors. The dissertation author was a primary co-author of this review.

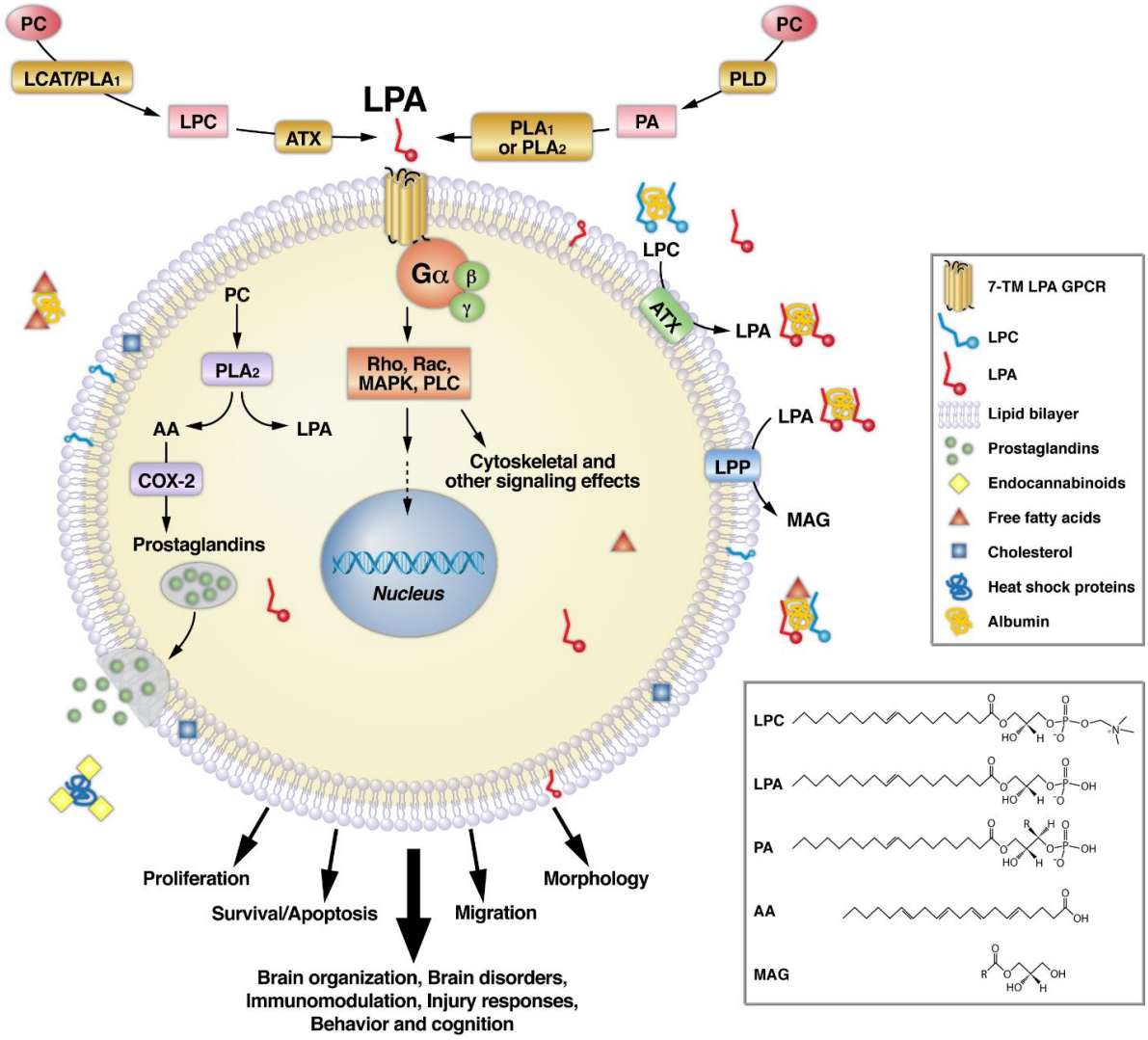

Figure 3.1: LPA is a bioactive lipid that signals through defined GPCRs within the nervous system.

Lipids comprise a significant portion of the central nervous system (CNS) and have differing structural, energetic, and bioactive signaling properties. Signaling lipids are often bound to carrier proteins such as albumin or heat shock proteins. LPA activates members of a family of G protein-coupled receptors (GPCRs) and influences multiple cellular processes including proliferation, survival, apoptosis, morphological change, and migration, as well as the production of other lipids such as prostaglandins through arachidonic acid (AA) conversion by cyclooxygenase 2 (COX-2). The synthetic pathways for LPA include conversion of phosphatidylcholine (PC) into lysophosphatidylcholine (LPC) by lecithin-cholesterol acyltransferase (LCAT) and phospholipase A (PLA) 1 enzymes, or by conversion of PC to phosphatidic acid (PA) by phospholipase D (PLD). LPC is then metabolized to produce lysophosphatidic acid (LPA) by the enzyme autotaxin (ATX). LPA can be broken down into monoacylglycerol (MAG) by a family of lipid phosphate phosphatases (LPPs). Other lipids in the CNS include the endocannabinoid family, fatty acids, cholesterol, and prostaglandins, which are beyond the scope of this review.

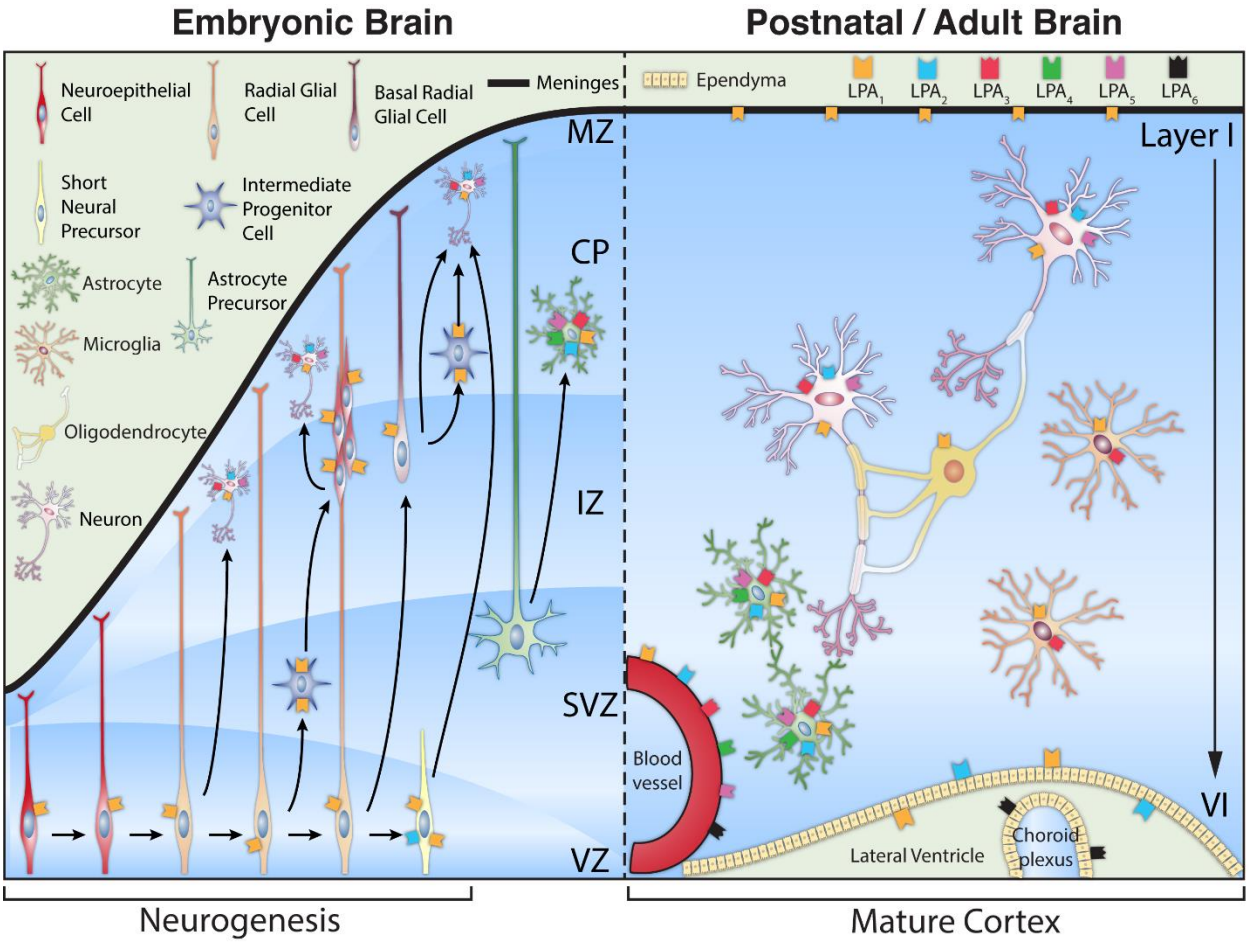

Figure 3.2: LPAR subtypes in the developing and mature cerebral cortex.

Reported LPAR subtype expression of the six LPA receptors, $LPA_{1\text{-}6}$, varies with developmental age and cell type. (**Left**) LPARs are expressed in neural progenitor cells (NPCs) and other developing cortical cells. These expression patterns vary as the progenitors arise in the ventricular zone (VZ) and differentiate as they migrate through the subventricular zone (SVZ), intermediate zone (IZ), to localize within the cortical plate (CP). In the embryonic brain, LPA-mediated processes include proliferation, interkinetic nuclear migration, neurite retraction, survival, morphological change, and cell migration. (**Right**) Most major cell types in the mature cortex express specific subtypes of LPARs. LPARs are also expressed in cells of the ependyma, blood-brain barrier, and meninges, which overlie the most superficial marginal zone (MZ). Postnatally, LPA signaling influences myelination, microglial and astrocytic responses, vascular stabilization, and higher cognitive processes.

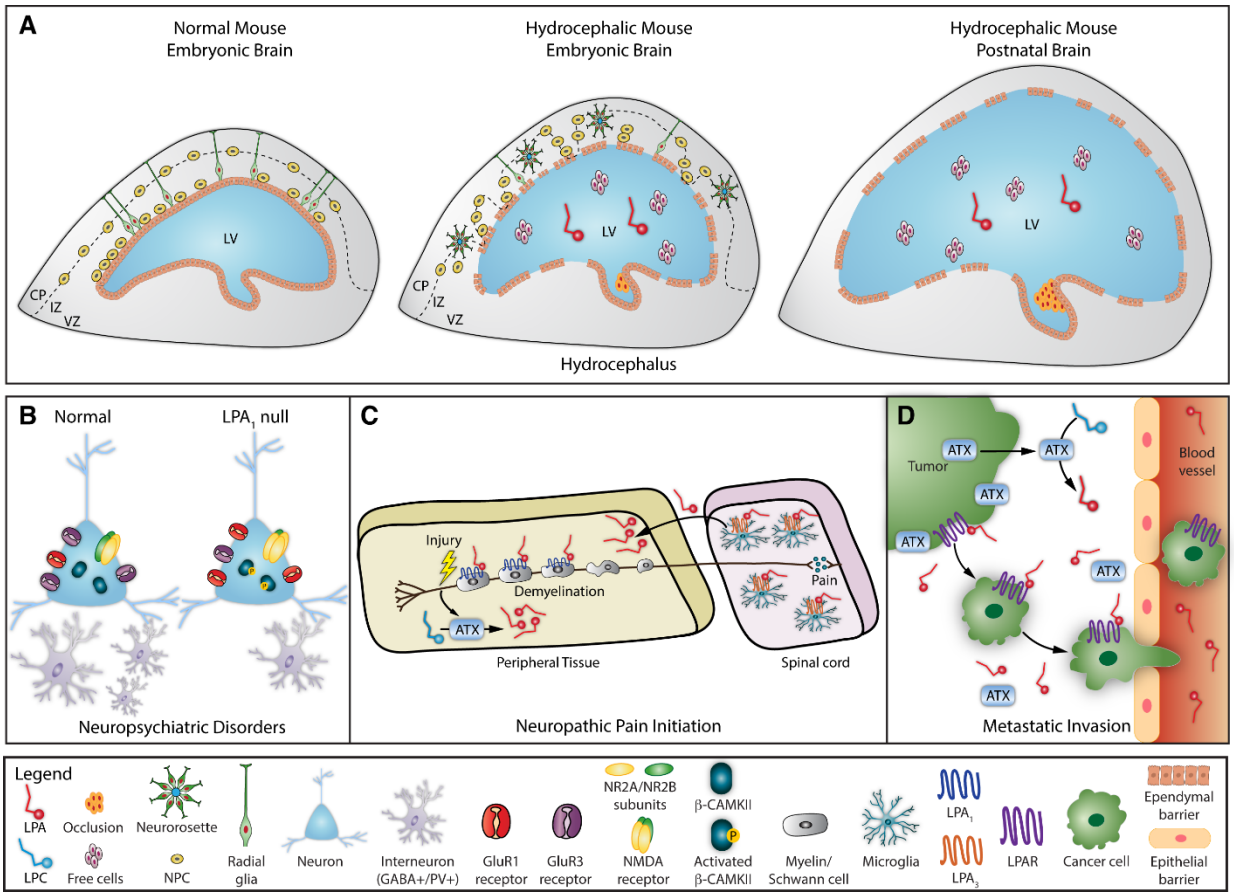

Figure 3.3: Dysregulated LPA signaling may lead to nervous system disorders.

Aberrant LPA signaling, whether produced by overactivation, altered LPA production/degradation, or changes in receptor expression, can disrupt the nervous system to produce sequelae relevant to human brain disorders. (**A**) LPA signaling has been linked to post-hemorrhagic hydrocephalus. A mouse model recapitulates multiple histological comorbidities seen in humans, including ventriculomegaly, formation of neurorosettes, disrupted NPCs within the ventricular zone (VZ), intermediate zone (IZ), and cortical plate (CP), loss of cell adhesion that leads to the presence of free-floating cells in the CSF, compromised ependymal lining, and ventricular occlusions. Hydrocephalus is often chronic, with increased CSF pressure, ventriculomegaly, and decreased brain mass persisting throughout postnatal life. (**B**) *Lpar1*-null mice display dysregulated neural signaling, with disruption of glutamatergic (AMPA and NMDA receptor expression and composition) and GABAergic (GABA+/PV+ neuron decreases) function, leading to significant cognitive impairments in animal models. (**C**) Nerve damage induces the production of LPA via ATX-mediated conversion of LPC. LPA stimulates LPA_3 on activated microglia, resulting in feed-forward LPA release that, in turn, can activate LPA_1 on Schwann cells, leading to downregulation of myelin proteins, progressive demyelination, and initiation of neuropathic pain. (**D**) Brain tumors can show overexpression of ATX and LPARs, leading to altered LPAR signaling. $LPA_{1\text{-}3}$ stimulation by increased environmental LPA induces cell migration and promotes cancer cell metastases.

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Chapter 4:

Ependymal loss through LPA_1 overstimulation initiates hydrocephalus in a novel neonatal mouse model

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ABSTRACT

Post-hemorrhagic hydrocephalus (PHH) is a common neurological condition treated with invasive neurosurgical interventions. PHH is associated with increased intracranial pressure (ICP), resulting in dramatic ventriculomegaly with concomitant motor and cognitive deficits, and is especially prevalent in premature infants. Here we report a novel mouse model of hydrocephalus that maps neurodevelopmentally to premature human infants, identifying a receptor-driven cellular mechanism of disease initiation. We discovered that lysophosphatidic acid (LPA), a signaling lipid present in the blood, induces high ICP levels and ventriculomegaly 7 days following injection into the ventricular system, through a mechanism altogether distinct from fetal and adult PHH. LPA acutely disrupted the ependymal cells that generate CSF flow, followed by phagocytosis, cell death, and ventricular surface denudation. Ependymal death and subsequent PHH development can be prevented by knockout or pharmacological blockade of the LPA_1 and LPA_3 receptors, suggesting novel therapeutic targets toward preventative therapies.

BACKGROUND

Hydrocephalus is a common neurological condition that affects 1 in 1000 people worldwide (Chi et al., 2005; Dewan et al., 2018). It is typically a chronic condition associated with persistent migraines, cognitive deficits, epilepsy, and motor dysfunction (Ding et al., 2001; Lacy et al., 2008; Laurence and Coates, 1962; Vinchon et al., 2012a). Neurosurgical interventions have reduced the lethality of hydrocephalus, but current treatment paradigms, such as inserting a shunt into the ventricles of the brain to physically drain excess CSF, are palliative and only serve to mitigate symptoms (Cherian et al., 2004; Shooman et al., 2009; Walid and Robinson, 2012). Clinically, hydrocephalus is characterized by an accumulation of cerebrospinal fluid (CSF) pressure in the brain and subsequent enlargement of the fluid-filled ventricles, termed ventriculomegaly (Bergsneider et al., 2006).

This study highlights a novel postnatal model of hydrocephalus that recapitulates several clinical hallmarks of premature post-hemorrhagic hydrocephalus (**Table 1**). While fetal and adult cases of post-hemorrhagic hydrocephalus (PHH) occur, PHH is particularly common in premature infants with fragile cerebral vasculature (Robinson, 2012; Tully and Dobyns, 2014). Approximately 16% of premature infants suffer from severe intraventricular hemorrhage, with 20% of these infants requiring a permanent shunt (Robinson, 2012). Despite the overwhelming clinical need, models representing this developmental stage are sorely lacking. This is exacerbated by the knowledge that early postnatal brains are dramatically different from both fetal and adult brains in cellular composition, receptor expression patterns, and morphology.

Many animal models of hydrocephalus exist, including genetic mutants and obstructive models that physically restrict CSF flow (Bigio et al., 2011). Of the genetic models, several studies have demonstrated that removing the ependymal cells that line the ventricular system or

inducing ciliary dysfunction often generates spontaneous hydrocephalus (Banizs et al., 2005; Ibañez-Tallon et al., 2004; Jimenez et al., 2001; Tissir et al., 2010; Wang et al., 2016). Additionally, compromised ependymal cells have been observed in human hydrocephalus patients (Dominguez-Pinos et al., 2005; Fukumizu et al., 1995; McAllister et al., 2017; Sival et al., 2011). Ependymal cell health is particularly important to CSF homeostasis, as the cilia on ependymal cells beat in a synchronized manner to generate fluid CSF flow around the central nervous system (Faubel et al., 2016; Hagenlocher et al., 2013). Intracranial pressure is difficult to measure in small animals, so ventriculomegaly is commonly used to diagnose hydrocephalus for *in vivo* studies. However, innovations on utilizing intracranial pressure monitoring in mice and rats are on the horizon (Moazen et al., 2016; Murtha et al., 2012), and we have adapted clinical ICP monitoring protocols to measure CSF pressure in early postnatal mice.

While genetic studies contribute to our understanding of hydrocephalus, congenital cases are often attributed to other comorbidities, such as spina bifida, aqueductal stenosis, and meningitis (Cinalli et al., 2011; Munch et al., 2012; Shaheen et al., 2017; Zhang et al., 2006). After birth, hydrocephalus usually develops following severe hemorrhage or brain trauma, which is incredibly common in premature infants (Robinson, 2012). Prior models exist detailing the effects of bloodborne factors on hydrocephalus in embryonic or adult brains (Grondona et al., 1996; Park et al., 2016; Shim et al., 2013; Yung et al., 2011). However, there are few previously existing models detailing PHH in the premature or neonatal brain. Here, we have identified lysophosphatidic acid (LPA) as a key initiating factor for neonatal PHH in a mechanism distinct from its fetal and adult activities.

LPA is a signaling lipid that binds to 6 canonical G protein-coupled receptors, designated LPA_1-$LPA_{6(}$ Yung et al., 2014). LPA is produced from lysophosphotidylcholine (LPC) by the

enzyme autotaxin and circulates in the bloodstream bound to carrier proteins such as albumin (Aoki et al., 2002; Sonoda et al., 2002). Of particular relevance, bloodborne LPA levels are significantly elevated in cases of hemorrhage or trauma (Aoki et al., 2002; Sano et al., 2002). Therefore, severe hemorrhage introduces large concentrations of LPA into the ventricular system, potentially wreaking havoc on LPA receptor (LPAR)-expressing cells and altering healthy CSF dynamics.

RESULTS

Intraventricular LPA exposure induces high-pressure hydrocephalus in neonatal mice

Previously, LPA has been demonstrated to induce fetal PHH when injected in the ventricles of E13.5 mice (Yung et al., 2011), which correlates to late first trimester in humans (**Fig. 1a**). However, the developing brain differs dramatically from the postnatal brain. In order to study PHH in a therapeutically accessible population of great clinical need, we utilized early postnatal mice at 8 days old (P8) to emulate the brain development phase of premature infants less than 34 weeks in gestation (**Fig. 1a**) (Clancy et al., 2001; Clancy et al., 2007). To simulate hemorrhage, 5µL of vehicle or 5mM stock LPA was stereotactically injected into the lateral ventricles of littermates, to a final concentration of 80-800µM (**Fig. 1b**, methods). Brain tissue was harvested 7 days following the procedure ("P8+7"), stained with hematoxylin and eosin (H&E), and analyzed for ventriculomegaly and histological abnormalities (**Fig. 1c**). Most notably, LPA-injected mice demonstrate a ventricular volume that is more than 10 times larger than vehicle-injected or uninjected lateral ventricles (**Fig. 1d**). This severe ventriculomegaly, accompanied by cortical thinning and destruction of the corpus callosum, was observed in 100% of LPA-injected animals. Intriguingly, this phenotype was not due to overgrowth and occlusion of the rostral aqueduct, as has been observed in fetal LPA-induced hydrocephalus (Yung et al., 2011). Therefore, this model describes a novel mechanism for initiation of communicating or idiopathic PHH.

While ventriculomegaly is commonly assessed in models of PHH, increased intracranial pressure (ICP) – the second clinical hallmark of hydrocephalus – is difficult to assay in small animals. To this end, we developed a novel protocol for measuring ICP in mouse pups (**Fig. 1b**).

By inserting a fiberoptic pressure monitor into the lateral ventricle of sedated mice, we were able to collect CSF pressure readings from P8+7 uninjected, vehicle-injected, and LPA-injected animals, demonstrating a significantly increased ICP range in LPA-exposed animals (**Fig. 1e**).

LPA-induced hydrocephalus is chronic and does not resolve without intervention. If allowed to survive untreated, more than 80% of LPA-exposed mice die within 12 weeks of age (11 weeks post-injection) (**Fig. 1f**). These mice are typically underweight, exhibiting hunched posture, ungroomed fur, and reduced movement. Additionally, this model affects male and female mice indiscriminately (**Supplementary Fig. 1**).

Ependymal dysfunction occurs quickly

As this model recapitulates multiple clinical aspects of human PHH (**Table 1**), we began to characterize the earliest hallmarks of disease initiation. To determine which brain regions or cell types were affected first, we harvested brains at several timepoints, including 3, 6, and 24 hours post-injection. Sections were stained with H&E for histological analysis or acetylated tubulin (AcTub) and S100 antibodies to identify ependymal cilia and cell bodies, respectively (Didier et al., 1986). It was immediately apparent that the ependymal cells were selectively affected by LPA injection (**Fig. 2a**). This phenotype progressed over time, with cell and nuclear rounding evident by 3 hours post-LPA injection, apparent loss of basement membrane adhesion 6 hours post-LPA injection, and significant depletion of the ependymal monolayer at 24 hours post-LPA injection (**Fig. 2b**). Ciliary health declines in tandem with cellular morphology changes, as AcTub immunolabeling of the cilium declines and disappears at 3-6 hours while ependymal cell bodies (S100β) are lost by 24 hours (**Fig. 2c**). This loss of ependymal cell bodies

was quantified by measuring S100 fluorescence surrounding the lateral ventricles (**Supplementary Fig. 2**).

Ependymal cilia degenerate and are phagocytosed

We utilized electron microscopy to further interrogate ependymal health over time. Transmission electron microscopy (TEM) demonstrated perturbations of ciliary morphology, displaying frequent loss of membrane integrity and occasional distortion of the 9+2 microtubule axoneme 6 hours following LPA exposure (**Fig. 3a-d**). Scanning electron microscopy (SEM) also showed loss of ciliary tufts and disruption of the smooth ependymal lining 6 hours post-surgery (**Fig. 3e-f)**. The remaining cilia appear kinked and disorganized. This gradual depletion led to a craggy and uneven ventricular surface that was fully depleted of cilia 24 hours following LPA exposure (**Supplementary Fig. 3**).

Examination of these early timepoints also revealed a large number of non-ependymal cells on top of the ependymal barrier. SEM investigation at 3 and 6 hours post-LPA exposure captured several cells with an elongated morphology phagocytosing ependymal cilia (**Fig. 3g-h**). We discovered that these invaders stained heavily with an ionized calcium-binding adapter molecule 1 (Iba1) antibody (**Fig. 3i-j**), identifying them as macrophages and/or microglia. Intriguingly, the Iba1+ cells that reside within the choroid plexus in vehicle-injected samples are reduced in LPA-injected samples, suggesting they may have migrated from the choroid plexus bloodstream to the ventricular surface upon insult. The presence of inflammatory F480+ macrophages and microglia in the whole brain 24 hours post-injection was quantified by flow cytometry (**Fig. 3k**). These data imply that, since cells of the innate immune system remove

apoptotic cells (Devitt and Marshall, 2011), affected ependymal cells may be actively cleared from the ventricular wall prior to the development of hydrocephalus.

Ependymal cells die within 24 hours

Desiring to learn more about this dramatic loss of ependymal cell integrity, we continued to investigate the mechanism of ependymal cell loss. Using ISEL+ DNA end-labeling, a modified version of the TUNEL assay, we discovered that DNA fragmentation occurs as early as 3 hours post-LPA exposure (**Fig. 3l-o**). Activation of apoptotic signaling cascades was assessed by cleaved Caspase 3 (Cas3) staining, which was present in ependymal cells 6 hours after LPA exposure (**Fig. 3p-s**). This DNA fragmentation and subsequent apoptosis was highly specific to the ependymal monolayer of the ventricular system. As LPAR overstimulation can initiate programmed cellular apoptosis after tissue injury (Funke et al., 2012; Holtsberg et al., 1998), these data suggest a mechanism for LPA-induced ependymal damage.

Ependymal cells express LPARs

Interested in the correlation between PHH development and LPAR expression patterns, we performed RNAscope against *Lpar1-6* expression on P8 tissue sections. We discovered dense *Lpar1* expression and highly specific *Lpar3* expression within the ependymal monolayer (**Fig. 4a,b**), while LPA_2 and LPA_4 receptors demonstrated low and non-specific mRNA expression in the ventricular zone, LPA_5 demonstrates no ventricular area expression, and LPA_6 is highly expressed in vasculature and choroid plexus with low ependymal expression (**Supplementary Fig. 4**). These expression patterns differ greatly from previously reported embryonic and adult data (Ohuchi et al., 2008; Allen Brain Atlas, 2015), suggesting that the

mechanism of PHH and individual susceptibility may change as the subject ages. This conclusion is supported by the fact that, while LPA injection causes severe ventriculomegaly after 7 days in 100% of subjects injected at P4 or P8, only 50% of P14 mice are affected, and adult mice do not exhibit any significant ventriculomegaly (**Supplementary Fig. 5**), correlating to reduced LPAR expression in the ependyma as subjects age.

LPA_1 and LPA_3 are key mediators of PHH sequelae

Since LPA specifically damages ependymal cells prior to the development of hydrocephalus, we tested whether these results are attributable to the activation of specific LPARs. To this end, we injected LPA into the lateral ventricles of LPA_1-LPA_5 single knockout mice and quantified ventriculomegaly after 7 days. While PHH developed in most LPA_2, LPA_4, and LPA_5 knockouts, only 40% of LPA_1 -/- and 60% of LPA_3 -/- mice demonstrated significant ventriculomegaly (**Fig. 4c,d**). Affected LPA_1-null animals also suffered from less severe ventriculomegaly (**Fig. 4d**), and protected LPA_1 knockouts exhibited normal ICP levels compared to controls (**Fig. 4e**). These results implicate LPA_1, and to a lesser extent LPA_3, in mediating LPA-induced ventriculomegaly and PHH.

Partial protection against LPA-initiated ventriculomegaly and PHH by genetic removal of LPA_1 or LPA_3 supported the possibility that pharmacological interventions could prevent LPA-induced PHH. To this end, we acquired LPA_1 antagonist AM095. We injected 1mg/kg of antagonist intracranially, waited 15 minutes, then administered LPA to the same locus. After 7 days, brains were harvested, sample identity blinded, and ventriculomegaly quantified. For this experiment, LPA was suspended in vehicle containing lipid-free bovine serum albumin (BSA) and injected at a lower concentration to create a more physiologically relevant system (see

methods). While this new methodology resulted in less pronounced ventriculomegaly (**Fig. 4f**), it still produced consistent hydrocephalus. Most remarkably, AM095 treatment resulted in significant protection from hydrocephalus (**Fig. 4f**).

Further investigation revealed that LPA_1 knockout, LPA_3 knockout, or pharmacological inhibition of LPA_1 with AM095 protected against PHH initiation by reducing ependymal damage. More than half of LPA_1 knockout animals demonstrated significant ependymal protection, while LPA_3 knockout ependymal cells were depleted 24h post-LPA exposure (**Fig. 4g**, quantified in **Supplementary Fig. 2**). Additionally, pretreatment with LPA_1 antagonist AM095 protected the ependymal monolayer from depletion 24 hours post-injection (**Fig. 4g**). Protected LPA_1 and LPA_3 knockout animals as well as AM095-treated cohorts had normal ventricular and cortical morphology 7 days post-surgery (**Fig. 4h**). These data suggest that, while both LPA_1 and LPA_3 are involved in LPA-induced hydrocephalus, LPA_1 receptor signaling may be the key mediator of ependymal deterioration following LPA overexposure.

Mechanism of LPA-mediated PHH

Since at-risk neonatal infants are often kept in the hospital for observation, this novel postnatal model represents a clinically accessible population of great need. LPA released into the ventricular system following severe hemorrhage overstimulates ependymal LPA_1 receptors, resulting in specific damage, degeneration, and removal of ependymal cells. This ependymal loss reduces cilia-driven CSF flow and removes the CSF-parenchyma barrier, causing CSF accumulation which leads to ventriculomegaly and chronic hydrocephalus. This loss can be prevented by LPA_1 knockout, LPA_1 blockade, and to a lesser extent LPA_3 knockout (**Fig. 5**). The involvement of LPAR signaling in post-hemorrhagic ependymal damage, combined with the

high bloodborne concentrations of LPA (and its precursor, LPC), presents an intriguing druggable target, especially since LPA_1 and $LPA_{1/3}$ antagonists are currently available and in clinical trials for other indications. Indeed, the efficacy of LPA_1 inhibitor AM095 in preventing LPA-induced ventriculomegaly is a first step toward these pharmacological goals. Administration of an LPAR antagonist within a few hours of hemorrhage could protect against future sequelae, reducing hydrocephalus incidence and severity in early postnatal infants.

DISCUSSION

We report a new mouse model for PHH generated via intraventricular LPA exposure during early postnatal life. It employs a medically accessible mechanism – LPA receptor signaling – at ages that are more clinically relevant to a human PHH at-risk patient population: premature infants in neonatal intensive care units who often suffer from intracranial or intraventricular hemorrhage. The mouse model described here replicates multiple aspects of PHH reported in humans of equivalent stages of development, including elevated ICP and ventriculomegaly (**Table 1**). This model requires LPA exposure within the cerebral ventricles, as can occur naturally during intracranial hemorrhage, which overactivates ependymal cell LPA_1 and, to a lesser extent, LPA_3, resulting in ciliary dysfunction and reduced motility along with ependymal cell damage, death, and removal. Ependymal cell loss, ventriculomegaly, and PHH can be prevented to a significant extent by LPA_1 removal or blockade, and to a lesser extent by LPA_3 removal (**Fig. 5**).

These data are among the first to model hydrocephalus in mice at an age that neurobiologically corresponds to premature human infants at risk for PHH (Kahle et al., 2016; Clancy et al., 2001; Mazzola et al., 2014). In a previous PHH model, LPA was shown to induce fetal hydrocephalus when injected into the ventricles of embryonic E13.5 mice, in an LPA_1-dependent manner. This model recapitulated multiple comorbidities found in human fetal hydrocephalus, and involved mechanisms that affected mitotic and early post-mitotic LPA_1-expressing neural progenitor cells (NPCs) (Park et al., 2016; Yung et al., 2011). These conditions fundamentally contrast with the new postnatal PHH model reported here, which responds to LPA exposure at a developmental stage after neurogenesis has ceased within the cerebral cortex and the subjacent ganglionic eminence, implicating direct ependymal cell effects

in this later model. Both models produce hydrocephalus with 100% penetrance and ultimately disrupt ciliated ependymal cells, consistent with models of PHH involving reduction of cilia-driven CSF flow and removal of the CSF-parenchyma barrier (Jimenez et al., 2001; Wang et al., 2016; Banizs et al., 2005; Tissir et al., 2010; Ibañez-Tallon et al., 2004), causing CSF accumulation which, in turn, leads to ventriculomegaly and chronic hydrocephalus (Faubel et al., 2016; Lun et al., 2015; Brinker, Stopa, Morrison, & Klinge, 2014). A key difference, however, is that the fetal model manifests alterations in NPC fate that reduce ependymal cell production and induce acqueductal stenosis, whereas the new postnatal model directly affects the ependymal cells resulting in their dysfunction, death, and removal without causing physical CSF blockages.

ICP is an important clinical hallmark of PHH which is difficult to measure in small animals. As a result, ventriculomegaly is commonly used as a proxy for hydrocephalus. This circumstance leaves open the question of whether animal models actually produce increased ICP. We therefore developed an ICP monitoring protocol for use in early postnatal mice utilizing a slim fiberoptic pressure cable that emulated clinical approaches. Importantly, to our knowledge, this new postnatal model is the first to demonstrate increased ICP beyond the more common endpoint of ventriculomegaly alone. Use of this model and ICP measuring technique should allow more accurate assessment of PHH-related variables including possible therapeutics.

The complete penetrance of intraventricular LPA exposure in producing postnatally-induced hydrocephalus showed two notable features. First, the CNS damage and severity of hydrocephalus followed a general dose response, whereby higher concentrations of LPA produced more severe PHH sequelae, particularly the degree of ventriculomegaly. Higher concentrations of LPA would be expected to access more LPA receptors as well as activate more LPA receptor subtypes. This may in part explain the incomplete rescue of LPA_1 or LPA_3 null

mutants, the rare but existent rescue of other receptor subtype null mutants, as well as the variable pharmacological inhibition by Ki16425 or AM095 in the face of a near-maximum LPA dose. Models utilizing reduced LPA dosing or formulation may be superior towards identifying new medical interventions, targeting not only LPA receptor subtypes, but also downstream signaling pathway molecules like GPCR Kinase 2 (GRK2, GPCRK2) that inhibits LPA_1 activation in neural cells (Herr, Herr, Lee, Noguchi, & Chun, 2011). Practical implications of dose-dependent effects could be patient stratification in future clinical trials based upon the severity of intraventricular hemorrhage, whereby proof-of-concept studies would be more likely to succeed in patients with low-grade IVH who are still at risk of long-term disability (Mazzola et al., 2014; Klebermass-Schrehof et al., 2012; Dorner, Burton, Allen, Robinson, & Soares, 2018; Matthieu Vinchon, Baroncini, & Delestret, 2012).

The second feature of this neonatal PHH model was the progressive nature of dysfunction, leading ultimately to the death and removal of ciliated ependymal cells. Examination at multiple timepoints delineated temporal stages of ependymal damage and raises questions about which stages might be therapeutically tractable. From 3-24 hours *in vivo,* significant ciliary changes were identified that included ciliary membrane and axoneme disruption, followed by ependymal cell death and removal.

It is possible, however, that some aspects of ciliary dysfunction might be reversible. In this regard, a notable feature of the new postnatal model was the presence of inflammatory phagocytes engulfing cilia, within 3 h following LPA injection. These cells appeared to be macrophages, based upon their morphology and on changes observed in the choroid plexus, however some may also be microglia in view of Iba1 expression in both. It is conceivable that

phagocytosis of some cilia might occur at a point that could be rescued in the absence of innate immune cell infiltration, a possibility enabled by this model that can be examined in the future.

The involvement of LPAR signaling involving at least LPA_1 and LPA_3 are promising targets for medical intervention, since these receptors are transmembrane GPCRs that as a family mediate many human medicines. Additionally, at least one LPA_1 antagonist has entered human Phase II clinical trials for fibrosis, with several others under development for medical indications including cancer (Palmer et al., 2018; Stoddard & Chun, 2015). The superior efficacy of the LPA_1 inhibitor AM095 in preventing LPA-induced ventriculomegaly supports further development of LPA receptor antagonists with improved pharmacokinetic profiles for use in model systems and future clinical trials. Considering that IVH is observed in 15-20% of premature infants (Robinson, 2012; Stoll et al., 2015), a case might be made for a prevention trial in the most susceptible infants at increased risk during earliest GAs. Possible combined therapies utilizing LPA receptor antagonists with suitable anti-inflammatory agents could also be considered, all towards medical interventions to reduce PHH sequelae and the need for neurosurgical interventions.

METHODS

Animal models

P8 littermates of C57BL/6J and Balb/c background were used for this study. *Lpar1-5* knockout mice (J J Contos, Fukushima, Weiner, Kaushal, & Chun, 2000; James J Contos et al., 2002; Ye et al., 2005; Sumida et al., 2010; Lin, Rivera, & Chun, 2012) were crossed onto C57BL/6J or Balb/c backgrounds several generations before use. Pups were fostered with WT mothers when their original mothers demonstrated aggression or when knockout mothers could not leave the breeding colony. All procedures were conducted in accordance with IACUC guidelines of The Scripps Research Institute and Sanford Burnham Prebys Medical Discovery Institute.

LPA preparation and concentration

Powdered 18:1 LPA (Avanti Polar Lipids 857130P) was dissolved with methanol, then aliquoted into tubes, vacuum dried, and stored at -20C. LPA was reconstituted by sonicating in HBSS, resulting in a 5mM stock. 5µL of this stock was administered intracranially, resulting in approximate concentrations of 800µM in CSF or 80µM in whole brain. However, we anticipate 75-90% loss between reconstitution and injection, as LPA sticks to pipette, tube, and syringe surfaces, resulting in approximately 100µM in CSF or 10µM in whole brain.

Recently, our lab has observed significantly decreased loss of lipid concentration by storing LPA in a liquid form after reconstituting in PBS with 0.01% BSA. For the final inhibitor experiment, 500µM of stock LPA in 0.01% BSA was utilized, resulting in an approximate concentration of 80µM in CSF (before losses). These preparation and storage methods enable

the use of lower LPA stock concentrations for similar phenotypic results, and will be utilized for future work.

Surgery

P8 pups were immobilized on a stereotactic frame using a custom head rest and sedated with either IP-administered Nembutal (40 mg/kg) or gaseous isoflurane. The skin atop the head was opened 0.5cm from the bregma and the needle inserted through the skull at approximately 3.2mm caudal x 0.7mm lateral x 1.2mm deep. Mice were injected into the right lateral ventricle with 5µL of vehicle (Hank's balanced salt solution (HBSS), Gibco 14175-095) or 18:1 LPA (Avanti Polar Lipids 857130P) using a 35-36G needle and a glass 10µL Nanofil syringe (WPI). For drug experiments, mice were pretreated by intracranial injection of 2.5µL containing 1mg/kg AM095 (MedChem HY-16029), 1mg/kg Ki16425 (Cayman 355025-24-0), or vehicle (PBS with 0.01% BSA and 5% DMSO), 15 minutes elapsed, then mice were injected in the same location with 5µL of 18:1 LPA. Post-operative flunixin meglumine (1 mg/kg, Merck 0061-0851-03) was administered subcutaneously for 2 days and mice monitored daily for health until tissue harvest.

Intracranial pressure recording

A Biopac fiberoptic pressure cable was inserted through the barrel of a 21G needle with filed-down bevel. The sensor was wetted and calibrated in ultrapure water before each use. Pressure recordings were taken by retracting the sensor into the needle barrel, puncturing the skull of a heavily sedated mouse, and extending the pressure probe into the lateral ventricle. The probe was allowed to equilibrate for 5 seconds before recording every 5th measurement from the Opsens control panel, to a total of at least 10 measurements which were averaged together. To

prevent fluid escape and pressure release during recording, the edge of the needle was sealed around the skull with putty or wax. After recording, the probe was carefully withdrawn and brain tissue collected.

Histology

Whole brains were harvested from experimental animals at the timepoints indicated. For paraffin embedded sections, brains were fixed in formalin acid alcohol (4% formaldehyde + 5% glacial acetic acid in 70% ethanol), paraffinized in a TissueTek VIP, and embedded with a TissueTek TEC. Frozen sections were fixed in 4% paraformaldehyde in PBS, then cryoprotected with 10% and 20% sucrose in PBS and embedded in O.C.T. (Tissue-Tek 4583) or Neg-50 (ThermoFisher 6502). Sections were cut with 10µm thickness on a Leica microtome or cryostat.

A Zeiss Imager.M2 was used to obtain all images. For brightfield imaging, brain sections were deparaffinized and rehydrated through xylene and ethanol washes, stained with H&E, dehydrated in ethanol and xylenes, then coverslipped and preserved with DPX. ISEL+ was performed with the 2.5 brown detection kit (ACDbio 322310) and HRP labeling (Vector ImmPRESS MP-7401) per manufacturer's instructions.

For immunohistochemistry applications, deparaffinized or frozen sections were washed in tris-buffered saline pH 7.4 (TBS), blocked in TBST (0.1% Triton X-100 in TBS) with 5% normal goat serum (NGS), incubated with primary antibody in TBST with 1% NGS overnight, washed in TBST, incubated with secondary antibody diluted 1:1000 in TBST, then washed in TBS with DAPI nuclear counterstain. Slides were coverslipped with Vectashield (Vector Labs H-1000) and kept at 4°C until imaging. Antibody concentrations used include S100A/B 1:300 on paraffinized tissue (Novus Biologicals NB200-538), AcTub 1:250 on paraffinized tissue

(Sigma T6793), cleaved Cas3 1:200 on frozen tissue (Cell Signaling #9664), and Iba1 1:250 on frozen tissue (Wako 019-19741).

Electron microscopy was performed at The Scripps Research Institute with a Philips CM100 TEM and Hitachi S4800 SEM, under direction by the core staff.

Ventriculomegaly quantification

Lateral ventricle volume was measured in coronal sections collected every 100µm, from the most anterior to posterior sections containing lateral ventricle. The ventricular area of each section was quantified in Adobe Photoshop CS6, then added to create a volume approximation. Choroid plexus was included during quantification, as the free-floating nature of this organ causes it to occupy inconsistent locations in the ventricle.

Brain immune cell isolation and quantification

24 hours post-vehicle or LPA injection into P8 mouse ventricles (P8+1), animals were perfused with PBS and brains harvested. The Miltenyi gentleMACS Octo dissociator was used to create single cell suspensions from whole brains. Immune cells were then isolated by discontinuous Percoll (GE Health17-0891-02) gradient before fixation in 0.1% PFA. Cells were incubated with anti-CD16/32 (Fc receptor block, eBioscience 14-0161) before staining in Brilliant Stain Buffer Plus (BD Biosciences 566385) with antibody cocktail to identify macrophages and activated microglia ($CD45^{+}CD11b^{+/hi}F4/80^{+}Ly6G^{-}$). Antibodies were purchased from either BD Biosciences (BD) or eBioscience and were as follows: CD45-BV711 (BD 30-F11), CD11b-BV510 (BD M1/70), F4/80-AF488 (eBioscience BM8), and Ly6G-PE-Cy7 (eBioscience 1A8). Liquid counting beads (BD) were added immediately before data were

obtained on a BD FACSAria Fusion flow cytometer and analyzed using FlowJo v10.4 software (Tree Star, Inc.).

Statistical analysis.

Statistical analyses were performed in GraphPad Prism 7. One-way ANOVA was used to compare each experimental group to uninjected and vehicle groups. Dot plots were utilized reporting mean (center bar) and standard error of the mean (SE, error bars).

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Chapter 4, in part, was submitted to eLife. **Lummis NC**, Sánchez-Pavón P, Kennedy G, Frantz A, and Chun J. "$LPA_{1/3}$ overactivation induces neonatal post-hemorrhagic hydrocephalus through ependymal loss and ciliary dysfunction," *eLife*. (Submitted Nov 2018). The dissertation author was the primary investigator and author of this paper.

Table 4.1. Clinical post-hemorrhagic hydrocephalus phenotypes recapitulated by this neonatal LPA-induced mouse model.

Sequelae reported in clinical studies following cohorts of hydrocephalic patients are compared to phenotypes observed in this neonatal model of PHH. Intraventricular hemorrhage was mimicked by injecting LPA directly into the lateral ventricle of P8 mice. Patients treated with shunts had far greater survival than untreated patients, highlighting the need for therapeutic intervention.

Hydrocephalus Characteristics	Clinical Studies	Neonatal Model
Bleeding/hemorrhage	Yes	Mimicked
Ventricular dilation	Yes	Yes
Elevated CSF pressure	Yes	Yes
Early lethality	~75% untreated[5], ~20% treated[6,11]	80% by 12 weeks
Loss of ependymal layer	Yes[19-22]	Yes
Ciliary defects	Yes[62-65]	Yes
Aqueductal occlusion	Sometimes[30,64]	No
Monocyte infiltration	Implicated in shunt failure[66-68] & systemic inflammation[69]	Yes
Motor dysfunction	~50%[4,70]	Reduced mobility

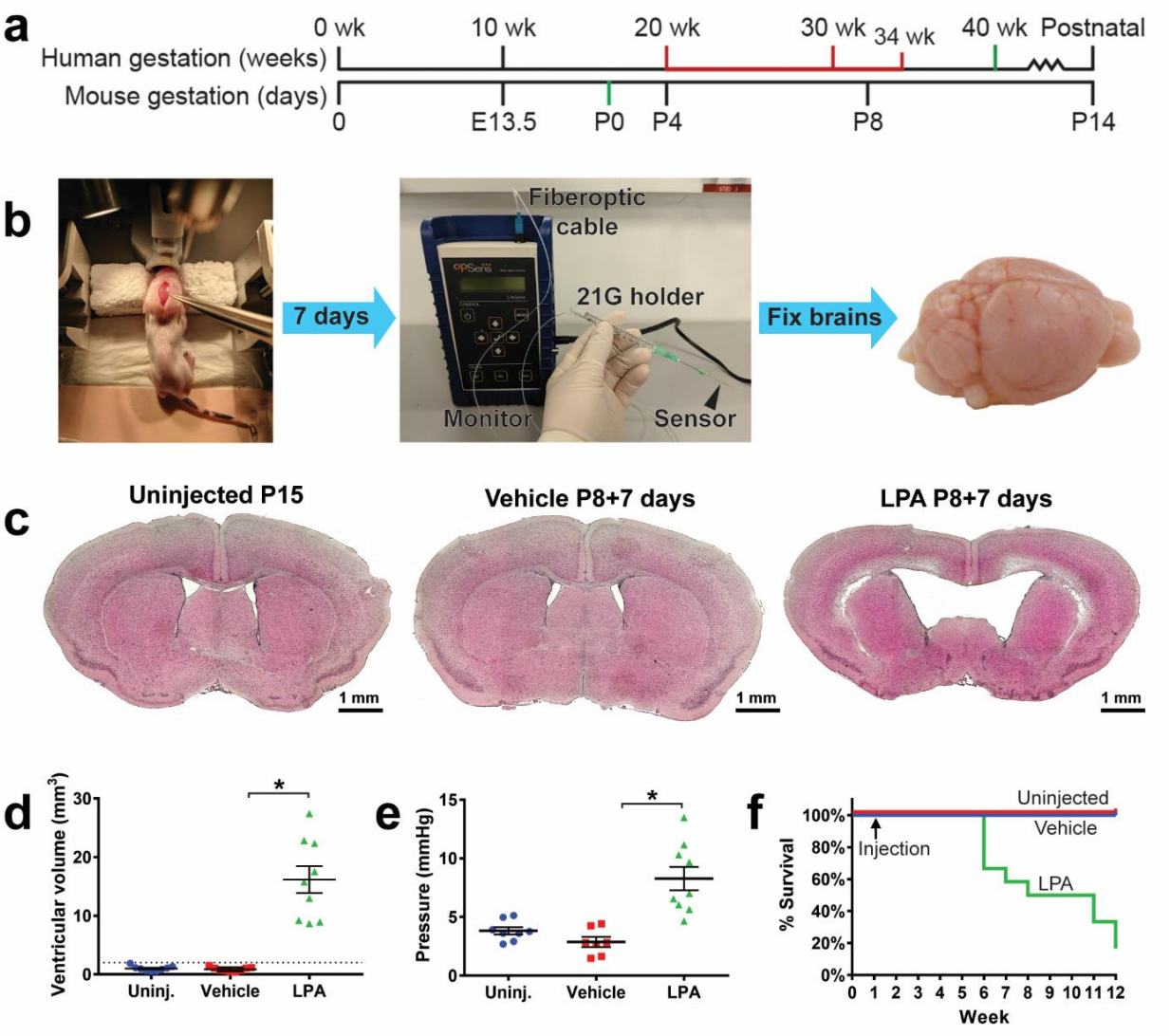


Figure 4.1: Intracerebral LPA exposure leads to hydrocephalus in neonatal mice.

(a) Approximate correlative stages of human and mouse brain development, highlighting full term birth (green) and ages at high risk for PHH (red). (b) Schematic of this neonatal model of LPA induced hydrocephalus. Mice receive stereotactic intracranial injection at P8 (left, forceps point to approximate location of injection), followed by terminal intracranial pressure measurement (center) and brain harvest 7 days later ("P8+7", right). (c) Intracerebral injection of LPA produces ventriculomegaly. Representative brain sections from uninjected, vehicle-injected, and LPA-injected P8+7 mice are shown. (d) Quantification of increased lateral ventricle volume. The dashed line indicates 2 standard deviations above the vehicle mean (n=10). (e) Corresponding increase in intracranial pressure in the brains of P8+7 mice injected with LPA (n=9) as compared to brains from uninjected (n=8) or vehicle-injected (n=7) mice. (f) Kaplan-Meier survival curve for mice injected at P8 showing 80% lethality at 10 weeks (n=7 uninjected, n=9 vehicle, n=11 LPA). *p<0.0005

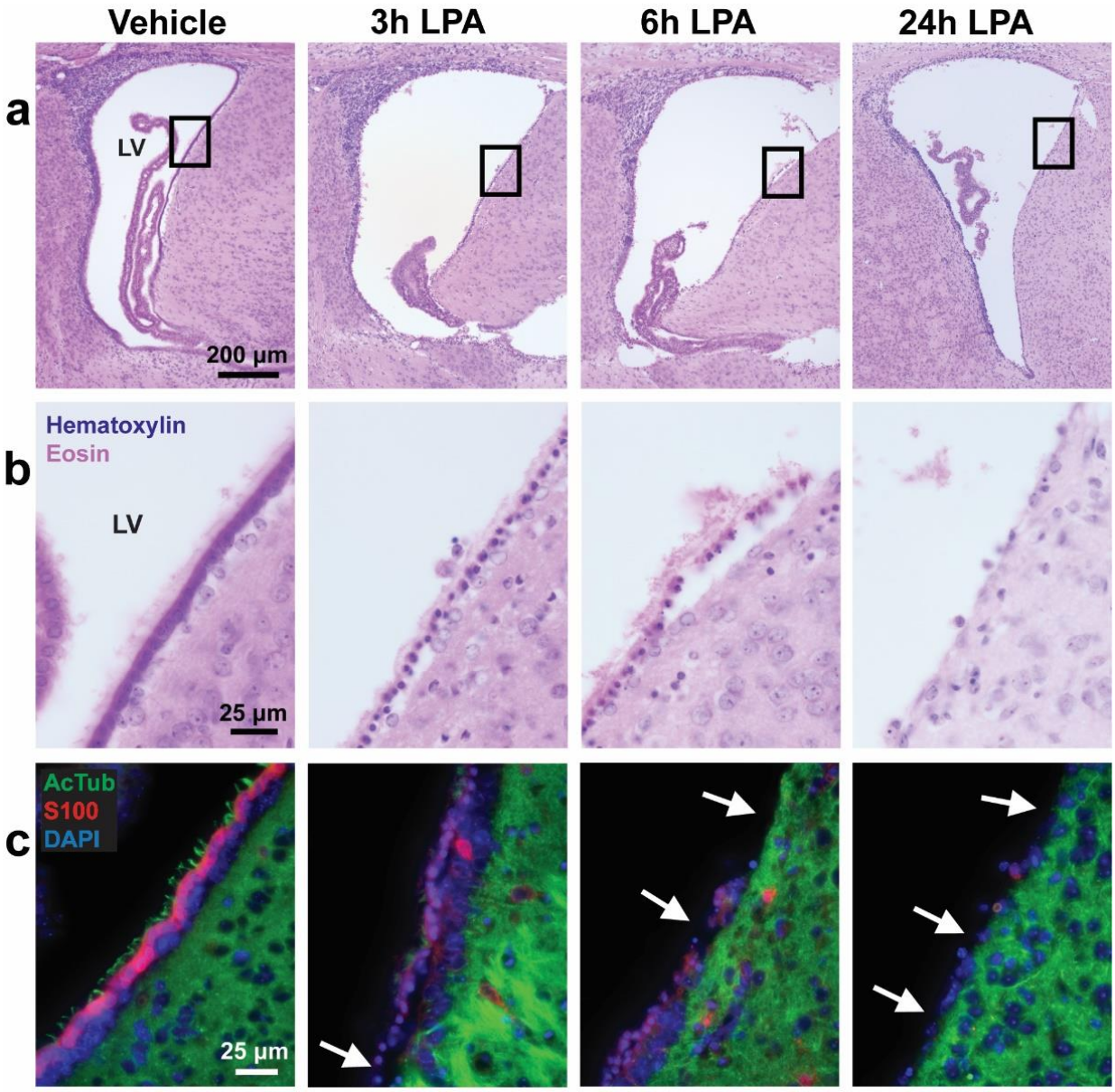
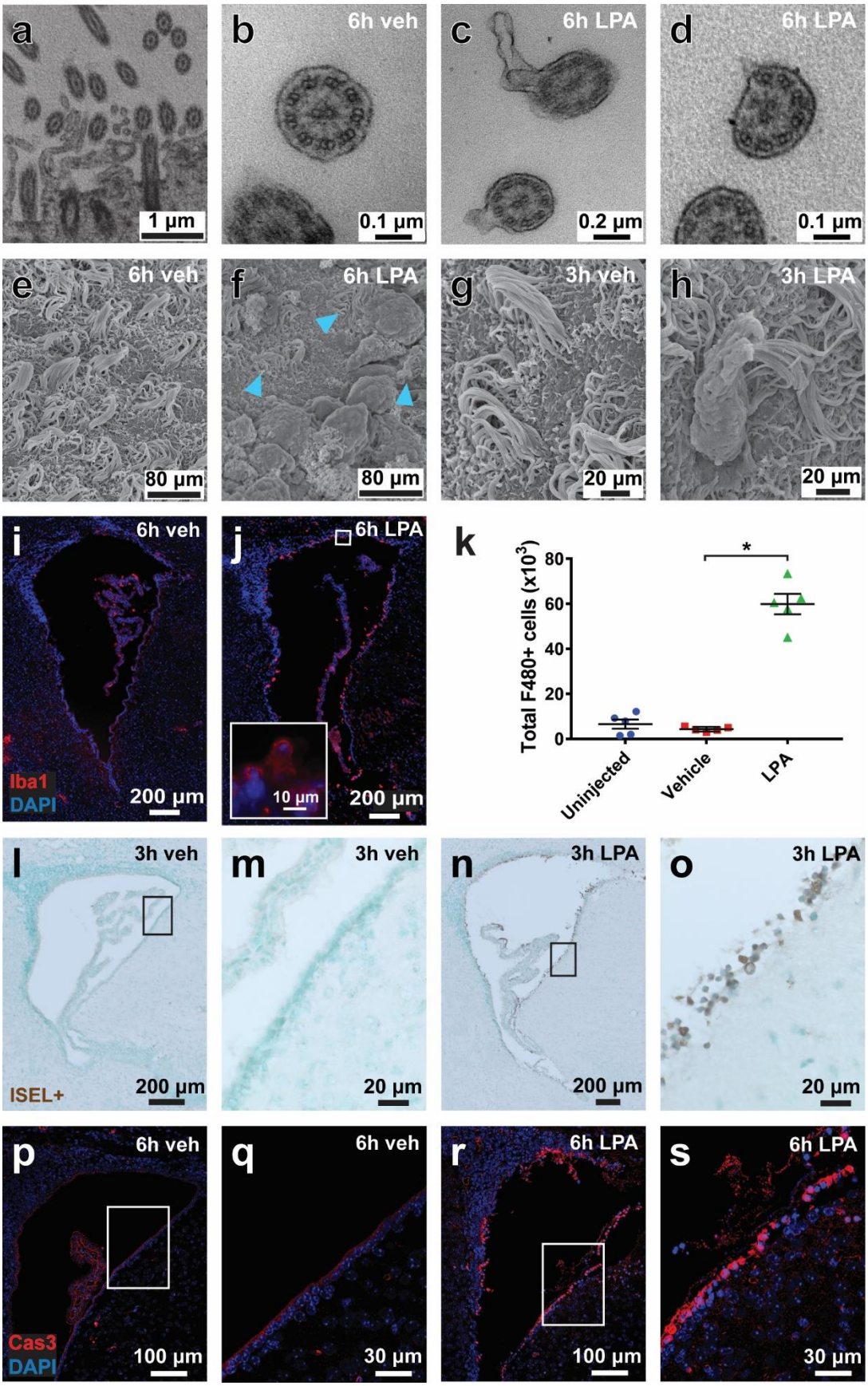


Figure 4.2: LPA affects ependymal integrity.

(a) Images of H&E-stained lateral ventricles 3, 6, and 24 h post-LPA exposure (10x). A representative vehicle-injected lateral ventricle (6 h post-injection) is shown for comparison. (b) 63x magnified regions of the boxed areas shown in (a). (c) Cilia and cell bodies of lateral ventricle ependymal cells immunostained with AcTub (green, cilia) and S100 (red, cell body) with DAPI nuclear counterstain (blue). Arrows point to denuded sections of the ventricular wall. LV = lateral ventricle.

Figure 4.3: Ependymal cell disruption precedes death.

(a) TEM scan of ciliary tuft with several distinct cilia adjacent to ependymal membrane. (b) Ciliary membrane and axoneme structure from a wildtype vehicle-injected mouse. (c) Disrupted ciliary membrane and (d) axoneme observed 6 hours post-LPA exposure. (e) Intact ciliated ependymal surfaces were observed with SEM on vehicle-exposed brains, while (f) LPA-exposed brains show progressive ciliary loss and presence of cellular debris. Blue arrowheads point to remaining ciliary tufts 6 hours after LPA injection. (g) At three hours post-injection, vehicle-exposed cilia were unperturbed whereas (h) phagocytotic cells were observed engulfing shriveled and disorganized ependymal cilia exposed to LPA. (i-j) LPA exposure causes an influx of macrophages/microglia into the lateral ventricles 6 hours post-injection. (i) Macrophage cells, as identified by Iba1 immunostaining, are present in the vascular choroid plexus of vehicle injected mice. (j) LPA produces an influx of Iba1+ cells that accumulate on the ependymal wall of the lateral ventricles. (10x with 63x inset). (k) Flow cytometric analysis of cells isolated from the brains of uninjected, vehicle injected, and LPA injected mice. Brains from uninjected, vehicle injected, and LPA injected mice (24 hours post-injection) were dissociated and isolated cells stained with F480 to identify macrophages and microglia. The total number of F480+ cells is shown (*$p<0.0001$, n=5 brains). (l-o) LPA exposure causes selective ependymal cell DNA damage within 3 hours as demonstrated by the ISEL+ assay (fragmented DNA = brown, nuclei = light green). (l-m) Brains exposed to vehicle are unaffected whereas (n-o) LPA exposure causes selective ependymal DNA damage (10x with boxes enlarged to 63x). (p-s) Cleaved caspase-3 (red, with blue nuclear counterstain) immunolabeling in (p-q) vehicle and (r-s) LPA-injected brains shows selective ependymal apoptosis 6 hours following LPA exposure (20x with boxes enlarged to 63x).


a
1 µm
b
6h veh
0.1 µm
c
6h LPA
0.2 µm
d
6h LPA
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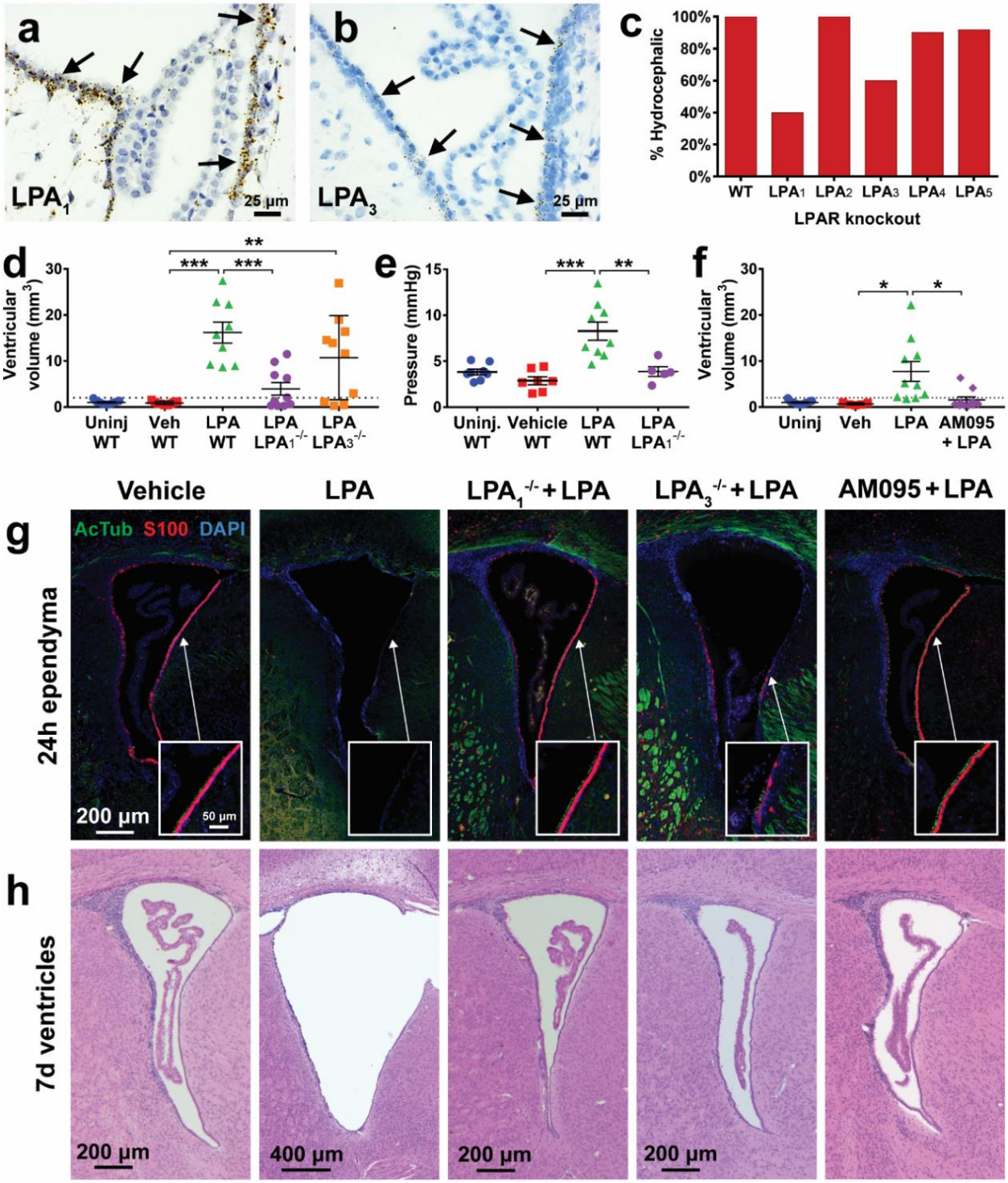
e
6h veh
80 µm
f
6h LPA
80 µm
g
3h veh
20 µm
h
3h LPA
20 µm
i
6h veh
Iba1
DAPI
200 µm
j
6h LPA
10 µm
200 µm
k
Total F480+ cells (x10^3)
80
60
40
20
0
*
Uninjected
Vehicle
LPA
l
3h veh
ISEL+
200 µm
m
3h veh
20 µm
n
3h LPA
200 µm
o
3h LPA
20 µm
p
6h veh
Cas3
DAPI
100 µm
q
6h veh
30 µm
r
6h LPA
100 µm
s
6h LPA
30 µm

Figure 4.4: LPAR selectivity of hydrocephalus development.

Specific LPAR expression in the ependyma was demonstrated by RNAscope for (a) *Lpar1* and (b) *Lpar3,* (brown RNA probes with blue nuclear counterstain). (c) LPA was injected into specific LPA receptor knockout mice and the incidence of hydrocephalus reported, as determined by ventriculomegaly analysis (n=10 for LPA_1-LPA_4, n=11 for LPA_5). (d) Quantification of ventriculomegaly in LPA_1 and LPA_3 knockout mice 7 days following 5mM LPA exposure compared to controls (**p<0.001, ***p<0.0001, n=10). (e) Protected LPA_1 knockouts show normal ICP levels 7 days post-injection (**p<0.001, ***p<0.0001, n=8 uninjected, n=7 vehicle, n=9 LPA, n=5 $LPA_1^{-/-}$). (f) Mice injected with a lower concentration of LPA (500uM in 0.01% BSA) demonstrate significant ventriculomegaly that can be blocked by LPA_1 selective inhibitor AM095 (*p<0.05, n=9 uninjected, n=5 vehicle, n=10 LPA, n=10 AM095+LPA). (g) AcTub (green) and S100 (red) ependymal staining 24 hours post-injection (10x mag with 63x insets). The loss of LPA_1 or LPA_3, or pharmacological inhibition of LPA_1 with AM095, provides protection against LPA-mediated ependymal cell loss. (h) H&E stained lateral ventricle sections 7 days post-injection, demonstrating unperturbed ventricular morphology following LPA_1 loss, LPA_3 loss, or AM095 pretreatment.


a
LPA1
25 µm
b
LPA3
25 µm
c
% Hydrocephalic
100%
80%
60%
40%
20%
0%
WT
LPA1
LPA2
LPA3
LPA4
LPA5
LPAR knockout
d
Ventricular volume (mm3)
Uninj WT
Veh WT
LPA WT
LPA LPA1-/-
LPA LPA3-/-
e
Pressure (mmHg)
Uninj. WT
Vehicle WT
LPA WT
LPA LPA1-/-
f
Ventricular volume (mm3)
Uninj
Veh
LPA
AM095 + LPA
Vehicle
LPA
LPA1-/- + LPA
LPA3-/- + LPA
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24h ependyma
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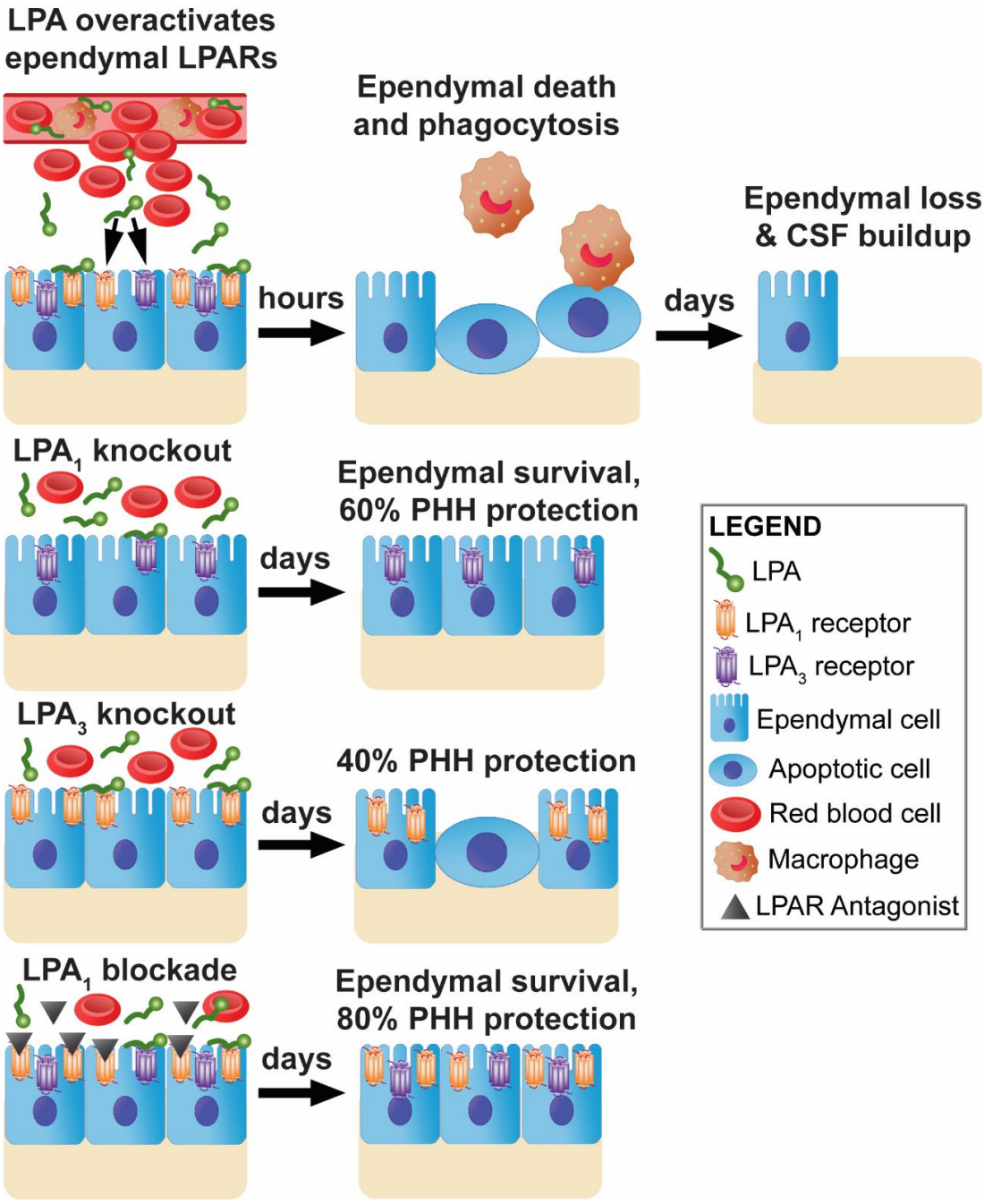
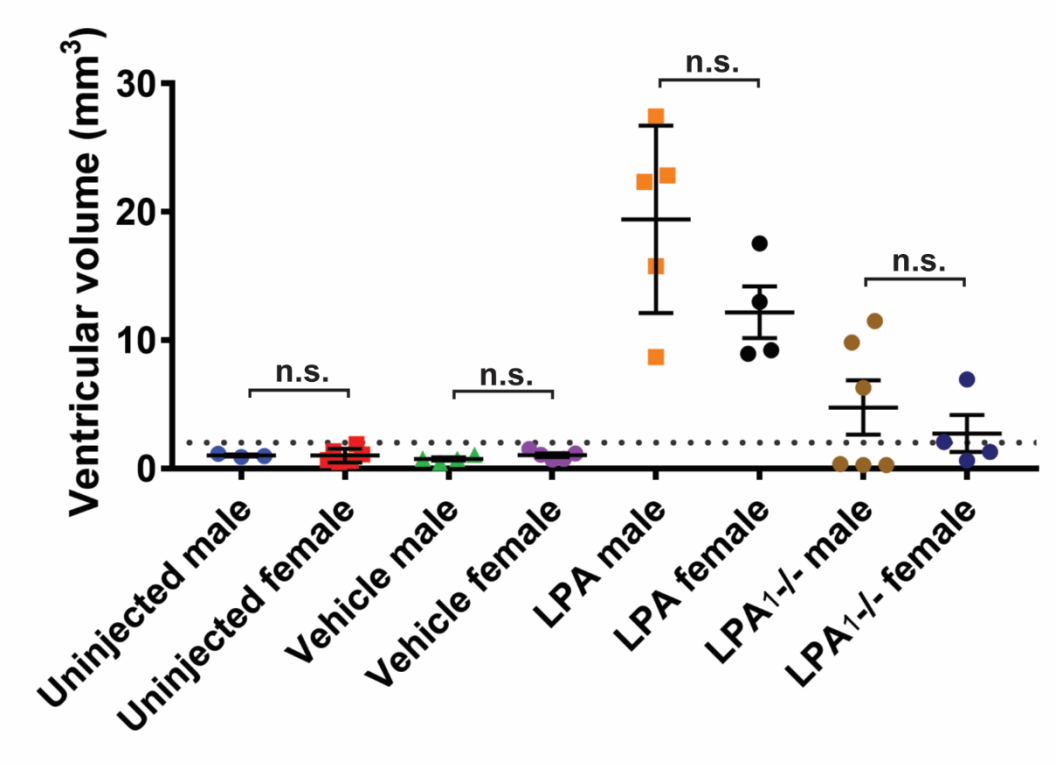


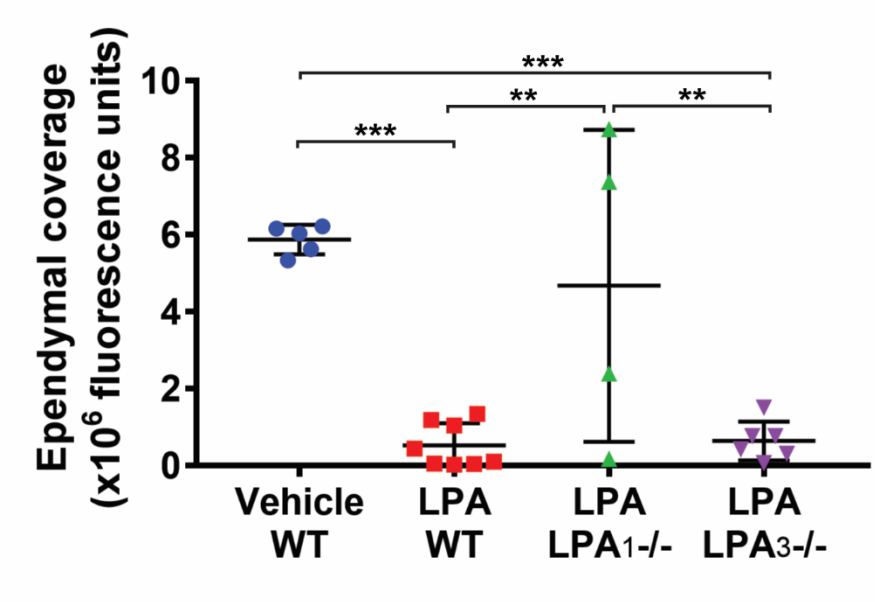
Figure 4.5: Mechanism for LPAR signaling in postnatal hydrocephalus.

Following a severe hemorrhagic event, LPA is released into the CSF. LPA binds to LPA_1 and LPA_3 receptors on the ependymal cells that line the ventricles, causing GPCR overstimulation. After 6 hours, these signaling events cause the ependymal cells to become apoptotic, resulting in ciliary dysfunction and phagocyte recruitment. LPA exposure produces a near total ependymal cell loss after 24 hours. With the lack of cilia-driven flow, CSF builds up in the ventricular space, leading to ventriculomegaly and chronic hydrocephalus. These events can be prevented with the genetic deletion of LPA_1 or LPA_3, or through pharmacological inhibition of LPA_1 with the selective antagonist AM095, demonstrating that this effect occurs through specific LPARs.



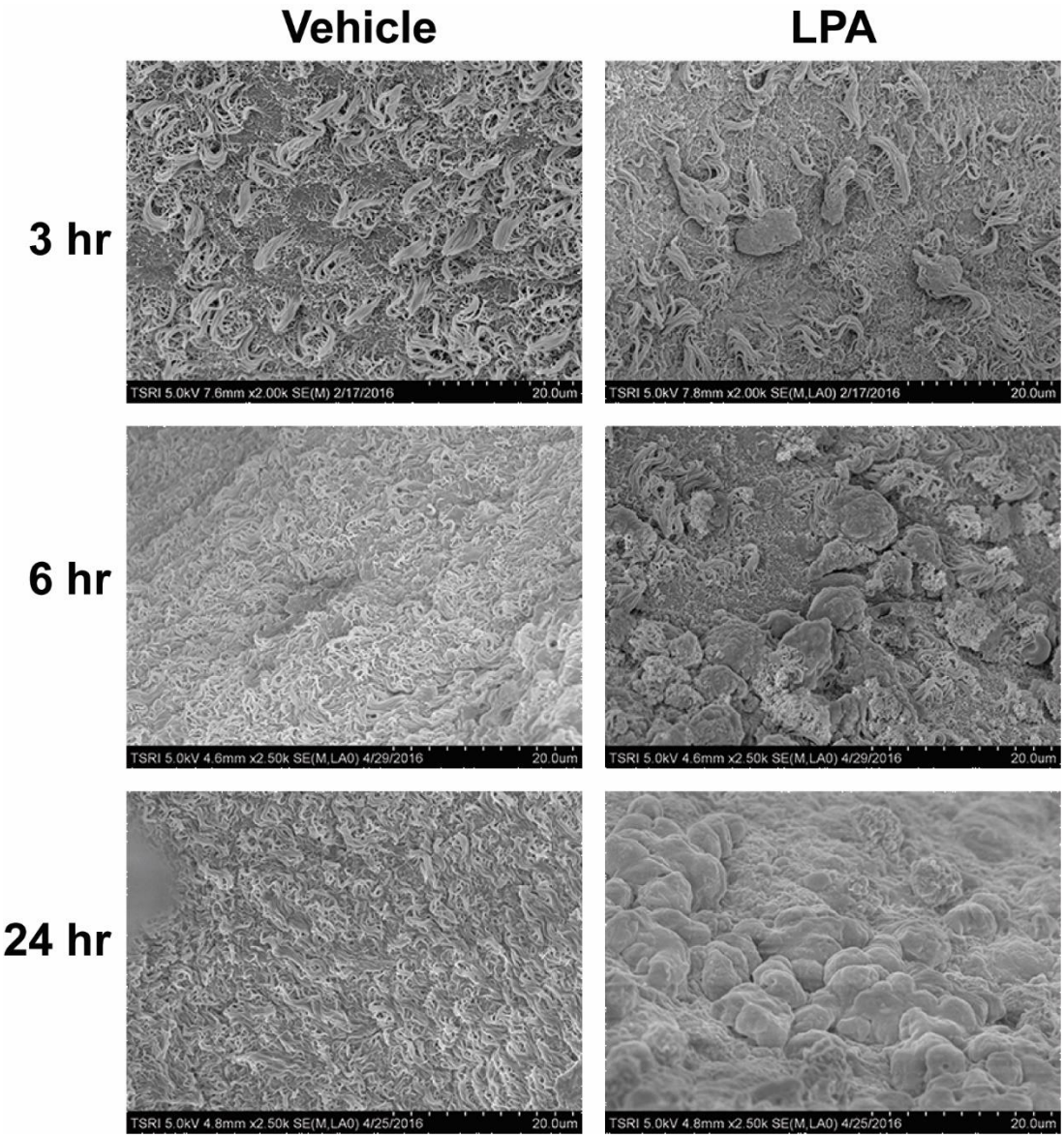
Supplementary Figure 4.S1: LPA-induced PHH is independent of sex.

Ventricular volume of uninjected, vehicle-injected, or LPA-injected WT and LPA_1-/- mice were separated based on sex. There are no significant differences in mean volume or distribution.



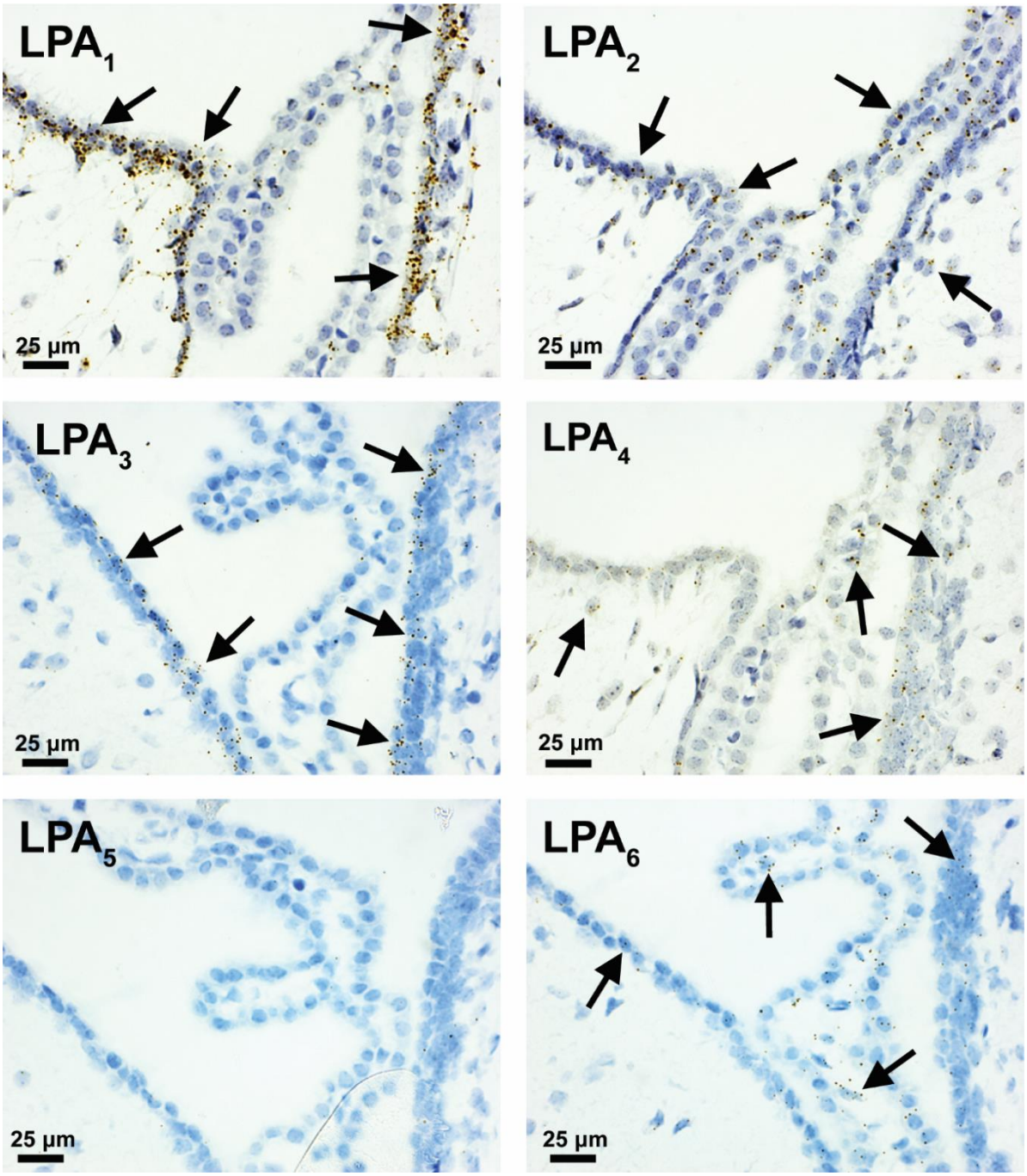
Supplementary Figure 4.S2: LPA-induced ependymal depletion.

The area of S100 fluorescence surrounding the lateral ventricles 24 hours post-injection was quantified using Particle Analysis in ImageJ (with Fiji plugin). 5 sequential sections were quantified for each data point, each 200µm apart, accounting for 1mm of lateral ventricle coverage. *$p<0.0001$



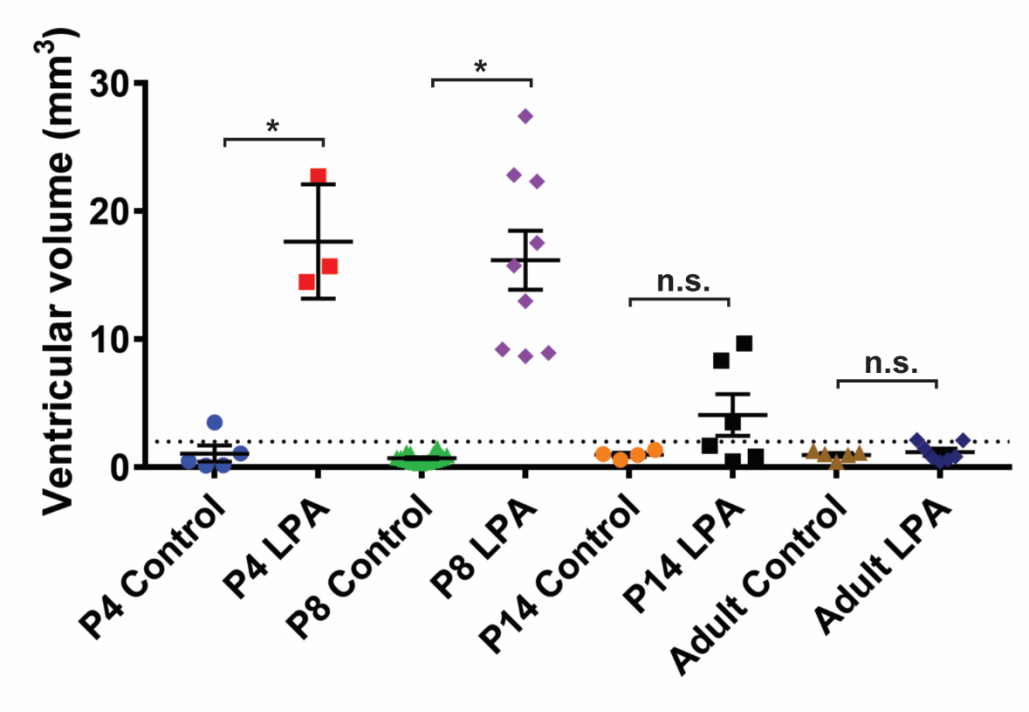
Supplementary Figure 4.S3: SEM cilia coverage of ventricular surface.

SEM images demonstrating the gradual depletion of cilia and loss of integrity of the ependymal wall over time after LPA exposure. At 3 hours, the wall is recognizable as ependymal cells, at 6 hours significant disruption occurs but ciliated ependymal cells are still visible, and at 24 hours the surface is uneven and very few visible cilia remain.



Supplementary Figure 4.S4: LPAR RNA expression in the ventricular region.

LPAR expression was determined by RNAscope (brown RNA probes with light blue nuclear counterstain) for *Lpar1-Lpar6* in the lateral ventricle area. *Lpar1* has the highest levels of ventricular expression, including specific ependymal expression. *Lpar2* is non-specifically expressed at low levels in many cells. *Lpar3* is only expressed in the ependymal monolayer. *Lpar4* is expressed in ependymal cells, vasculature, and parenchymal cells. *Lpar5* exhibits little to no expression. *Lpar6* is found in vascular cells, the choroid plexus, and exhibits low expression in the ependyma. Performed with Grace Kennedy.



Supplementary Figure 4.S5: Temporal susceptibility of LPA-induced PHH.

LPA was injected into the lateral ventricles of mice at several ages, and ventricular volume was quantified 7 days post-surgery. Compared to uninjected and vehicle-injected controls, 100% of mice injected at P4 or P8 developed severe ventriculomegaly. However, less than 50% of mice injected at P14 developed morphological abnormalities following LPA injection, while adult mice were completely unaffected. Adult data contributed by Aaron Frantz.

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Chapter 5:

Characterization of LPA-induced hydrocephalus

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ABSTRACT

Post-hemorrhagic hydrocephalus (PHH) in premature infants is a common neurological disorder treated with invasive neurosurgical interventions. Intraventricular exposure to lysophosphatidic acid (LPA), a bloodborne signaling lipid, induces PHH in fetal mice through developmental mechanisms. This dissertation reports a new LPA-induced postnatal PHH model that maps neurodevelopmentally to premature infants, demonstrating ventriculomegaly with increased intracranial pressure. We have demonstrated that LPA acutely disrupts the ependymal cells that generate CSF flow, followed by phagocytosis, cell death, and ventricular surface denudation. Analyses of LPA receptor-null mice identified LPA_1 and LPA_3 as key mediators of PHH, and pharmacological blockade of LPA_1 prevented 80% of PHH, supporting the medical tractability of LPA receptor antagonists in preventing PHH in premature infants. This chapter supplements these discoveries previously discussed in Chapter 4 by further characterizing this animal model, including dose and strain-dependent effects, cell types affected by LPA-induced PHH, detailed analysis of ependymal changes, and behavioral studies.

INTRODUCTION

Post-hemorrhagic hydrocephalus (PHH) during the perinatal period is a common neurological condition that affects approximately 1 in 1000 live births (Dewan et al., 2018; Chi, Fullerton, & Gupta, 2005; Kahle, Kulkarni, Limbrick, & Warf, 2016). It is characterized by an accumulation of cerebrospinal fluid (CSF), enlargement of the fluid-filled ventricles (termed 'ventriculomegaly') (Bergsneider et al., 2006), increased intracranial pressure (ICP), cortical thinning, and a range of co-morbid neuroanatomical changes and functional deficits (**Table 1**). PHH is particularly common in premature infants that often have fragile cerebral vasculature (Robinson, 2012; Tully & Dobyns, 2014) and who are at risk for severe intraventricular (or intracranial) hemorrhage (IVH). Clinical assessments have reported 16% of premature infants suffer from severe IVH, resulting in 20-50% or more requiring a palliative, permanent shunt (Robinson, 2012; Walid & Robinson, 2012; Shooman, Portess, & Sparrow, 2009; Cherian, Whitelaw, Thoresen, & Love, 2004). Therapies to prevent PHH are currently lacking, a situation compounded by a paucity of adequate animal models that recapitulate human hydrocephalus and which are modifiable by mechanistically relevant, druggable targets.

One possible target is a class of lysophospholipid receptors, which mediates effects of the signaling lipid known as lysophosphatidic acid or LPA. LPA binds to at least 6 G protein-coupled receptors (GPCRs), designated LPA_1-LPA_6 (Yung, Stoddard, & Chun, 2014). LPA is produced from lysophosphatidylcholine (LPC) by the enzyme autotaxin and circulates in the blood within platelets or bound to carrier proteins such as albumin (Sonoda et al., 2002; Aoki et al., 2002). Of particular relevance, bloodborne LPA levels are significantly elevated during hemorrhage or trauma (Aoki et al., 2002; Sano et al., 2002). Therefore, severe hemorrhage introduces large concentrations of and/or prolonged exposure to LPA.

Chapter 4 established that LPA, when injected into the lateral ventricles of P8 mice to simulate infantile intraventricular hemorrhage, consistently produces ventriculomegaly (VM) and intracranial pressure increases characteristic of hydrocephalus (Bergsneider et al., 2006). This occurred primarily through overstimulation of LPA_1 and LPA_3 receptors expressed by the ventricular ependymal cells, a monolater of cells that are critical for maintaining CSF flow and homeostasis. This overstimulation resulted in ependymal death, increased intracranial pressure, and chronic hydrocephalus. However, further characterization is necessary to supplement these data. Here, we provide further evidence of changes induced by LPA-mediated PHH, including dose-dependent bilateral ventriculomegaly that is independent of strain or sex, induced astrocyte expression within the damaged cortical white matter tracts, more detailed characterization of ependymal and ciliary changes, and behavioral analyses of adolescent hydrocephalic mice.

RESULTS

Characterization of LPA-induced ventriculomegaly

LPA-mediated PHH is dose-dependent

In order to choose an appropriate efficacious dose for study of LPA-induced ventriculomegaly, we injected 5µL of several different concentrations of LPA into the lateral ventricles. These included 10mM, 5mM, 2.5mM, 1.25mM, and 0.625mM LPA. 0.25mg LPA stored at -20°C was reconstituted in HBSS, sonicated for 15 minutes, then (where appropriate) diluted and re-sonicated before injection (**Fig. 1a**). All 2.5mM, 5mM, and 10mM injected animals developed significant ventriculomegaly, while 33% of 1.25mM-injected mice were affected, and 0.625mM did not induce noticeably enlarged ventricle volumes. From these results, we chose 5mM LPA as the stock concentration for neonatal hydrocephalus studies to reliably reproduce severe PHH with 100% penetrance.

LPA-induced ventriculomegaly can be rescued by LPA_1 or LPA_3 knockout

With the tools to model neonatal PHH at our disposal, we were interested in determining the LPA receptor specificity of these effects. Since LPA receptor (LPAR) knockout animals were bred into C57BL/6J (LPA_4, LPA_5) and Balb/c (LPA_1, LPA_2, LPA_3) mouse strains, it was imperative to test the penetrance of LPA-mediated VM in both backgrounds. Cohorts were injected with 5µL of vehicle or 5mM LPA and brains were analyzed 7 days following this procedure. Ventricular volume quantification demonstrated no strain-dependent differences in VM degree or penetrance (**Fig. 1b**).

Following this validation experiment, we began injections of vehicle or 5mM LPA into the lateral ventricles of constitutive LPA_1-LPA_5 knockout mice. While LPA_2, LPA_4, and LPA_5 knockout animals did not demonstrate significant PHH protection, knockout of LPA_1 or LPA_3 resulted in reduced VM in a substantial portion of the population. These results are quantified in **Figure 1c**, demonstrating 60% protection in LPA_1 knockout animals and 40% protection in LPA_3 knockouts.

PHH development can be prevented by LPA_1 antagonism

Since knockout animals allowed us to identify LPA_1 and, to a lesser extent, LPA_3 receptor signaling involvement during PHH initiation, we were hopeful that pharmacological agents targeting these same receptors might prove efficacious in mitigating hydrocephalus. To this end, we obtained the LPA_1 receptor antagonist AM095 and $LPA_{1/3}$ dual antagonist Ki16425. In order to perform these experiments in more physiological conditions and enhance the solubility of these lipid-like compounds, we adopted a new vehicle containing 0.01% BSA in PBS, as described above. Antagonist solutions were stored at 100mM in 100% DMSO, then diluted for a solution containing 4mM drug with 4% DMSO and 0.01% BSA in PBS. This resulted in a 1mg/kg intracranial injection.

Wild type C57BL/6J mice were pretreated with 2.5µL of vehicle or antagonist, 15 minutes elapsed, then injected with 5µL of 500µM LPA at the same stereotactic coordinates. After 7 days, brains were collected and ventriculomegaly analyzed (**Fig. 1d**). Under these conditions, most LPA injected animals demonstrated significant ventriculomegaly, while 80% of AM095-treated animals and 40% of Ki16425-treated animals were protected from developing PHH. Additionally, the unprotected 20% of AM095-treated animals suffered from less severe

ventriculomegaly than its LPA-injected counterparts. This disparity may reflect a pharmacokinetic issue with Ki16425, as it is less stable than AM095 in aqueous solution, or it could be due to intrinsic mechanisms such as upregulation of degradative enzymes.

LPA induced ventriculomegaly is bilateral

Because the methodology of PHH initiation begins with an injection of the right lateral ventricle, we hypothesized that the effects of PHH may be more severe in the ipsilateral hemisphere. To this end, we quantified the ventricular volume within the left and right hemispheres separately and compared the results (**Fig. 1 e-f**). While some ependymal damage appeared more severe on the ipsilateral side, especially at the lower 500μM LPA concentration, the lateral ventricles were uniformly expanded 7 days following the procedure. A contributing factor to this effect may be the tearing of the corpus callosum that runs between the left and right lateral ventricles around P8+3 days, effectively connecting the lateral ventricle system as the disease progresses. A small exception to this effect was observed in LPA_3 knockout animals, where 3 out of 10 animals demonstrated significant ipsilateral ventriculomegaly.

CNS cell type changes following LPA exposure

Brains harvested 24 hours or 7 days after LPA or vehicle-injection were stained with DAPI (nuclei), NeuN (neurons), GFAP (activated astrocytes), and MBP (myelin), and whole-brain images were acquired (**Fig. 2**). While cell types were relatively unaffected 1 day following surgery, more damage was seen in brains with severe VM 7 days following LPA exposure. This damage was associated with an increased number of activated astrocytes. We also noticed tearing of the corpus callosum associated with ventricular enlargement, loss of corpus callosum-

associated white matter, and cortical thinning associated with white matter tracts. Further investigation is required to determine whether this white matter loss is specific or a result of gross ventricular expansion.

LPA exposure causes ependymal disruption and death

Ependymal cells are lost by 25h and incompletely regenerated after 7 days

In order to discern the mechanism of LPA-mediated hydrocephalus initiation, we harvested brains at several timepoints post-LPA injection. Histological staining showed an immediate disruption of the ependymal cell bodies lining the ventricular surface (**Fig. 3a-b**). Ependymal cells demonstrated condensed nuclei and a more round morphology 3 hours post-LPA exposure. After 6 hours, these cells were further perturbed, resulting in an uneven ventricular surface and detachment from the basal lamina. 24 hours post-LPA exposure, the ventricular surface appears fully depleted of ependymal cells, with parenchymal cells directly contacting the CSF.

These phenomena were further investigated with immunohistochemistry. Tissue sections were deparaffinized and stained with acetylated tubulin (AcTub) to visualize structural ciliary proteins and S100 for ependymal cell body localization (**Fig. 3c**). While cilia are still visible at 3 and 6 hours in histological stains, AcTub staining diminishes at 3 hours post-LPA exposure and is almost completely lost at 6 hours, suggesting a loss of structural integrity. Additionally, ependymal S100 staining was almost completely depleted after 24 hours, with only a few fluorescent cells remaining in the corners of the ventricles or in proximity to the attachment site of the choroid plexus. These data further support the histological evidence of LPA-induced ependymal loss. Intriguingly, at the 7 day timepoint, many more S100-stained ependymal cells

were observed than at 24 hours, mostly adjacent to the corners of the lateral ventricles. This suggests that, while acute ependymal damage may be significant enough to cause a chronic hydrocephalic state, these cells may naturally regenerate over time despite the increasing CSF pressure.

Ependymal cells express membrane-bound LPA_1

Since LPA_1 signaling appears to be involved in hydrocephalus development (**Fig. 1c-d**), we were interested in its localization within the P8 brain. While RNAscope analysis allows us to assess the expression of LPA_1-LPA_6 (see Chapter 3, Supplementary Figure 4), especially considering the difficulty in obtaining GPCR antibodies with high specificity, intracellular transcripts do not necessarily correlate to the presence of functional protein. For this experiment, we used a validated LPA_1 antibody (Invitrogen PA1-1041) and costained with AcTub to observe LPA_1 localization at high resolution (**Fig. 4**). These results demonstrated membrane localization, suggesting functional transmembrane activity. This localization was strongest on the apical surface of the ependymal cells facing the CSF, but was absent from ependymal cilia as determined by the lack of AcTub costaining.

LPA induces ciliary abnormalities prior to ependymal loss

In order to study the LPA-exposed ependymal phenotype in greater detail, we performed electron microscopy (TEM) on sections of lateral ventricle. This especially allowed us to observe the structure of ependymal cilia. A typical cross-section of motile cilia contains 9 microtubule pairs in a ring attached to 2 central microtubule singlets, referred to as the axoneme (**Fig. 5a**). This structure, pulled and pushed by dynein motors, is responsible for ciliary beating.

Following LPA exposure, we noticed increased instances of ciliary perturbations, including disrupted membranes (**Fig. 5b**) and lost or skewed microtubules (**Fig. 5c**). These abnormalities were quantified at 3, 6, and 24 hours post-LPA exposure (**Fig. 5d**), and more detailed statistics are reported in **Table 1**. The most significant abnormalities were observed at 6 hours post-injection, the same timepoint that loss of AcTub expression was observed, suggesting that ciliary deconstruction may occur at this stage. Differences between vehicle and LPA-exposed samples were negligible by 24 hours. This makes sense, as ependymal cell death has already occurred at this point, and only the surviving cells could be quantified.

Innate immune cells phagocytose the ependymal monolayer

Iba1-expressing phagocytes are recruited to dying ependymal cells

During our investigation, we noticed several non-ependymal cells present on the ventricular surface. As immune cells are often recruited to tissues that are unhealthy or dying, we performed several leukocyte antibody stains, identifying these invaders as Iba1+ macrophages or microglia (**Fig. 6a-c**). While Iba1+ macrophages exist naturally within the vascular choroid plexus that floats in the lateral ventricle (**Fig. 6a**), these choroid plexus cells are depleted in LPA-treated brains, correlating to a massive increase in Iba1+ cells attached to the ventricular surface (**Fig. 6b**). These cells were quantified by averaging the number of fluorescent Iba1+ cells adherent to the ventricular surface in serial sections (**Fig. 6c**). Invading phagocytes peaked 6 hours post-LPA exposure, when the ependymal cells are significantly damaged, and were reduced at 24 hours when the ependymal cells have been cleared from the ventricular surface.

Ependymal cilia are phagocytosed

These phagocytes were observed more closely using scanning electron microscopy (SEM) and TEM. While the vehicle-treated ventricular surface was coated uniformly in tufts of ependymal cilia (**Fig. 6d**), several elongated cells were observed engulfing cilia in LPA-treated samples (**Fig. 6e**). These cells were cluttered with dense endocytic vesicles (**Fig. 6f**). TEM also captured a phagocytotic cell adjacent to a tuft of cilia that is in the process of digesting a perfect cross-section of ciliary axoneme (**Fig. 6g**).

Ciliary dysfunction in hydrocephalus

A brain slice culture system for ependymal cilia visualization

AcTub staining and EM evidence suggests that ependymal cilia disruption occurs 3-6 hours post-LPA exposure, preceding ependymal cell body loss by several hours. To study the activity and function of cilia more directly, we developed an *in vitro* culture system. For each experiment, brains were collected from 2-3 P8 animals and cut into 250µm-thick sections with a vibratome. Sections containing lateral ventricle were incubated with membrane-permeable dyes for 5 min, including 5µg/mL Hoechst nuclear stain, 5µg/mL Lectin DyLight 488 for ependymal staining, and 5µg/mL CM-DiI. While CM-DiI is a soluble non-specific membrane dye, this dye attached to ependymal cilia with higher affinity than other cellular membranes, likely due to their surface area and proximity to solution. Following 2 3-minute washes in high-glucose DMEM, brains were transferred to a glass-bottomed 12-well plate containing 1mL media. Brain sections

were weighed down with harp slice grids (commonly used in electrophysiology) and incubated for 1 hour before transfer to the microscope.

In order to capture ciliary beating, we needed a device to acquire fluorescent images with at least 100fps. The Nikon N-SIM with A1R confocal resonance scanning system allowed us to achieve this. This microscope has a resonance scanner that acquires simultaneous images in all 3 of the required channels. A z-stack of the dyed lateral ventricle surface at 20x, with Lectin-coated ependyma and spots of DiI-stained cilia, can be seen in **Figure 7a**. Additionally, a band scan that limits image acquisition to a portion of the available scanning area allowed us to capture images of the ependymal surface at 113.2 fps. A still image example of this surface is displayed in **Figure 7b**. This new methodology allows us to monitor ependymal morphology and ciliary function in a live system, providing a powerful tool to observe ependymal changes to different stimuli.

Ciliary beating is disrupted 3-6h following LPA exposure

Using this system, we were interested in tracking ciliary movement speed and patterns to determine if LPA exposure directly affects ciliary function. We set up a 12-well plate with ventricular slice culture, acquired a series of baseline images at t0, then treated the wells in triplicate with vehicle, 1μM LPA, 10μM LPA, or 100μM LPA. Timelapse images were acquired every 30 minutes for 6 hours, when *in vivo* cilia demonstrate significant perturbation. These images were analyzed with Imaris tracking software. This software allowed us to track fluorescent cilia with a specified CM-DiI radius and travel distance. Additionally, we could export videos with colored dots overlaying the tracked points and gray lines outlining individual ciliary paths (**Fig. 7c**), along with spreadsheets containing statistics on every tracked point

including speed, size, and travel distance. Points were further excluded by minimum tracking duration (50ms) and x-y coordinates, to prevent inclusion of cilia with unreliable tracking or counting of the same cilium multiple times.

One complication involved the significant variance on number of trackable cilia and cilia speed between untreated wells at t0. Because of this, it is important to compare each well to its baseline untreated statistics at t0. Additionally, 2 of the 100µM LPA replicates began immotile and were excluded from analysis. The percent change over baseline in number of motile cilia and speed of ciliary movement is shown in **Figure 7d-e**. Using these criteria, treatment with 10µM LPA resulted in significant reduction in average ciliary beat speed (**Fig. 7d**) and number of motile cilia (**Fig. 7e**) compared to vehicle-treated controls. However, these experiments will need to be repeated to ascertain a dose-dependent mechanism with greater statistical power. Additionally, tracking over 24 hours will allow us to observe changes in ependymal function, coverage, and morphology in real time.

Ciliary arrest is sufficient to cause ventriculomegaly

Scientific literature provides significant evidence that abrogation of ependymal cilia function is sufficient to cause ventriculomegaly and hydrocephalus (Banizs et al., 2005; Ibañez-Tallon et al., 2004; Jimenez et al., 2001; Rodriguez et al., 2012; Tissir et al., 2010; Wang et al., 2016). However, we wanted to test this hypothesis ourselves. To this end, we acquired sodium metavanadate and sodium orthovanadate. Vanadate is a chemical which prevents ATP catalysis by dynein, a molecular motor whose movement drives ciliary beating (Doerner et al., 2015; Gibbons et al., 1978). While metavanadate and orthovanadate have the same function, their chemical properties vary significantly. Sodium orthoanadate is a competitive phosphatase

inhibitor with reversible activity and a 2 hour half-life in aqueous solution (Kawano et al., 2001). Conversely, sodium metavanadate forms an irreversible charge transfer reaction with the target ATPase (Gasviani, 2006) and has an *in vivo* half-life spanning tens to hundreds of days, depending on the tissue (Bingham et al., 2012). They are both soluble up to 1mM in water, although orthovanadate requires the water to be adjusted to pH 10 and heated for 15 minutes at 70°C.

We first tested this hypothesis by incubating brain slices with 1, 10, or 100µM vanadate, with duplicate replicates. Images of beating cilia were acquired at time 0, then wells were treated with vanadate and monitored every 30 minutes for 5 hours. Ciliary arrest was achieved in all 100µM-treated samples within 1 hour, while the results from lower concentrations were inconclusive with inconsistent ciliary motility between replicates.

Following these results, we injected 5µL of 10 or 100 µM stock of vanadate into the right lateral ventricle of wildtype C57 animals and waited 7 days to observe PHH-related phenotypes. Both metavanadate and orthovanadate was used. Injection of sodium metavanadate resulted in one case of severe ventriculomegaly, while no orthovanadate-treated mice developed hydrocephalus (**Fig. 7f**). Metavanadate-induced VM was accompanied by survival of ependymal cilia as well as a fully intact ependymal barrier (**Fig 7fg-h**). These results suggest that arrest of ependymal ciliary motility is sufficient to induce PHH without affecting ependymal viability.

Intraventricular LPA causes blood-brain barrier leakage

Previous studies have suggested that LPA signaling can control blood-brain barrier (BBB) permeability (On et al., 2013; Herr, unpublished). We were interested if LPA introduced to the ventricular space could induce BBB leakage, further exacerbating the hemorrhagic state.

To determine whether LPA induces acute BBB leakage, we injected LPA or vehicle into the lateral ventricles of P8 mice, waited 30 minutes, then injected sedated mice with 1.2µL of 0.05 mg/mL dye per gram mouse (60 mg/kg) directly into the heart. Two dyes were combined into this solution: 330 Da fluorescein and 3000 Da tetramethylrhodamine-conjugated dextran. After 15 minutes of cardiac beating, brains were harvested and fixed with 4% paraformaldehyde, crosslinking the dyes to parenchymal and endothelial tissue. As fluorescein is a nonpolar low molecular weight compound, it is able to diffuse into the brain parenchyma without assistance, but the larger dextran compound requires a leaky or compromised BBB to enter the brain. Indeed, dextran leakage was observed in LPA-injected but not vehicle-injected brains (**Fig. 8a**). This leakage was quantified in **Figure 8b** by measuring fluorescence intensity of ventricular images in ImageJ.

This experiment was also performed by injecting dyes into the heart 7 days after LPA exposure, when brains are significantly hydrocephalic. However, no leakage was observed in either LPA or vehicle-injected brains (data not shown). These results suggest that BBB leakage is transient following LPA exposure, and not a contributing factor to CSF volume increases in chronic PHH.

LPA-induced PHH does not affect mobility

Motor difficulty is one of the most common side effects reported by hydrocephalus patients (Bergsneider et al., 2006; Zhang et al., 2006). Interested in assessing the effects of neonatal PHH on juvenile mouse behavior, we kept cohorts of uninjected, vehicle-injected, and LPA-injected animals for 12 weeks, monitoring weight, survival, and behavior. Hydrocephalus began causing lethality in animals at 6 weeks of age (5 weeks post-injection), with 20% survival

at 12 weeks of age (**Fig. 9a**). This complicated behavioral analysis, as adult mice establish a more reliable baseline of variation, and pups' motor coordination is not developed enough to perform motor tests until approximately 8 weeks of age. To circumvent this, mice were tested from 7-8 weeks of age (starting 6 weeks after injection). Open field and rotarod tests were performed over the course of 2 weeks

During the first day of weeks 1 and 2, mice were tested in the open field box for 10 minutes. Distance traveled (**Fig. 9b**), average movement speed (**Fig. 9c**), and time spent resting (**Fig. 9d**) were quantified with computer analysis of infrared laser coordinates. While a few hydrocephalic mice underperformed, 2 mice were hyperactive compared to controls. Additionally, the most stationary mouse, who spent almost half of the open field time resting at the same coordinates, died before week 2. While hydrocephalic mice appeared more stationary inside the cage and were often docile when handled, their ability to move in a novel environment was unhindered.

Rotarod trials were performed 3 consecutive days during weeks 1 and 2. First, Mice were tested on the accelerating rotarod, with speeds increasing from 4-40rpm in 300 seconds. Trials were performed in triplicate, with the average rotarod training time for week 1 reported in **Figure 9e**. This test showed no significant differences between uninjected, vehicle-injected, or LPA-injected cohorts. At the end of week 2, basic motor skills were tested with a constant slow rotation speed of 5rpm for 120 seconds (**Fig. 9f**). As this test does not exhaust the animals, trained mice should be able to remain on the rotarod for the entire testing period. All surviving hydrocephalic animals performed well. While mice within 1 week of death were often completely incapable of performing on the rotarod, most hydrocephalic mice performed similar to control mice, able to remain on the accelerating rotarod for 60-120 seconds.

LPA-injected LPA_1 or LPA_3 knockout animals did not display significant differences from wild type controls in rotarod or open field testing. In fact, they appeared to perform worse on the rotarod test (data not shown). These mice were tail-clipped and genotyped after behavioral testing to prevent this minor injury from interfering with behavioral testing or inducing foster mother rejection before weaning. As uninjected and vehicle-injected littermates all genotyped as wild-type or heterozygous, more controls are necessary to determine if these results are inherent to the knockout strain.

Despite their mobility, I did notice several hydrocephalic mice gripping the rotarod with a skewed and open-pawed gait, often leaping forward at intervals instead of walking at a regular pace. Therefore, further behavioral analysis such as grip or gait tests may be informative. Additionally, these tests were performed early in life and only a few weeks after the development of hydrocephalus, whereas motor difficulty often develops in patients after an extended period suffering from chronic hydrocephalus. Perhaps rotarod testing will show greater differences in adult mice induced to have a less severe form of hydrocephalus with a longer survival time.

Mechanism of LPA-induced post-hemorrhagic hydrocephalus

This dissertation provides significant evidence toward lysophosphatidic acid signaling being a key initiating factor of post-hemorrhagic hydrocephalus. LPA is released into the CSF during intraventricular hemorrhage signaling on LPA_1 and LPA_3 receptors present on ependymal cell membranes. As LPA concentrations in the CSF are normally very low, this overactivation induces ependymal damage, ciliary dysfunction, phagocytosis, and severe ependymal cell loss. Ependymal cells serve as a significant driver of CSF flow and function as a barrier separating the

CSF and parenchyma, so loss of these cells is sufficient to induce CSF accumulation, leading to intracranial pressure increase and chronic hydrocephalus (**Figure 10**).

METHODS

Animal models

Healthy P8 littermates of C57BL/6J and Balb/c background were used for this study. *Lpar1* and *Lpar3* knockout mice were generated in-house and crossed onto Balb/c backgrounds several generations before use (Contos et al., 2000; Ye et al., 2005). Pups were fostered with WT mothers when their original mothers demonstrated aggression or when knockout mothers could not leave the breeding colony. All procedures were conducted in accordance with the IACUC guidelines of TSRI and SBP.

Surgery

P8 pups were immobilized on a stereotactic frame using a custom head rest and sedated with either IP-administered Nembutal (40 mg/kg) or gaseous isoflurane. The skin atop the head was opened 0.5cm from the bregma and the needle inserted through the skull at approximately 3.2mm caudal x 0.7mm lateral x 1.2mm deep. Mice were injected into the right lateral ventricle with 5µL of vehicle (Hank's balanced salt solution (HBSS), Gibco 14175-095) or 18:1 LPA (Avanti Polar Lipids 857130P) using a 35-36G needle and a glass 10µL Nanofil syringe (WPI). For drug experiments, mice were pretreated by intracranial injection of 2.5µL containing 1mg/kg AM095 (MedChem HY-16029), 1mg/kg Ki16425 (Cayman 355025-24-0), or vehicle (PBS with

0.01% BSA and 5% DMSO), 15 minutes elapsed, then mice were injected in the same location with 5µL of 18:1 LPA. Post-operative flunixin meglumine (1 mg/kg, Merck 0061-0851-03) was administered subcutaneously for 2 days and mice monitored daily for health until tissue harvest.

Dye leakage experiments were performed by injecting intracranial vehicle or LPA, waiting 30 minutes, then opening the cardiac cavity of sedated mice and injecting 1.2µL/g (or 60mg/kg) dye mixture into the heart. Sodium fluorescein (Sigma F6377) and TMR-dextran (Life Technologies D-3308) were used for this experiment. These dyes circulated for 15 minutes before collection of brain tissue and fixation in 4% PFA. Brains were frozen following sucrose protection and sectioned in low light to prevent photobleaching. Lateral ventricle images were acquired on the Zeiss.M2 Imager and quantified for fluorescence intensity using ImageJ.

Histology

Whole brains were harvested from experimental animals at the timepoints indicated. For paraffin embedded sections, brains were fixed in formalin acid alcohol (4% formaldehyde + 5% glacial acetic acid in 70% ethanol), paraffinized in a TissueTek VIP, and embedded with a TissueTek TEC. Frozen sections were fixed in 4% paraformaldehyde in PBS, then cryoprotected with 10% and 20% sucrose in PBS and embedded in O.C.T. (Tissue-Tek 4583) or Neg-50 (ThermoFisher 6502). Sections were cut with 10µm thickness on a Leica microtome or cryostat.

A Zeiss Imager.M2 was used to obtain all images. For brightfield imaging, brain sections were deparaffinized and rehydrated through xylene and ethanol washes, stained with H&E, dehydrated in ethanol and xylenes, then coverslipped and preserved with DPX.

For immunohistochemistry applications, deparaffinized or frozen sections were washed in tris-buffered saline pH 7.4 (TBS), blocked in TBST (0.1% Triton X-100 in TBS) with 5%

normal goat serum (NGS), incubated with primary antibody in TBST with 1% NGS overnight, washed in TBST, incubated with secondary antibody diluted 1:1000 in TBST, then washed in TBS with DAPI nuclear counterstain. Slides were coverslipped with Vectashield (Vector Labs H-1000) and kept at 4°C until imaging. Antibody concentrations used include S100A/B 1:300 on paraffinized tissue (Novus Biologicals NB200-538), AcTub 1:250 on paraffinized tissue (Sigma T6793), Iba1 1:250 on frozen tissue (Wako 019-19741), LPA_1 1:400 on frozen PLP-fixed sections (Invitrogen PA1-1041), NeuN 1:1000 on paraffinized tissue (Millipore MABN140), GFAP 1:1000 on paraffinized tissue (Sigma G3893), and MBP on paraffinized tissue (Neuromics CH22112).

Electron microscopy was performed at The Scripps Research Institute with a Philips CM100 TEM and Hitachi S4800 SEM, under direction by the core staff.

Ventriculomegaly analysis

Lateral ventricle volume was measured in coronal sections collected every 100µm, from the most anterior to posterior sections containing lateral ventricle. The ventricular area of each section was quantified in Adobe Photoshop CS6, then added to create a volume approximation. Choroid plexus was included during quantification, as the free-floating nature of this organ causes it to occupy inconsistent locations in the ventricle.

Brain slice culture for live imaging

Brains were collected from 2-3 P8 animals, kept in iced HBSS with bubbled oxygen, and cut into 250µm sections with a Leica VT1000 S vibratome. Sections containing lateral ventricle were incubated with membrane-permeable dyes in high-glucose DMEM at 37°C for 5 min.

These dyes included 5µg/mL Hoechst nuclear stain (Invitrogen H3570), 5µg/mL Lectin DyLight 488 (Vector DL-1174) for ependymal staining, and 5µg/mL Vybrant CM-DiI (Invitrogen V22888). While CM-DiI is a soluble non-specific membrane dye, this dye attached to ependymal cilia with higher affinity than other cellular membranes, likely due to their surface area and proximity to solution. Following 2 3-minute washes in DMEM, brains were transferred to a glass-bottomed 12-well plate (MatTek P12G-1.5-14-F) containing 1mL high-glucose DMEM and weighed down with harp slice grids (ALA Scientific HSG-5BD MEA). Sections were incubated for 1 hour to induce adhesion before transfer to the Nikon N-SIM with A1R confocal scanner, which was also equipped with a plate heating chamber. This microscope has a resonance scanner that acquires simultaneous images at 405, 488, and 568 wavelengths. By performing a band scan, we were able to capture 10s timelapse images at 113.2 fps, which allows for visualization of ciliary beating. These videos were analyzed with Imaris to track the movement of CM-DiI stained cilia. Points were excluded for colocalization with Hoechst or Lectin 488, restricted by frame-to-frame distance to track the back-and-forth movement of cilia, and filtered by tracking duration to prevent quantification of cilia beating out of frame. Tracking analysis video was exported at 23fps (1/5 speed).

Behavior

The open field test was performed on Monday of testing weeks 1 and 2. Mice were acclimated to the dark testing room for 30 minutes before testing began. Then mice were placed in the open field box for 10 minutes and movement tracked through a laser grid system. Boxes were cleaned with ethanol and dried before and after each group. Data were exported to an Access database and analyzed with PAS reporter.

The accelerating rotarod test was performed on Wednesday, Thursday, and Friday of testing weeks 1 and 2. This device accelerates from 4 to 40rpm in 300 seconds. Mice were placed on the stationary rotarod for 10s before testing began to assure proper balance. If a mouse fell before 10s elapsed on the moving rod, they were retried. During week 1, mice were trained on the accelerating rotarod. Trials were performed in triplicate, with a significant break between each test (at least 15 minutes). During week 2, mice were considered trained and only tested once per day. On the final day (Friday of week 2), following the accelerating rotarod trials, mice were tested for motor competency on a fixed-speed rotarod moving at 5rpm for 120s.

Statistical analysis.

Statistical analyses were performed in GraphPad Prism 7. One-way ANOVA were used to compare each experimental group to uninjected and vehicle.

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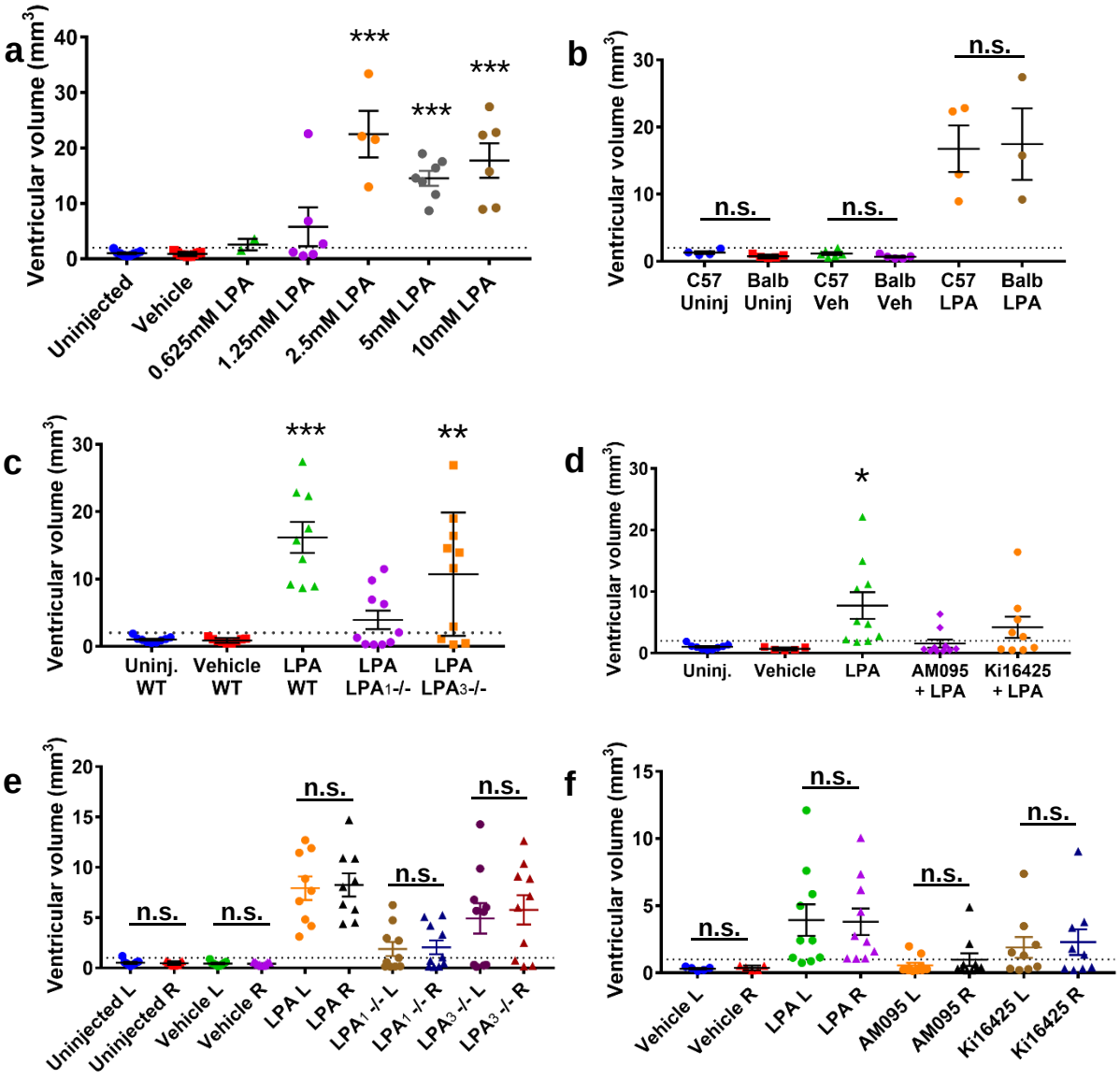

Figure 5.1: LPA_1 mediates LPA-induced ventriculomegaly.

Ventricular volume was quantified by adding the lateral ventricle area of serial sections collected 100µm apart, from the anterior opening of the lateral ventricles to the posterior closing of the ventricular aqueducts. (a) LPA induces ventriculomegaly in a dose-dependent manner. 2.5-10mM LPA causes severe ventriculomegaly with 100% penetrance, while lower concentrations have less dramatic effects with low PHH incidence. (b) Uninjected, vehicle-injected, and LPA-injected mice from C57BL/6J and Balb/c backgrounds have the same ventricular volume distribution. (c) Incidence of LPA-induced ventriculomegaly can be reduced with constitutive LPA_1 or LPA_3 knockout. (d) 500µM LPA in 0.01% BSA induces consistent ventriculomegaly which can be prevented with LPA_1-specifc antagonist AM095 and slightly reduced with $LPA_{1/3}$ antagonist Ki16425. (e-f) Ventricular volume was split by left (contralateral to injection site) and right (ipsilateral) lateral ventricle size. Ventricular expansion was typically uniform, with only a few observed ipsilateral phenomena.
*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$

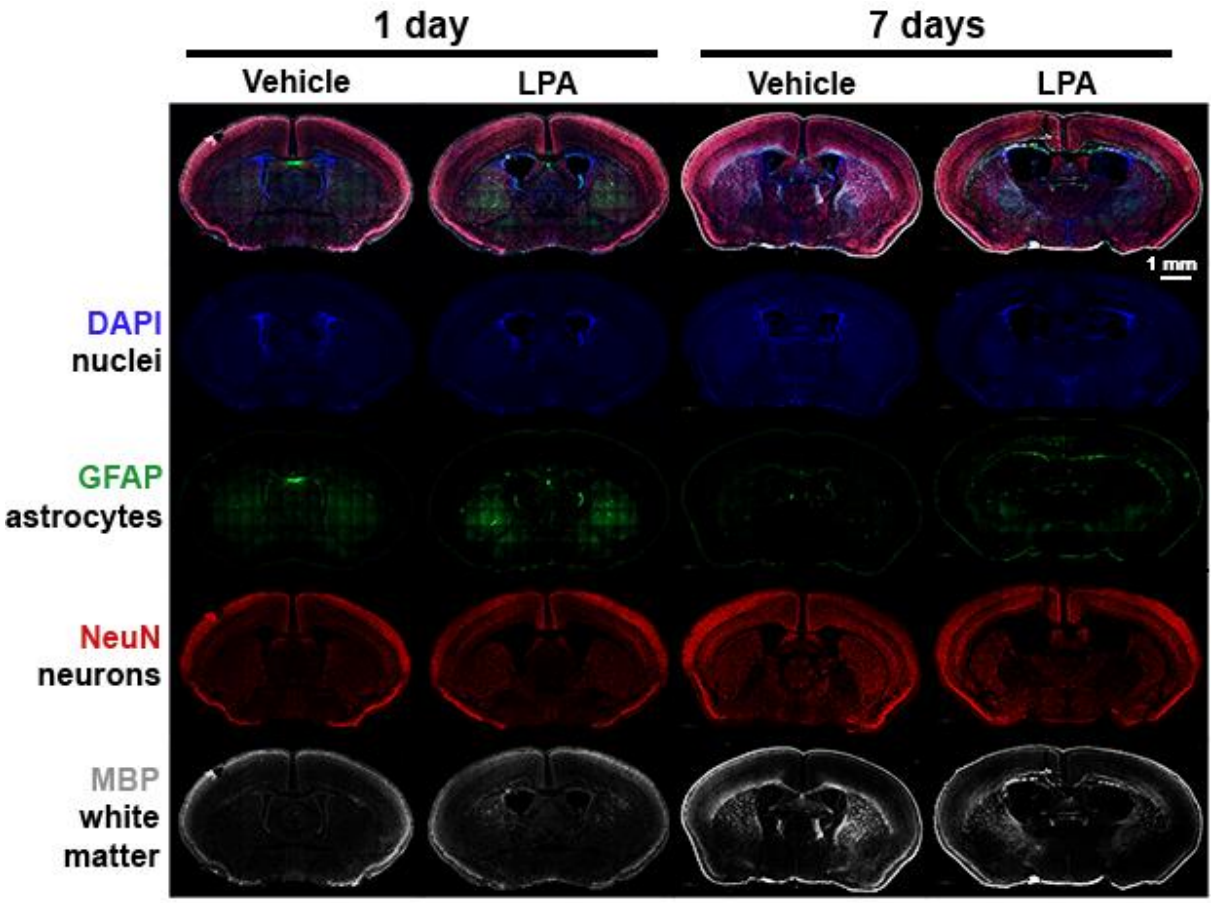

Figure 5.2: Cellular composition of LPA-exposed PHH brains.

Whole-brain tiles combined from 20x images were acquired from tissue sections of mice 24 hours ("P8+1") or 7 days ("P8+7") post-surgery. The top row is a composite of the dyes and antibody stains as follows. Row 2: DAPI, a nuclear dye; Row 3: GFAP, a marker for astrocytes and developing ependyma (P8+1, but not P8+7); Row 4: NeuN, a neuronal nuclei marker; Row 5: MBP, a marker for myelin basic protein found in myelinated white matter tracts.

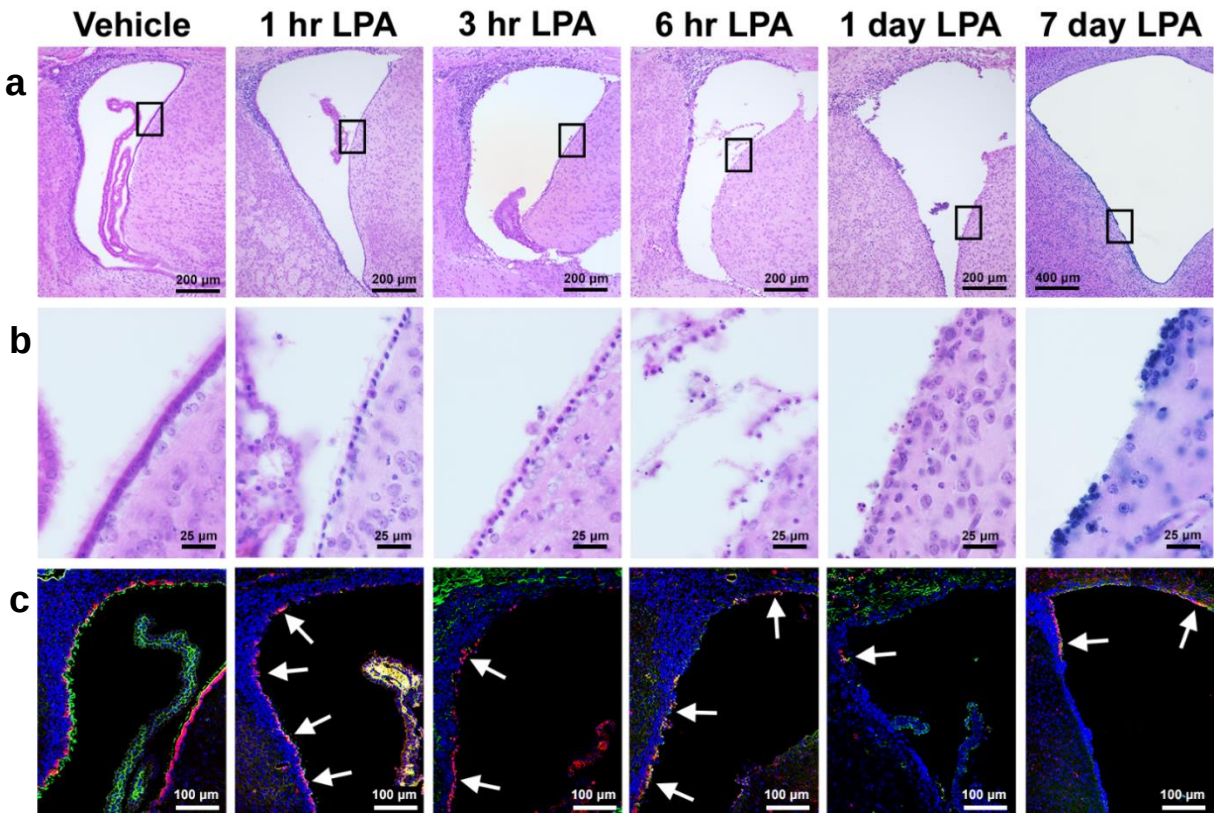

Figure 5.3: LPA induces ependymal cell loss.

(a) Lateral ventricles stained with H&E, with black boxes enlarged in (b). (c) S100 ependymal cell body marker and AcTub ciliary marker stains, with DAPI nuclear costain. Arrows mark intact S100-stained cell bodies. Ependymal cells begin rounding up 3 hours post-LPA exposure, lose contact with the basal membrane at 6 hours, and are significantly depleted 24 hours following injection. These ependymal cells appear to partially regenerate 7 days following LPA exposure during PHH progression.

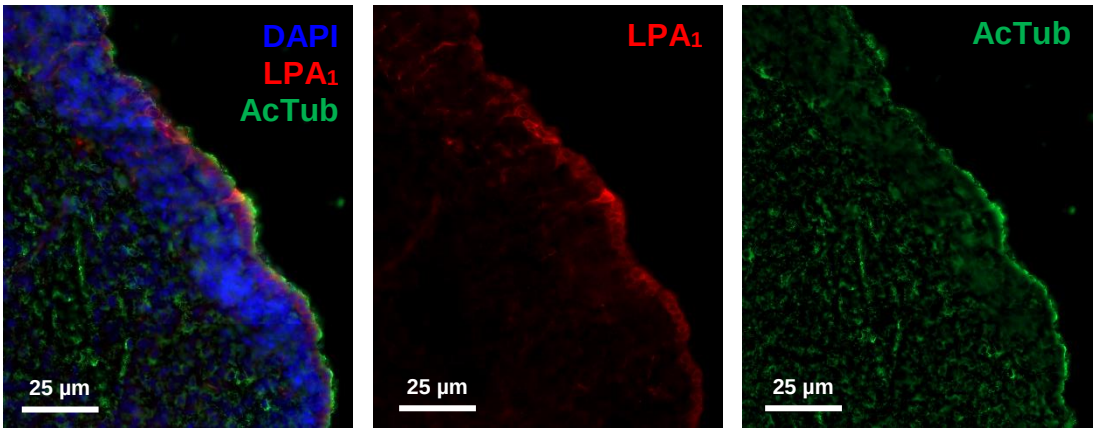

Figure 5.4: LPA_1 is expressed on the ependymal membrane.

Tissue sections fixed with PLP were antigen retrieved and co-stained with LPA_1 and AcTub antibodies. LPA_1 staining lines the ependymal membrane and is brightest at the apical edge but does not colocalize with AcTub.

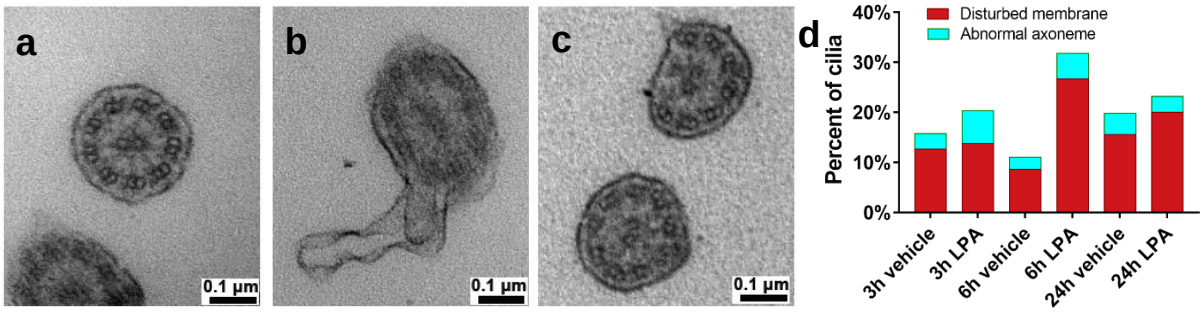

Figure 5.5: LPA induces ciliary disruptions 6 hours following injection.

(a) A representative TEM cross-section of a motile cilium from the lateral ventricle ependyma. (b) An example of a cilium with disturbed membrane morphology. (c) A normal cilium paired with a cilium missing one of the 9 outer microtubule pairs. (d) Quantification of ciliary abnormalities observed in vehicle or LPA-injected brains at several timepoints.

Table 5.1: Quantification of ciliary disruptions.

Numerical data showing the total cilia quantified. Percentages of cilia demonstrating abnormal properties were graphed in Figure 5c.

	Total Cilia Counted	Normal Axoneme	Disrupted Axoneme	Normal Membrane	Disrupted Membrane
Veh 3h	158	153, 96.8%	5, 3.2%	138, 87.3%	20, 12.6%
LPA 3h	123	115, 93.5%	8, 6.5%	106, 86.2%	17, 13.8%
Veh 6h	127	124, 97.6%	3, 2.4%	116, 91.3%	11, 8.7%
LPA 6h	443	420, 94.8%	23, 5.2%	325, 73.4%	118, 26.6%
Veh 24h	513	491, 95.7%	22, 4.3%	433, 84.4%	80, 15.6%
LPA 24h	289	280, 96.9%	9, 3.1%	231, 79.9%	58, 20.1%

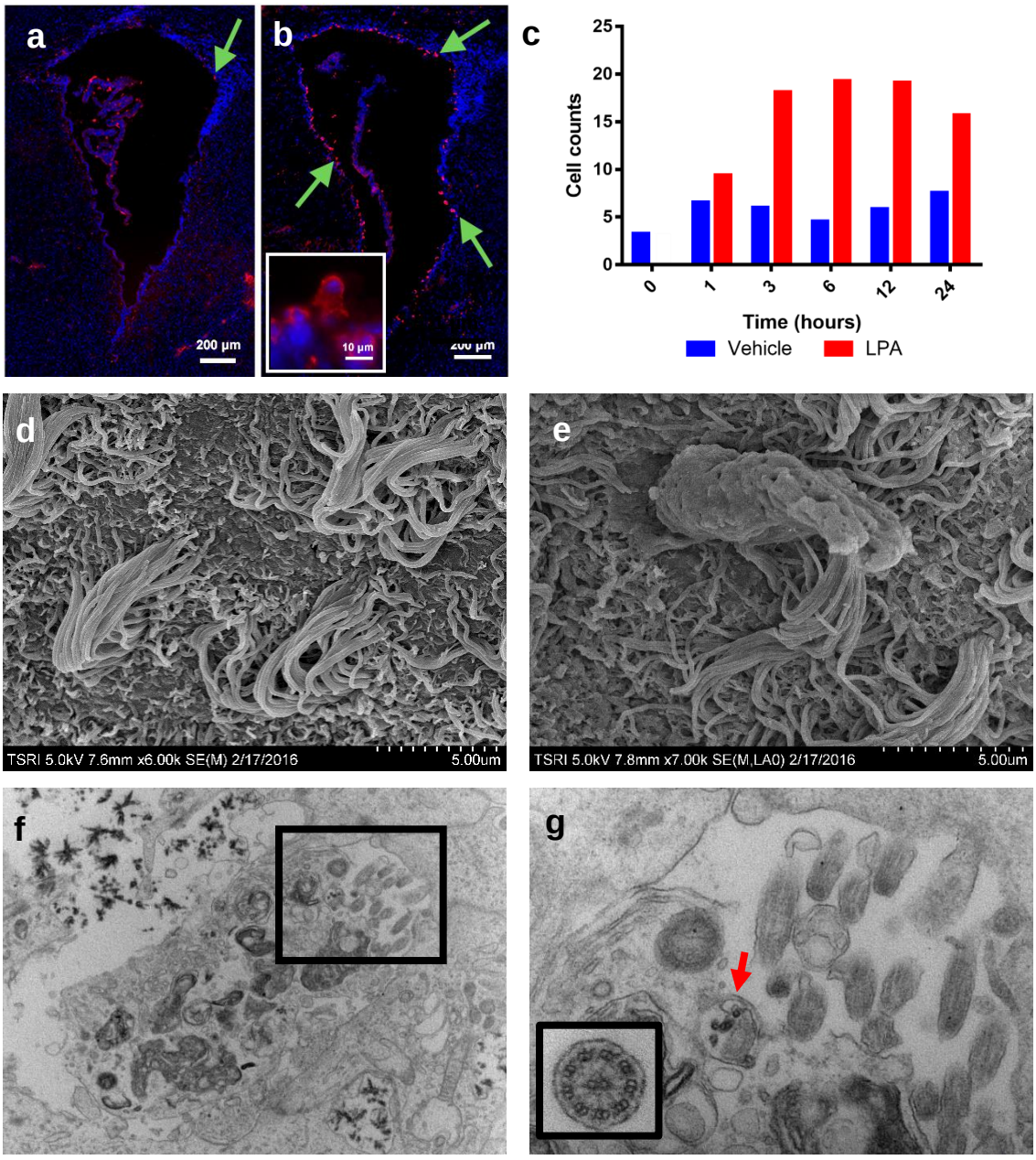

Figure 5.6: Ependymal cilia are phagocytosed.

Iba1 antibody staining was performed with DAPI nuclear costain to identify macrophages and microglia in (a) vehicle-injected and (b) LPA injected brains 6 hours post-surgery. (c) Number of Iba1+ cells adherent to the ventricular surface were counted from tissue sections harvested at 3-24 hours post-injection. (d) SEM image of ependymal cilia tufts. (e) SEM image of a phagocyte engulfing ependymal cilia 3 hours following LPA injection. (f) TEM image of a phagocyte engulfing a ciliary tuft, with box expanded in (g). (g) TEM phagocyte captured actively phagocytosing ependymal cilia, comparing a normal ciliary cross-section (inset) to a phagocytosed cilium (red arrow).

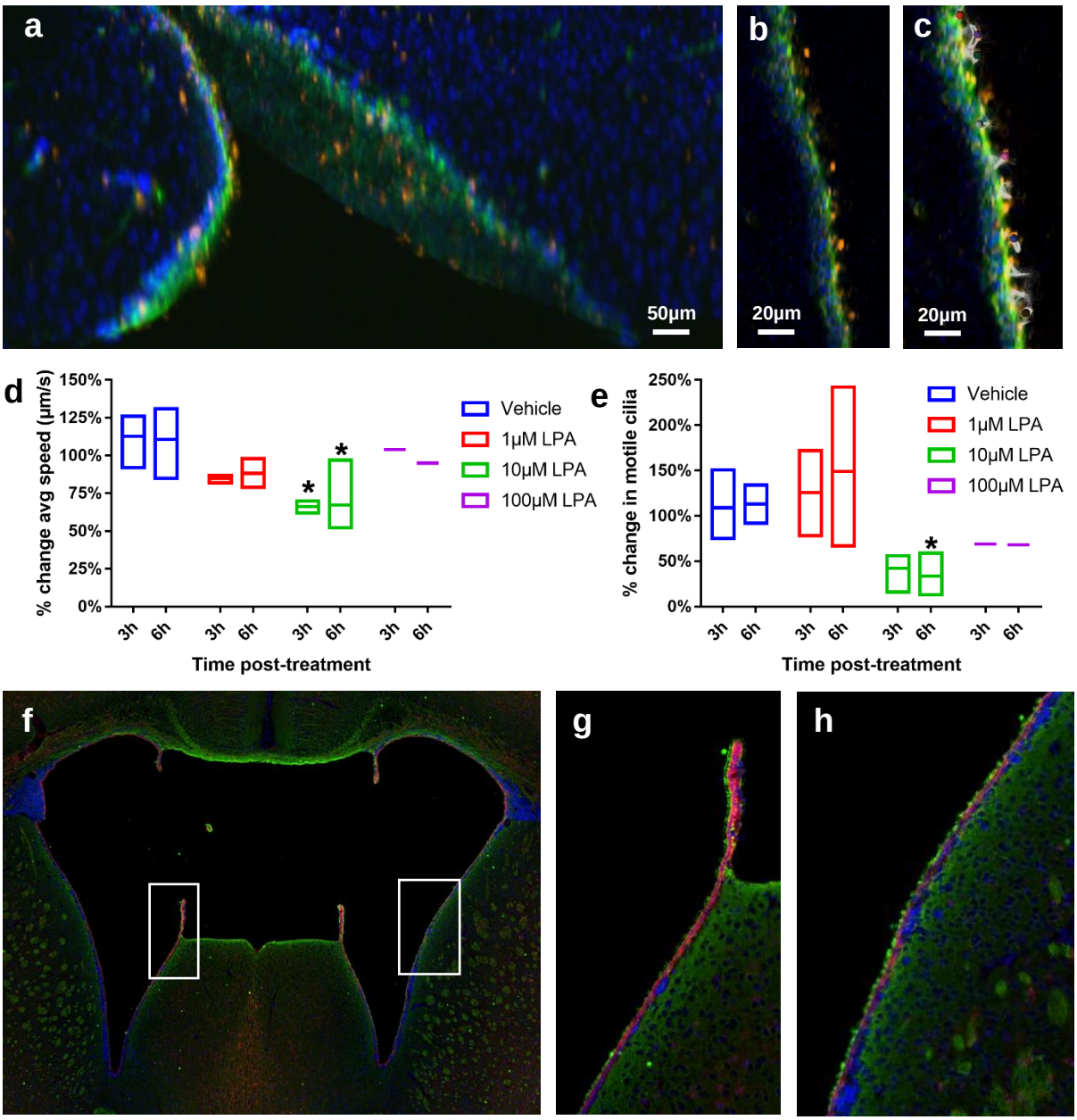

Figure 5.7: Ciliary dysfunction in hydrocephalus.

(a) 20x z-stack of a 250µm brain section in a live culture system, stained with Hoechst (blue), Lectin (green) and CM-DiI (red). (b) Example frame from a 10s timelapse video of ciliary beating. Acquired with Paloma Sanchez-Pavon. (c) Tracking analysis of the video in (b), with colored orbs superimposed on cilia and gray lines simulating the travel path of each tracked cilium. (d) Quantification of the change in average ciliary motility 3 and 6 hours post-treatment compared to untreated baseline. (e) Quantification of the number of tracked cilia per well. (f) S100 (red), AcTub (green), and DAPI (blue)-stained lateral ventricles, with severe ventriculomegaly and combined ventricles 7 days post-vanadate injection. (g,h) Despite severe VM due to ciliary arrest, intact S100-stained ependymal monolayers and AcTub-stained cilia are observed.*$p<0.05$

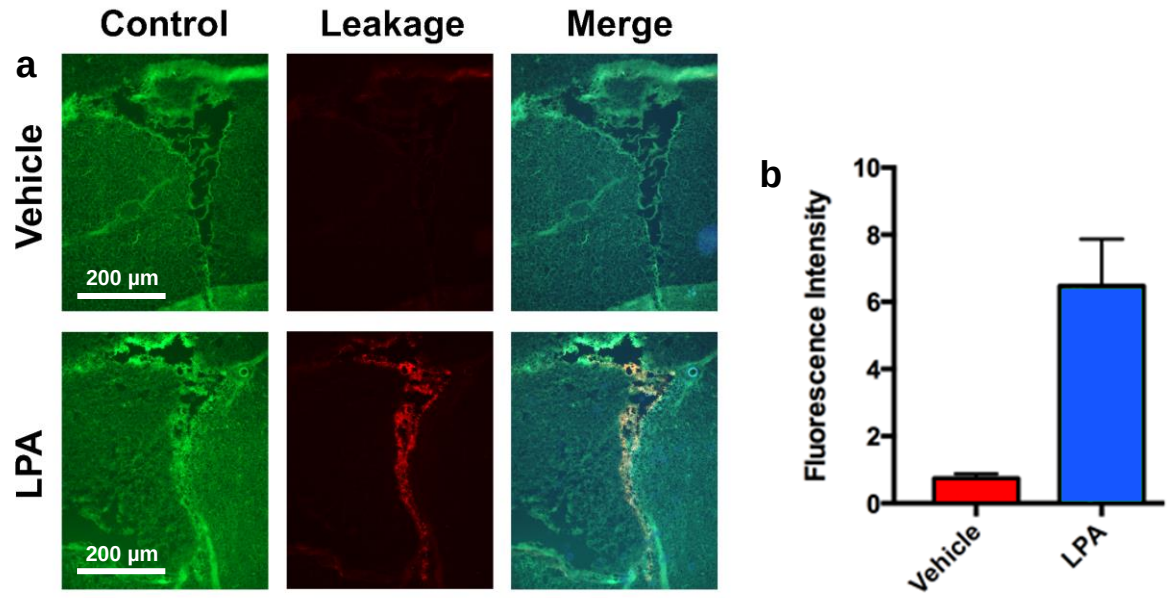

Figure 5.8: LPA induces transient BBB leakage.

(a) 300 Da fluorescein (green) and 3000 Da dextran (red) cardiovascular leakage into the lateral ventricle 30 minutes after vehicle or LPA injection. (b) Fluorescence intensity quantification of red dye leakage in the lateral ventricle.

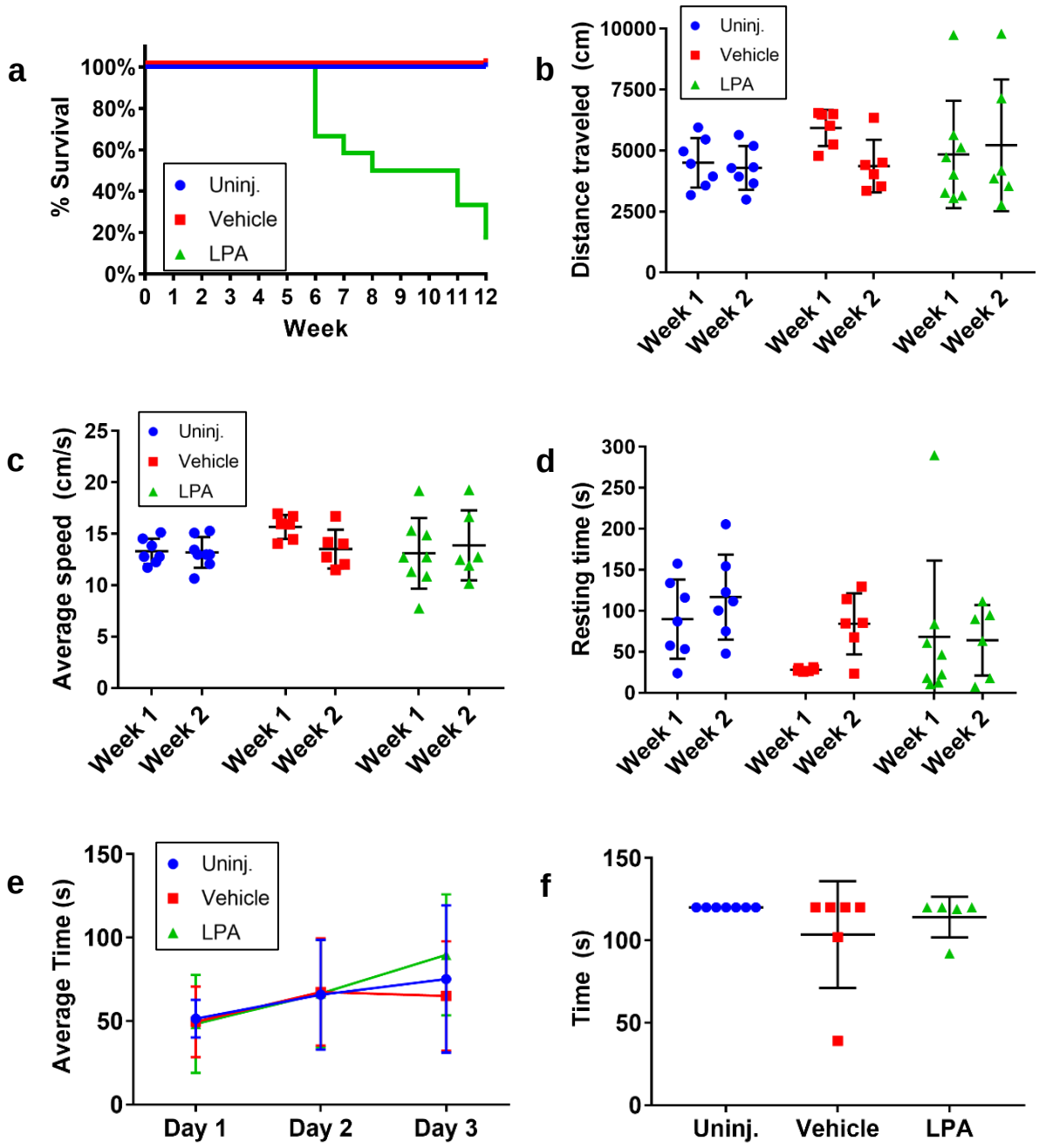

Figure 5.9: Effects of PHH on murine survival and motility.

(a) Kaplan-Meier survival curve of uninjected, vehicle-injected, or LPA-injected WT mice up to 12 weeks of age. (b) Distance traveled in 10 minute open field tests at 7 and 8 weeks of age. (c) Average movement speed in the open field test. (d) Time spent resting at the same coordinates during the open field test. (e) Average time spent on the accelerating rotarod for 3 consecutive days at 7 weeks of age (6 weeks post-surgery). (f) Time spent on the rotarod spinning at 5rpm during a final motor capability test at 8 weeks of age, with maximum duration of 120 seconds.

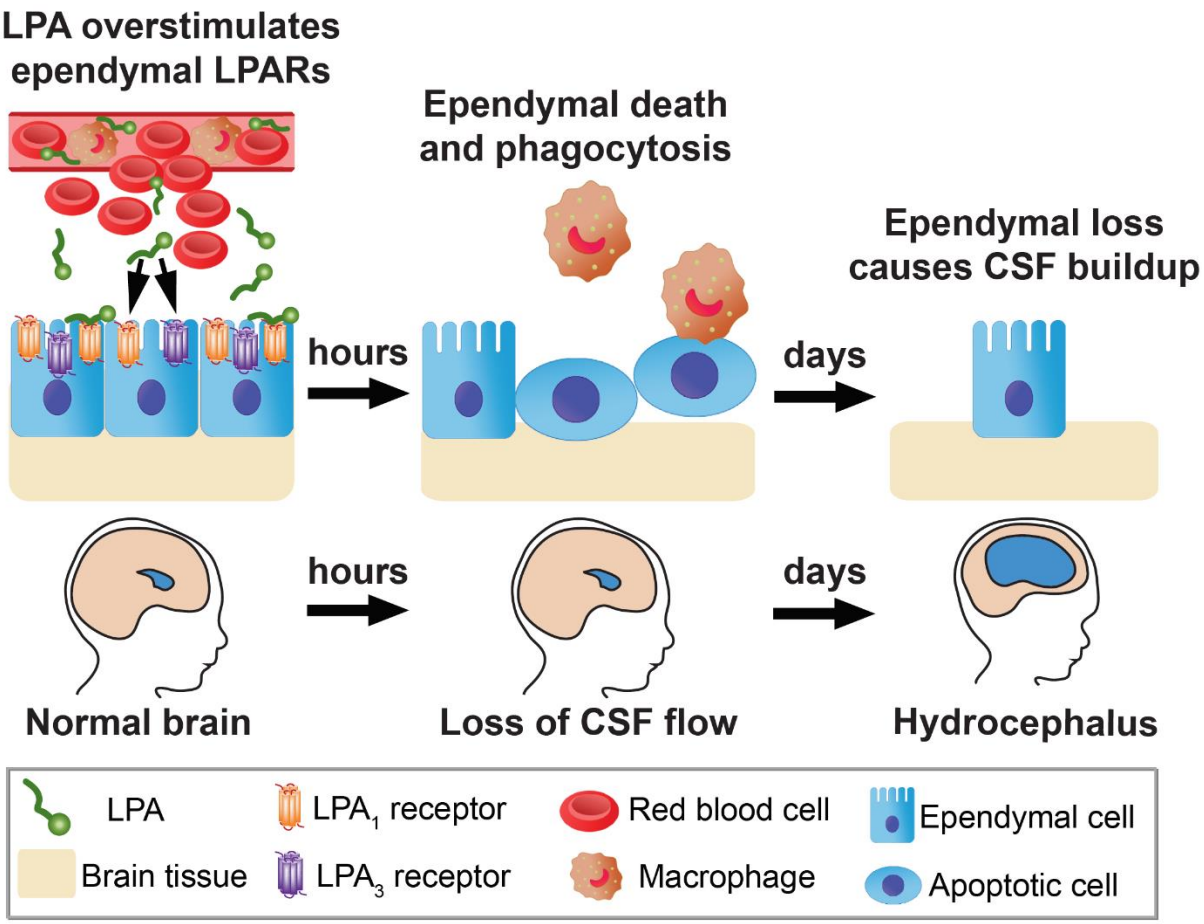

Figure 5.10: Mechanism of LPA-induced PHH.

Lysophosphatidic acid is introduced into the cerebrospinal fluid during intracranial hemorrhage, signaling on the apical wall of ependymal cells. Over the next 24 hours, ependymal cells deteriorate, ciliary function is compromised, and ependymal cell bodies are lost to apoptosis and phagocytosis. This ependymal cell loss reduces CSF flow, resulting in an accumulation of fluid that leads to intracranial pressure increase and chronic hydrocephalus.

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Chapter 6:

CONCLUSION

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This chapter discusses data presented in Chapter 4: *Ependymal loss through LPA_1 overstimulation initiates hydrocephalus in a novel neonatal mouse model* and Chapter 5: *Characterization of LPA-induced hydrocephalus.*

Discussion

The novel model described in this dissertation replicates the primary clinical post-hemorrhagic hydrocephalus phenotypes in neonatal mice, demonstrating elevated CSF pressure and dramatic ventriculomegaly in a highly reproducible manner. These data are among the first reports modeling hydrocephalus at a timepoint that correlates to premature infants, a high-risk group with significant unmet medical need. Previously, our lab demonstrated that LPA induces fetal PHH when injected in the ventricles of E13.5 fetal mice. Although LPAR stimulation recapitulates the same phenotype in fetal and postnatal pups, the fetal mechanism appears to be mediated through altered neural progenitor fate which occludes CSF pathways, while the postnatal model directly affects the ependymal cells responsible for generating CSF flow. Additionally, LPAR expression patterns vary between fetal, neonatal, and adult mice. For instance, high levels of LPA_1 expression begin in neural progenitors, transition to early ependymal cells, and remain in neurons and glial cells, while LPA_3 is sparse in fetal and adult brains but demonstrates high specificity in the early postnatal ependyma. These spatiotemporal differences highlight the importance of targeting distinct cellular populations and receptors for hydrocephalus cases of different etiology.

Utilizing postnatal mice also allows for intracranial pressure measurements of mice afflicted with PHH, which have not been reported in previous mouse models of hydrocephalus. While mouse ventricles are small, adapting a slim fiberoptic pressure cable allows access to the ventricular space to record ICP fluctuations. Additionally, as similar fiberoptic devices are used in medical practice to record human ICP, transitioning this method into common laboratory use could lead to countless clinically-relevant innovations.

Studying early postnatal models of hydrocephalus advances a significant area of unmet need. Outside of traumatic brain injury, severe hemorrhage is most common in prematurely born

infants, resulting in a large population of young PHH patients. These newborns are often kept under observation in hospital settings, allowing for fast therapeutic action.

The elucidation of LPA_1 as a key mediator of ependymal degeneration opens the gateway for possible pharmacological intervention. This is supported by the significant PHH protection demonstrated byLPA_1 knockout animals and with pretreatment of LPA_1 antagonist AM095 (Chapter 4, Figure 4; Chapter 5, Figure 1). As LPAR inhibitors are currently in clinical trials for indications such as fibrosis and cancer (Chapter 2), these drugs could be readily adapted to other pathologies such as hydrocephalus. Ependymal degeneration becomes histologically apparent 3 hours following LPA exposure, resulting in specific ependymal death that culminates 24 hours post-injection. This suggests that administration of an LPA_1 antagonist within a few hours following intracranial hemorrhage could prevent PHH development in a significant number of cases while mitigating off-target toxicities.

Outside of preventative medicine, this study also highlights potential therapeutic targets. The importance of ciliated ependymal cells in PHH initiation suggests that regeneration of the ependymal barrier may restore CSF flow to mitigate hydrocephalus sequelae. Additionally, the presence of inflammatory monocytes following LPA injection is intriguing. While these immune cells may be trying to clear out damaged tissue, sustained inflammation often propagates brain damage. Therefore, immune interventions may prove useful in treating developing or chronic cases of hydrocephalus. In the end, these studies serve to further our understanding of the initiation and progression of hydrocephalus, specifically for understudied neonatal populations at high risk for PHH. Further study is necessary to facilitate development of novel therapies which reduce patient reliance on repeated invasive neurosurgeries.

Future Directions

LPA concentration and preparation

Recent studies have shown that radiolabeled LPA reconstituted from vacuum-dried stock in saline solution demonstrates significant losses during each transfer step (Kihara, unpublished). This means that, starting from a 5mM stock, approximately 95% of lipid is lost when reconstituted and pipetted from the low-retention stock tube (~250μM) and a further 50% of lipid is lost during injection through the glass syringe (~125μM delivered into the CSF). As LPA is a polar compound, it is liable to stick to surfaces or form micelles at high concentrations. Bovine serum albumin (BSA) is a natural bloodborne carrier for LPA which significantly increases its bioavailability in aqueous solution, reducing non-specific binding and disrupting the formation of lipid clusters and micelles. Therefore, these losses can be mitigated by storing LPA in aqueous solution with 0.1-0.001% BSA, and by coating tubes and syringes with BSA solution before injection. As inclusion of BSA significantly increases the bioavailability of LPA, a lower stock concentration can be used for PHH induction. 500 μM LPA in 0.01% BSA was utilized for the LPAR inhibitor experiments, and BSA-containing vehicle will be utilized for future experiments. Current work involves characterizing the dose response of LPA-mediated PHH with different preparation methods, storage conditions, and BSA concentrations.

Blood-induced PHH

These studies have shown that LPA is sufficient to induce PHH with the assumption that LPA introduced into the CSF following hemorrhage is a key initiating factor of communicating PHH. However, in order to prove these claims, LPAR null mice or antagonists should be able to

prevent true blood-induced hemorrhagic hydrocephalus. We attempted to induce PHH with a single bolus of blood, plasma, or serum derived from cardiac puncture of an adult mouse into the lateral ventricles of P4 and P8 mice. However, this volume was not sufficient to induce PHH. In order to examine the effects of LPAR inhibition on blood-induced hydrocephalus, we may need to develop a model of PHH induced by a true hemorrhagic event. Instead of a single 5µL bolus of plasma, we may need to inject several small doses of blood over time, as severe hemorrhagic events last for several hours and introduce hundreds of microliters of blood into the CSF. Another option is to induce hemorrhage by stereotactic injection of a compound that disrupts the blood-brain barrier, such as bacterial collagenase, and assessing if PHH incidence is reduced in LPA_1 knockout animals or following treatment with LPAR antagonists.

LPAR knockout mice

Significant efforts were dedicated to acquiring ventriculomegaly data from all in-house constititive LPA_1-LPA_5 knockout animals. Our lab recently acquired LPA_6 mice, the last canonical LPAR, but due to difficulties in rederiving this strain we have been unable to inject LPA_6 knockouts. As LPA_6 is higly present in CNS vasculature, including the choroid plexus, and demonstrates weak expression within the ependymal monolayer, this receptor is of interest to future studies. These mice are currently breeding and we hope to achieve these results soon.

Since knockout experiments elucidated two involved receptors, LPA_1 and LPA_3, we hypothesized that these receptors were both acting within the postnatal ependyma, possibly in a redundant fashion. To answer this question, we attempted to breed these strains together. However, knockout parents of either strain have difficulty breeding, requiring double heteroztgous grosses, and the resulting knockout pups had limited viability. We injected

approximately 300 pups from LPA_1/LPA_3 crosses, but only 3 $LPA_{1/3}$ double null pups survived. In order to test possible $LPA_{1/3}$ interactions, conditional knockouts may be required.

We attempted to test a few different conditional LPA_1 knockout strains, but so far none of them have proevn efficacious in preventing hydrocephalus. LPA_1 nestin-cre (a developmentally active neural progenitor driver) developed full hydrocephalus. We also tried tamoxifen-inducible LPA_1 FoxJ1-cre (a developing ependymal cell marker), but these mice develop spontaneous hydrocephalus at an increased rate, so LPA-injected phenotypes could not be accurately assessed. Further investigation into FoxJ1-cre or other ependymal-specific conditional drivers may facilitate future multiple-knockout studies.

Live culture

The development of a live brain slice culture system opens up many possibilities. Future experiments involve examination of ependymal morphology over time following exposure to different concentrations of LPA, in addition to further characterization of LPA-induced ciliary motility dysfunction. While previous live acquisition was limited to 6 hours due to techinical issues, future experiments could assess ependymal health and function over 24 hours or even longer.

Longitudinal studies

One limitation of the methods utilized in this study is that brains had to be collected at several timepoints without direct correlation between brain health and physiology. MRI imaging experiments would allow us to perform longitudinal studies following the health of mice as PHH

develops. Imaging brains once a week would give much more detailed information on the morphological effects of PHH.

Further behavioral characterization would also be informative. While hydrocephalic animals are certainly capable of motility, as evidenced by results in open field and rotarod testing, they are undoubtedly affected by PHH. Employing a different battery of tests, such as the grip test, gait analysis, or novel object recognition, may elucidate differences between hydrocephalic and control mice.

One caveat to behavioral testing is that the tested animals may be slightly too young for behavioral trials. Most animals treated with 5mM LPA died by 12 weeks of age, with lethality beginning at 6 weeks of age, limiting the timeframe for testing. Utilization of a lower dose of LPA that allows for longer survival with less severe hydrocephalus may make it possible to test mice at different developmental stages.

Employing MRI and/or further behavioral testing would be particularly interesting for thearapeutic trials. These experiments would allow us to determine whether pharmacological agents improve animal health in correlation with the severity of ventriculomegaly. While an efficacious preventative medicine would be a breakthrough in PHH treatment, patients suffering chronic hydrocephalus are also interested in therapies to improve quality of life.

LPAR signaling pathway characterization

While we have provided evidence toward LPAR overstimulation causing cleaved caspase3-mediated apoptosis, more detailed analysis of the signaling pathways initiated in LPA-exposed neonatal ependymal cells would be intriguing. Collection of ependymal cells with a UniPick system, laser capture, or flow sorting would allow western blot or RNA analysis of G

protein-mediated pathways. In addition to Cas3/Akt-mediated cell death, another pathway of interest includes Rho/Rock-mediated migration and cytoskeletal changes, as these signaling events may explain the reduced ependymal adhesion and loss of ciliary acetylated α-tubulin observed 3-6 hours following LPA exposure.

Immune studies

A large increase in inflammatory macrophage/microglia counts were observed 6-24 hours post-LPA exposure. While administration of the anti-inflammatory antibiotic minocycline and phagocyte-killing clodronate liposomes had no effect on hydrocephalus initiation (unpublished), the sustained presence of inflammatory monocytes could propagate brain damage. Future work involves characterizing immune involvement during PHH initiation and progression.

LPAR inhibitor treatment paradigms

The data presented here highlights LPA_1 signaling as a key mediator for ependymal death and subsequent hydrocephalus. Pretreatment with an LPA_1 antagonist proved efficacious in preventing or reducing subsequent ventriculomegaly, but further characterization would be informative. Dose responses of different LPAR inhibitors can help establish the lowest efficacious dose. These drugs were injected intracranially, but should be capable of crossing the BBB, so different methods of administration such as oral or intraperitoneal could be attempted. Additionally, timecourse experiments are necessary to determine the range LPAR inhibitior treatment could prove efficacious. The experiment presented here was preventative 15 minutes pre-hemorrhage, whereas therapeutics administered during hemorrhage or within a few hours post-hemorrhage would be more medically relevant.