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The role of Lysophosphatidic Acid in Traumatic Brain Injury outcomes

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The role of Lysophosphatidic Acid in Traumatic Brain Injury outcomes

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

By

Whitney S. McDonald

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ABSTRACT OF THE DISSERTATION

The role of Lysophosphatidic Acid in Traumatic Brain Injury outcomes

By

Whitney S. McDonald

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2015

Professor Neil G. Harris, Chair

Over a century of research efforts have been devoted to developing a therapy to recover loss of function after Traumatic Brain Injury (TBI). Despite these efforts there is still no FDA approved treatment that promotes functional recovery after injury. Every year, millions of new TBI cases occur and many TBI patients have persistent loss of motor and cognitive function leaving them incapable of living independent of caregivers. There is an urgent need for a novel therapeutic that addresses the functional loss after TBI and anti-LPA therapeutic may prove effective in this regard. Progression of secondary injuries like excitotoxicity and inflammation is predictive of functional outcomes for TBI patients. Lysophosphatidic Acid (LPA) signaling is a potent mediator of the above secondary injures, despite these facts, no study has identified the role of

LPA in outcomes after TBI in the adult. Fundamental issues regarding LPAs metabolic changes in the brain after injury, the effects of LPA signaling on neuroregeneration after TBI and ultimately whether intervening with Anti-LPA after TBI improves outcomes and functional recovery, require resolution in order to utilize LPA as a therapeutic target for TBI. The work herein is aimed toward gaining a more comprehensive understanding of the effects of LPA metabolism and signaling in TBI and to identify the effects of blocking LPA on injury outcomes.

The first study identified the spatial and temporal profile of LPA metabolism in the injured brain and associated those changes with markers of axonal injury (Beta-APP) and cell death (Fluro-JadeB) using MALDI mass spectrometry techniques. Within 3 hour after TBI there was an enhancement of LPAs bioactive unsaturated species, as well as an increase in LPAs intra- and extra-cellular precursors at the injury epicenter in association with blood. LPA metabolism was also increased in distal regions of the brain, throughout the white matter tracts and in the cerebellum. Intracellular precursor, PA, was increased in the peri-contusional cortical grey matter and ipsilateral thalamus within 1 hour after injury and intracellular LPA increased in at 3 hours after injury in association with neuronal death markers. Pronounced expression of LPA 20:1 species was observed in the sub-cortical white matter which correlated significantly with the spatial distribution of axonal injury marker beta-APP. The data provided evidence of an increase in bioactive phospholipid metabolism throughout the brain within 3 hour of injury and associated those changes with necrosis and axonal injury. The study also identified a critical window of intervention, to potentially attenuate the increase in LPA signaling. The data suggested that LPA metabolism is involved in the early pathogenic cascades of TBI.

The second study identified the effects of a one-time dose of Anti-LPA at 2 hours after injury, on secondary injury outcomes to underlie functional decline: cell death, axonal injury and inflammation. Anti-LPA intervention resulted in a reduction of white matter damage and inflammation, but had no significant neuroprotective effects and anti-inflammatory effects in the grey matter. Furthermore, anti-LPA treatment significantly improved sensorimotor function with some behavioral scores being insignificantly different from that of uninjured sham. The result suggested that a one-time dose of Anti-LPA reduces axonal injury and white matter inflammation but is insufficient on neuroprotection of the cortical grey matter. Since grey matter pathology is sensitive even to mild changes in the brain, additional treatments of Anti-LPA may be needed to provide adequate neuroprotective effects. Nevertheless, a one-time treatment of anti-LPA improved sensorimotor outcomes, likely through white matter preservation. Data obtained in parallel studies suggest a potential neurogenic mechanism may also be involved in improving outcomes.

The final study identified the effects of Anti-LPA treatment on the endogenous neurogenic response of the sub-ventricular zone (SVZ) and cortex to injury with a thymidine analog-labeling paradigm to identify acutely dividing cells between 1 and 7 days post injury (DPI) and the long-term fate of early dividing cells at 14 and 28 DPI. Anti-LPA treatment enhanced SVZ neurogenesis and reduced DCX+ neuroblasts populations in the cortex within 7 days of injury. However, at 28 days of injury there was a significant enhancement of dividing neuroblasts in the cortex of anti-LPA treated mice as compared to the vehicle group, with migratory patterning similar to neuroblast response in the uninjured sham group. Furthermore, there was enhanced neurogenesis with Anti-LPA treatment as characterized by NeuN+ dividing cells in the cortex

although not statistically significant at 28 DPI. Lastly, at 28 DPI there was marked reduction in SVZ neuroblast populations as well as a reduction in activated microglia as characterized by morphology of Iba1+ cells. The results provide evidence of the potent effects of LPA signaling on acute and long-term neurogenic response of the cortex and SVZ. The results demonstrate that blocking post-injury LPA signaling enhances cortical survival of immature neurons likely by reducing long-term cortical excitotoxicity and consequently suggest that Anti-LPA intervention may enhance neurogenesis. Lastly, Anti-LPA treatment after injury acts as a potent anti-inflammatory in the SVZ, which correlated with a reduction in SVZ response at 28 DPI. Additional work on the dosage effects of Anti-LPA on outcomes and the longer-term effects of Anti-LPA treatment on neurogenesis is needed. However, the results herein provide significant evidence of Anti-LPA intervention as a potentially effective treatment that improves functional outcomes after TBI.

The dissertation of Whitney S. McDonald is approved.

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PUBLICATIONS & PRESENTATIONS

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1. Chapter 1

1.1 Overview of Traumatic Brain Injury

1.1.1 Epidemiology:

Traumatic Brain Injury (TBI) is a significant health issue in the United States. Each year, approximately 2.5 million cases of TBI occur; in the United States alone TBI is responsible for 30% of all injury related deaths (Faul M, Xu L, Wald MM, 2010). TBI negatively effects the patients and their families emotionally and financially; costs for treating TBI patients totals $77 billion each year (Seifert, 2007). Nearly 90% of patients that incur their portion of this financial burden are considered moderate to severe TBI cases (Seifert, 2007). In addition to the financial strain, patients with moderate to severe TBI experience sustained physical, cognitive and emotional disabilities that persist throughout their lives (Brown, Elovic, Kothari, Flanagan, & Kwasnica, 2008; Faul M, Xu L, Wald MM, 2010; Guerrero, Thurman, & Sniezek, 2000; Jean A Langlois, 2003; Kirkpatrick, 2002; Langlois, Rutland-Brown, & Wald; Seifert, 2007), (Thurman, Alverson, Dunn, Guerrero, & Sniezek, 1999), (Langlois et al.). The majority of research efforts have focused on developing physical rehabilitation, psychological therapies and prophylactic devices to combat the effects of this injury but despite theses interventions many moderate to severe TBI patients remain physically and mentally disabled for the remainder of their lives (Narayan et al., 2002). Positive clinical outcomes depend in large part on treatments that lead to full functional recovery enabling patients to resume a level of normality (Narayan et al., 2002). Despite decades of research there is paucity of treatments and therapies that achieve this goal.

1.1.2 Pathophysiology:

The clinical manifestations of TBI range from concussion to coma and death as a result of mechanical impact or sudden and rapid movement of the head. This insult results in temporary or permanent impairments in cognition, motor and psychological functions. The injuries that cause these functional impairments can be subdivided into two categories; primary and secondary injuries.

1.1.2.1 Primary Injury:

Primary traumatic brain injury is immediate, occurs at the moment of head trauma; when an object makes contact with the head or due to inertial forces effecting the brain within the skull (Graham, 1996). Contact injuries can cause brain tissue distorting, stretching, cutting or compressing whereas brain injuries due to inertial forces may damage the brain at more dispersed regions causing diffuse axonal injury and brain swelling. Clinically, the most common manifestations of primary injuries are concussions; diffuse axonal injuries, intracranial hemorrhages, skill fractures and penetrating head injuries (Marshall, 2000). Although primary injuries are preventable they cannot be therapeutically targeted. Thus, secondary injuries are the main focus for many therapeutic interventions for TBI.

1.1.2.2 Secondary Injury:

Unlike primary injuries, secondary injures are progressive, manifesting within hours or days of the primary injury. Where the initial insult to the brain causes tissue damage the secondary injury is a more widespread degeneration of neurons. The cascades of secondary injuries include increase in intracranial pressure (Baethmann et al., 1998) impaired blood flow and metabolism,

accumulation of lactic acid and membrane permeability, depleted energy stores and widespread depolarization and excitotoxicity (Greve & Zink, 2009; Prins, Greco, Alexander, & Giza, 2013; Werner & Engelhard, 2007). TBI causes cascades of cellular changes that have lasting effects on functional outcomes. Some of the key secondary injuries that contribute to outcomes are the breakdown of the blood-brain barrier (BBB), retraction of axons, excitotoxicity mediated by altered calcium homeostasis and persistent inflammation including glial scar formation. All of which promote cell death, tissue damage and ultimately functional impairments. The progression of secondary injuries for TBI patients is essentially the deciding factors underlying recovery (Brown et al., 2008; Granacher, 2007; Grindel, 2003). Pharmacological interventions to modulate the signaling cascades underlying secondary injuries progression may enhance functional recovery.

1.1.3 Animal Models for TBI:

Animal models for traumatic brain injury have been developed to emulate the clinical manifestation of TBI in humans for both primary and secondary injuries in an effort to test potential therapies. The human condition is multifactorial and complex so specific models have been developed to mimic moderate to severe injuries, focal and distal injuries as well as brain trauma occurring during developmental and adult stages of life. Namely, the Controlled Cortical Impact (CCI) (Edward Dixon et al., 1991; Lighthall, 1988) model and the Fluid Percussion Injury (FPI) (Dixon et al., 1987; Dixon, Lighthall, & Anderson, 1988) are two of the most well-characterized models of TBI. In both models injury severity can be modulated in adult and developing rodents and reproducible functional impairments have been well characterized. The CCI model of injury will be used in this study.

1.2. Endogenous regenerative mechanisms

In recent decades the brains endogenous repair mechanisms has been highlighted as a promising therapeutic tools to enhance neurogenesis and functional recovery after injury via neural protective or neural replacement mechanism (S. Chen, Pickard, & Harris, 2003; Xiong, Mahmood, & Chopp, 2010). In theory, stem cells from the brains neurogenic niches could repair damaged cells and or replace dead cells after injury. However, the utility of this therapeutic mechanism lie in the process of progenitor cell migration to and survivability in the damaged cortex; the neuronal fate direction and neuronal differentiation of those neuroblast in the cortex; lastly the ability of those new cells to provide support to surrounding cells and or compensate for neuronal loss. Several steps in these processes have proven inefficient to promote functional recovery on its own and thus there has been recent, growing interest in therapeutics that enhance neurogenesis in an effort to enhance neuroregeneration after TBI

1.2.1. Neural progenitor cells in the Adult Brain

During CNS development, the lining of the ventricular zone houses layers of mitotically active neural progenitor cells (NPCs) and through a cascade of molecular events forms the fully developed brain (Bayer, Altman, Russo, Dai, & Simmons, 1991; Jacobson, 1991; Shen et al., 2008). Neurogenesis is the process in which progenitor cells proliferate and differentiate into new functional neurons and this process was thought to be restricted to the developing brain until the early 1960's when researchers discovered that neurons in the olfactory bulb were replenished and new cells in the hippocampus were generated in adult rodents (Altman & Das, 1965; Altman, 1962). Further research revealed after injury progenitor cells proliferate in the subventricular zone (SVZ) and migrate toward the injury site and neurogenesis of the sub-

granular zone (SGZ) contributes to learning and memory (Alvarez-Buylla & Lim, 2004; Lagace et al., 2010; T. D. Palmer, Willhoite, & Gage, 2000; Sundholm-Peters, Yang, Goings, Walker, & Szele, 2005; Yamashita et al., 2006). Recent evidence also suggests that the cortex may have a resident progenitor cell population; although evidence of a cortical progenitor niche is still highly controversial (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002a; J. Chen, Magavi, & Macklis, 2004; S S Magavi, Leavitt, & Macklis, 2000; Parent, Vexler, Gong, Derugin, & Ferriero, 2002b; R L Zhang, Zhang, Zhang, & Chopp, 2001). The ability for the adult brain to generate new cells is an accepted phenomenon of the SVZ and SGZ niches and many research efforts have aimed to understand the response of these regions to TBI as a way to utilize these cell populations to enhance neuroregeneration (J. Chen et al., 2004; Donega, van Velthoven, Nijboer, Kavelaars, & Heijnen, 2013; Eriksson et al., 1998; Gu, Brännström, & Wester, 2000; Kunlin Jin et al., 2003; Kernie, Erwin, & Parada, 2001; Kernie & Parent, 2010a; D. Sun, 2014).

1.2.1.1 Identifying the Neural Progenitor Cell (NPC) in vivo:

There is no specific phenotypic marker for NPCs and even the most primitive, quiescent NPC populations of the adult brain are just recently being discovered (Coskun et al., 2008; Lugert et al., 2010; D. K. Ma, Bonaguidi, Ming, & Song, 2009; Nakafuku, Nagao, Grande, & Cancelliere, 2008; Olausson et al., 2014). Due to the elusive molecular properties of NPCs, combinations of mechanisms are used to identify progenitor cells in vivo.

Progenitor cells that have the potential to contribute to neural replacement efforts are mitotically active. One of the highly developed techniques to identify dividing cells is the use of halogenated-thymidine analogs such as bromodeoxyuridine (BrdU) and chlorodeoxyuridine

(CldU) (Hancock, Priester, Kidder, & Keith, 2009; Sanjay S Magavi & Macklis, 2008; Philippe Taupin, 2007). Thymidine analogues incorporate into the DNA during S phase of the cell cycle. This approach allows for seamless identification of actively dividing cells in vivo using immunohistochemistry (Taupin, 2007). In addition, cells can be multi-labeled with a combination of cell specific markers to determine the phenotype of the dividing cells. Multiple thymidine analogs can be used in combination to characterize the rate of division and fate of daughter cells (Aten, Bakker, Stap, Boschman, & Veenhof, 1992). A combination of these techniques and immunohistochemistry markers to detect cell type can be used to identify progenitor cell activity in vivo.

1.2.1.2 Adult Subventricular Zone Neurogenesis

SVZ function:

Since neurogenesis was discovered in the adult brain significant research efforts have been devoted to characterizing the overall function of this niche. The SVZ, located along the lateral ventricle wall, houses the largest population of stem cells in the adult mammalian brain (J Altman & Chorover, 1963; Bernier, Vinet, Cossette, & Parent, 2001.; Rakic, 2001). Although SVZ functionality in the human is not studied in-depth, studies in the rodent brain have provided a rather comprehensive characterization of SVZ structure and functionality. In rodents, new neurons are generated from the SVZ to repopulate the olfactory bulbs. SVZ-neuronal precursors (commonly referred to as neuroblasts) migrate through the rostral migratory stream to the olfactory bulb in a chain migration pattern (Lois, García-Verdugo, & Alvarez-Buylla, 1996; C. Wang et al., 2011). Although the majority of the neuroblast arriving in the olfactory bulb undergoes apoptosis, the surviving cells become GABAergic granular and periglomerular

neurons and integrate into the circuitry (Carlén et al., 2002; Couillard-Despres et al., 2005). This processes provided promising evidence, especially in the neurodegeneration and repair field, that SVZ neuroblast could also act as neural replacements in other parts of the brain.

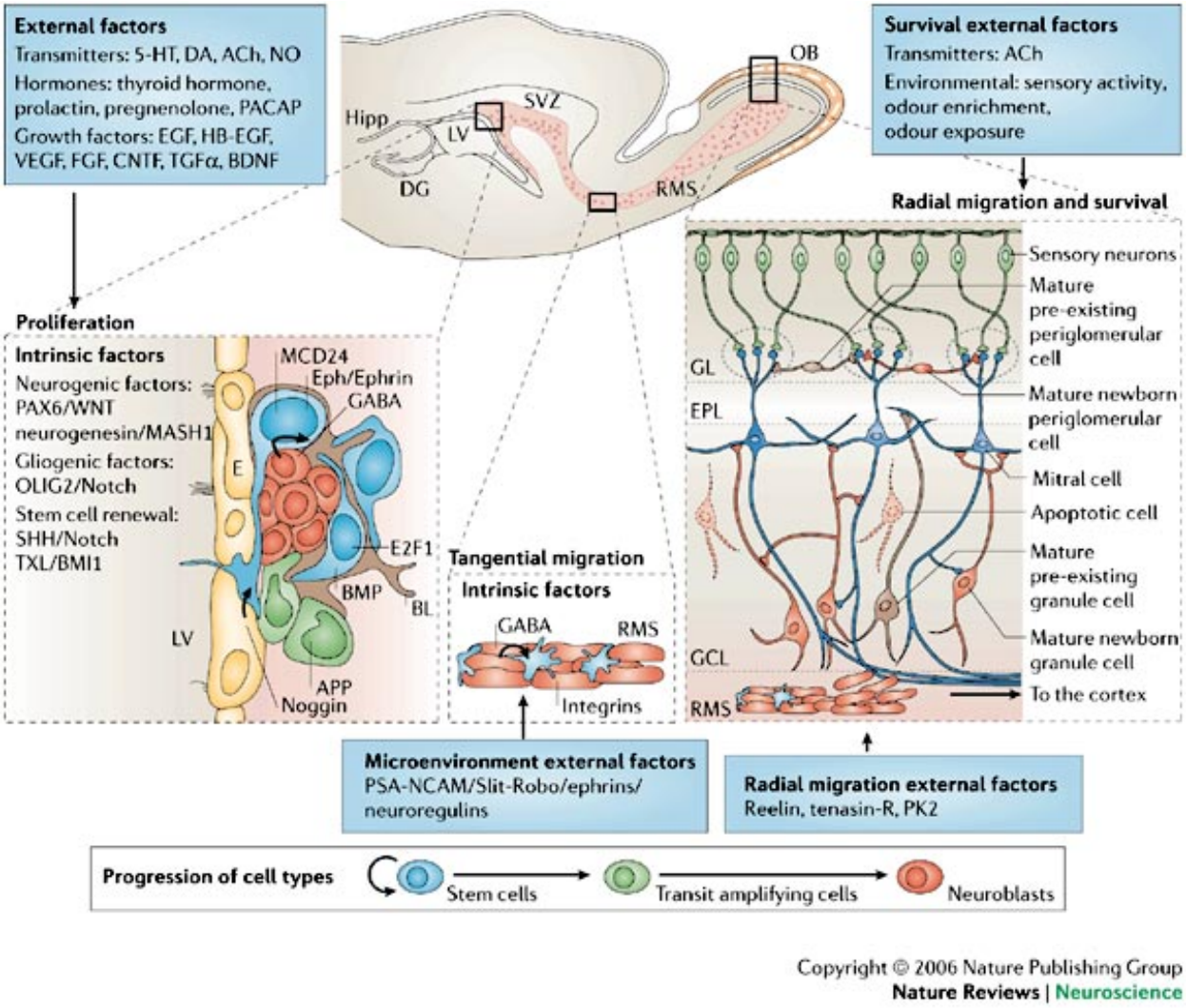

Figure 1: Summary diagram of SVZ architecture and factors controlling SVZ neurogenesis (adapted from Pierre-Marie Lledo, Mariana Alonso & Matthew S. Grubb, 2006).

SVZ Architecture:

Classification of SVZ cell types is important to the understanding of the molecular changes that underlie neurogenesis of the adult brain. Currently there are four main SVZ cell-types that have been identified; the ependymal cells (Type E), SVZ astrocytes (Type B), Transit Amplifying cells (Type C) and Neuroblasts (Type A). Each cell type is contained within a layer, the innermost layer housing the ependymal cells and the outer most layers containing the migratory neuroblasts (F Doetsch, García-Verdugo, & Alvarez-Buylla, 1997; F. Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; García-Verdugo, Doetsch, Wichterle, Lim, & Alvarez-Buylla, 1998; Hansen, Lui, Parker, & Kriegstein, 2010). There are many intrinsic and extrinsic factors that modulate proliferation, migration and survival of the SVZs' cellular niche. Studies have found that growth factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) enhance proliferation of transit-amplifying cells, while PSA-NCAM is crucial for neuroblast migration through the RMS (Bonfanti, 1994; L. Cao et al., 2004; Cremer et al., 1994; Greenberg & Jin, 2005; Kunlin Jin et al., 2002; Katoh-Semba et al., 2002; Kuhn, Winkler, Kempermann, Thal, & Gage, 1997; Zigova, Pencea, Wiegand, & Luskin, 1998) (**Figure** 1).

The ependymal cells (Type E), housed in layer 1 are mutliciliated cells that form a single-cell lining of the ventricular cavity. Type E cells are largely quiescent, express Nestin, Vimentin and CD133(Coskun et al., 2008; Danilov et al., 2009; F. Doetsch et al., 1999; Ihrie & Alvarez-Buylla, 2011; Nichols et al., 2013). These cells have little expression of Glial fibrillary acidic protein (GFAP) or Polysialylated-neural cell adhesion molecule (PSA-NCAM), which are markers for astrocytes and immature neurons respectively (Riquelme, Drapeau, & Doetsch,

2008). Studies have found that Type E cells retain stem cell characteristics in the post-natal brain and after transplantation and genetic lineage tracing the ependymal cells generated new neurons in the post natal SVZ (Coskun et al., 2008). The basal expansions of ependymal cells interact with astrocyte processes of layer 2 (Levison, Chuang, Abramson, & Goldman, 1993).

SVZ astrocytes (Type B), sometimes referred to as slowly dividing cells are housed in layer 2 of the SVZ and express GFAP and Nestin but not PSA-NCAM or Neuron-specific class III beta-tubulin (TuJ1) (F. Doetsch & Alvarez-Buylla, 1996). Type B is the second most abundance cell type in the SVZ and type B cell processes ensheath type A, neuroblast population (García-Verdugo et al., 1998). Type B cells play an essential role in modulating SVZ neurogenesis. A study using cytosine-β-d-arabinofuranoside (Ara-C) ablated all Type C and type A cells in the SVZ; in response to ablation of the dividing populations Type B cells were activated and produced more type C cells which then gave rise to type A cells (F Doetsch, García-Verdugo, & Alvarez-Buylla, 1999). This study was one of the first to demonstrate the crucial role of glial cell activation in SVZ functionality and indicated Type B cells as the true stem cells of the SVZ.

Transit Amplifying (Type C) are housed in layer 3. This cell population is the most actively dividing of all the SVZ cell types and also the most diverse (Brill et al., 2008, 2009; Gritti et al., 2002; Hack et al., 2005; Kohwi, Osumi, Rubenstein, & Alvarez-Buylla, 2005; Parras et al., 2004). Transit amplifying population lacks characteristics of a glial (GFAP+) or neuronal (PSA-NCAM+ or Tuj1+) phenotype and thus is consider a more immature cell type than Type A cells. Type C cells have been found to express markers of fate commitment (i.e. Pax6+ and Olig2+), while other type C cells express a more undifferentiated cell phenotypes (EGFR+ and Mash1+)

(Brill et al., 2008; J. Y. Kim et al., 2009; Y. Kim, Comte, Szabo, Hockberger, & Szele, 2009). Type C cells are also found in contact with ependymal cells and can be found in clusters near and sometimes in contact with neuroblasts. As previously described, type C cells are commonly known to give rise to type A cells, although some studies of have shown that Type B mitosis can give rise to type A neuroblasts (Danilov et al., 2009). Type C cells are also found to undergo both symmetrical and asymmetrical division, in which symmetrical division would enhance the cell population and asymmetrical division would enhance the type A neuroblast population (R. Zhang et al., 2004).

The neuroblast population (Type A) is in the final layer 4. Neuroblasts are the pro-neuronal progenitor cells, they are the most common cell type of the SVZ, and they express doublecortin (DCX), PSA-NCAM, Tuj1 but not GFAP (F Doetsch et al., 1997; Fiona Doetsch, Petreanu, Caille, Garcia-Verdugo, & Alvarez-Buylla, 2002; Koizumi et al., 2006; Sang et al., 2007). Neuroblasts are found in chains around the SVZ and the migratory patterning and marker expression similar to that of the migrating neuroblasts through the RMS (Gritti et al., 2002; James, Kim, Hockberger, & Szele, 2011; W. Sun, Kim, & Moon, 2010). Type A cells are also a highly targeted population of cells that migrate from the SVZ to the cortex in response to injury (Susarla, Villapol, Yi, Geller, & Symes, 2014; Yi et al., 2013; W. Zheng et al., 2011). DCX+ cell expression is highly utilized as an indicator for adult neurogenesis (J. P. Brown et al., 2003).

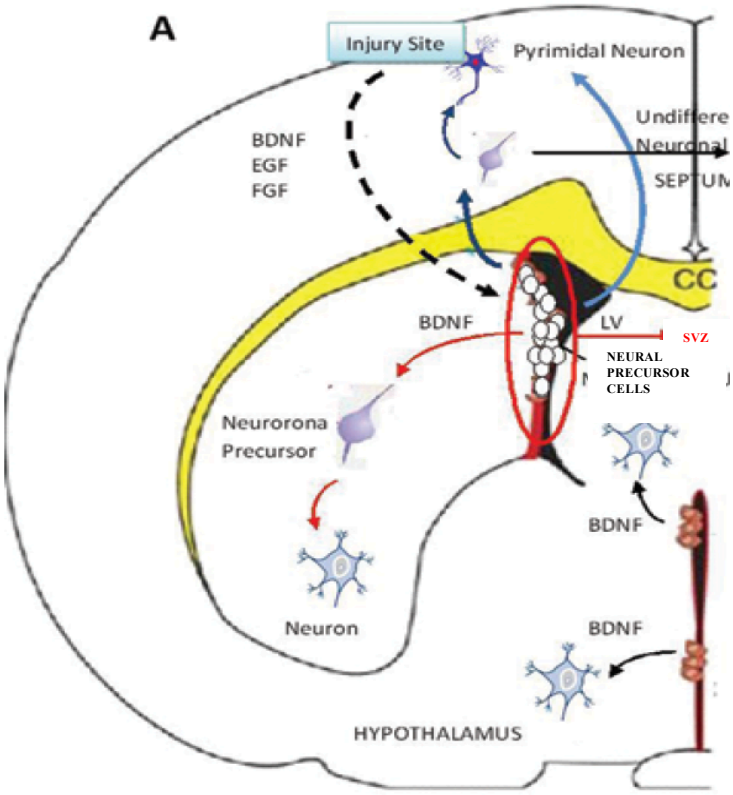

Figure 2: Diagrams showing injury-induced compensatory neurogenesis in the rodent SVZ after injury (adapted from Richardson et al 2010)

1.2.1.3 Evidence of the neurogenic response to cortical injury

The neurogenic response of the SVZ to stroke has been well characterized compared to what's known of this process in TBI. Stroke stimulates SVZ cell proliferation and induces migration of SVZ neuroblasts directed towards the site of injury (Arvidsson et al., 2002a; Dizon, Shin, Sundholm-Peters, Kang, & Szele, 2006; Goings, Sahni, & Szele, 2004; K Jin et al., 2001; Kernie et al., 2001; Parent, Vexler, Gong, Derugin, & Ferriero, 2002a; Ramaswamy, Goings, Soderstrom, Szele, & Kozlowski, 2005; R L Zhang et al., 2001). After TBI, a similar process occurs in which the SVZ niche is expanded and neuroblasts are observed migrating toward the site of the contusion (Richardson et al., 2010) (**Figure 2**). Furthermore, unlike neuroblast migration to the olfactory bulb in the uninjured brain, survival and neuronal differentiation of the

neuroblast arriving in the injured cortex is limited. Studies in stem cell transplantation into the brain after TBI reported a marked reduction in survival and little neuronal fate commitment of the transplanted progenitor cells (Hagan et al., 2003; Longhi, Zanier, Royo, Stocchetti, & McIntosh, 2005; Tate et al., 2002; Wennersten, Meier, Holmin, Wahlberg, & Mathiesen, 2004). Theses studies suggested that cortical environment after injury may be modulating the survival of progenitor cells in the cortex.

Although, it remains unclear if TBI-induced neurogenesis causes functional recovery, studies have shown that drugs that enhance neurogenesis are associated with improved outcomes after injury (Encinas, Vaahtokari, & Enikolopov, 2006; Garcia-Segura, Azcoitia, & DonCarlos, 2001; Dunyue Lu et al., 2007; D. Sun et al., 2009; Yoshimura et al., 2001). This suggests that improving the TBI-induced neurogenic response of the SVZ could aid in recovery. Therefore, although several studies have identified the robust response of SVZ cells to cortical trauma and suggests that this niche may be useful for neuroregeneration, the molecular underpinnings of the process modulating survival and fate commitment of progenitor cells is unknown.

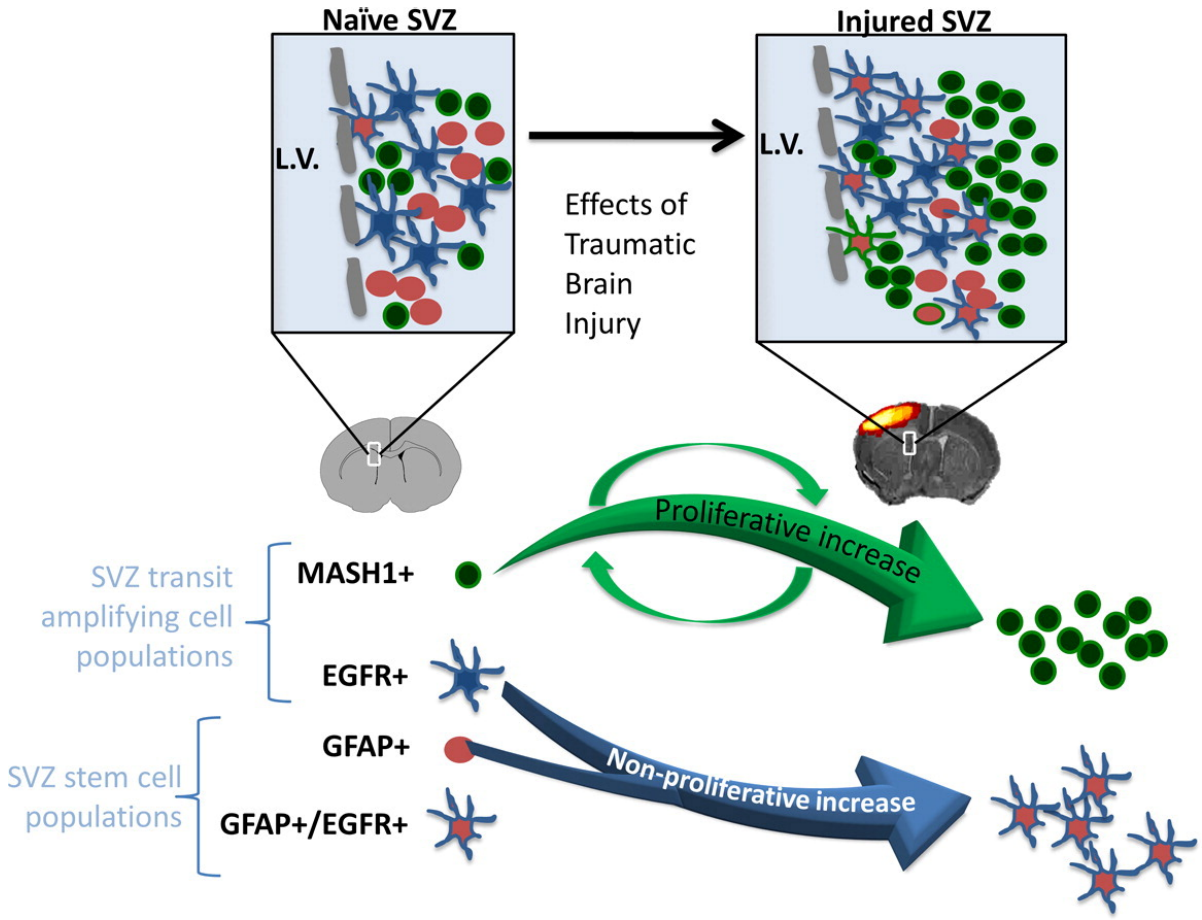

Figure 3: Diagram of SVZ niche expansion after TBI (adapted from Thomsen, et al 2014)

Injury expands the SVZ cell niche:

There is a significant increase in SVZ cell proliferation after stroke and CCI (Chirumamilla, Sun, Bullock, & Colello, 2002; Kernie & Parent, 2010b; Martí-Fàbregas et al., 2010; Ramaswamy et al., 2005; Richardson, Sun, & Bullock, 2007; Thomsen et al., 2014). Some studies have even found that this processes persists for months after the initial insult to the cortex (Leker et al., 2007; Parent et al., 2002b; Thored et al., 2006; R L Zhang et al., 2001) . Conflicting studies have demonstrated the transient effects of SVZ activation and suggested that injury severity may affect the time-course of sustained SVZ proliferation (R L Zhang et al., 2001; Rui Lan Zhang et al., 2011). Little is know of the effects of TBI on SVZ cells, however Miller, et al, recently

demonstrated that after injury GFAP+ and GFAP+/EGFR+ populations increased (Thomsen et al., 2014) (**Figure 3**). This suggests that injury induced dedifferentiation of the primarily quiescent type B population of the SVZ. Furthermore, this study also demonstrates that the transit amplifying type c population enhanced in the SVZ through proliferation, as defined by CLDU retention in Mash1+ cells (Thomsen et al., 2014) (**Figure 3**). The process of Type B expansion and type C proliferation is thought to maintain the brains endogenous repair mechanism after injury.

Injury enhances migration of neuroblasts:

As previously described, under physiological conditions SVZ neuroblasts migrate through the RMS to the olfactory bulb, and upon arrival some cells become functioning neurons (James et al., 2011; C. Wang et al., 2011). After injury neuroblasts migrate toward the site of injury, but not many survive or differentiate into functional neurons. (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002b; Kunlin Jin et al., 2003; Ohab, Fleming, Blesch, & Carmichael, 2006; Parent et al., 2002b). Studies in stroke have found that chemoattractive proteins and neurotrophic factors are involved in guiding neuroblasts to the lesion site (Emsley, 2003; Jaerve & Müller, 2012; Pontes, Zhang, & Hu, 2013). These effects are unknown in TBI, but it may be that a potent signaling molecule released at the site of injury may also be mediating the process of directing migrating neuroblasts Studies have also found that neuroblast migrate along blood vessels in close interaction with endothelial cells (Leventhal, Rafii, Rafii, Shahar, & Goldman, 1999; Thored et al., 2007; Yamashita et al., 2006; Rui L Zhang et al., 2009). This study suggests that molecular cues that may stimulate angiogenesis or modulate endothelial cell function may also be involved in signaling neuroblasts to migrate.

Injury limits survival and differentiation of neuroblast in cortex:

Although injury induces a significant response of SVZ cells causing them to proliferate and migrate to the injury site, the ultimate goal is for these cells to either 1) provide neural protective support of remaining cells in the peri-contusional cortex or 2) be neural replacements in the injury site (Jopling, Boue, & Izpisua Belmonte, 2011; D. Sun, 2014). However, studies have demonstrated that after injury many of the migrated neuroblast die or differentiate in to glia cells; that latter contributing to the inflammatory response or the glial scar (Benner et al., 2013; Gotts & Chesselet, 2005; Kernie & Parent, 2010a; Mierzwa, Sullivan, Beer, Ahn, & Armstrong, 2014; Susarla et al., 2014). Stem-cell transplantation studies have suggested that the lack of progenitor cell migration to and survival in the cortex after TBI may be due to inhibitory environmental cues (Hoane et al., 2004; D. Lu et al., 2001; Schouten et al., 2004; Tate et al., 2002). Therefore, there may be a potent signaling molecule aiding in inhibiting survival and neuronal differentiation of progenitor cells. Thus, understanding the molecular events that impede the survival and neuronal fate direction of neuroblasts is important to develop therapeutics that target the neurogenic niche of the brain for neuroprotective or neuralreplacement mechanisms in disease.

1.3 Lysophosphatidic Acid:

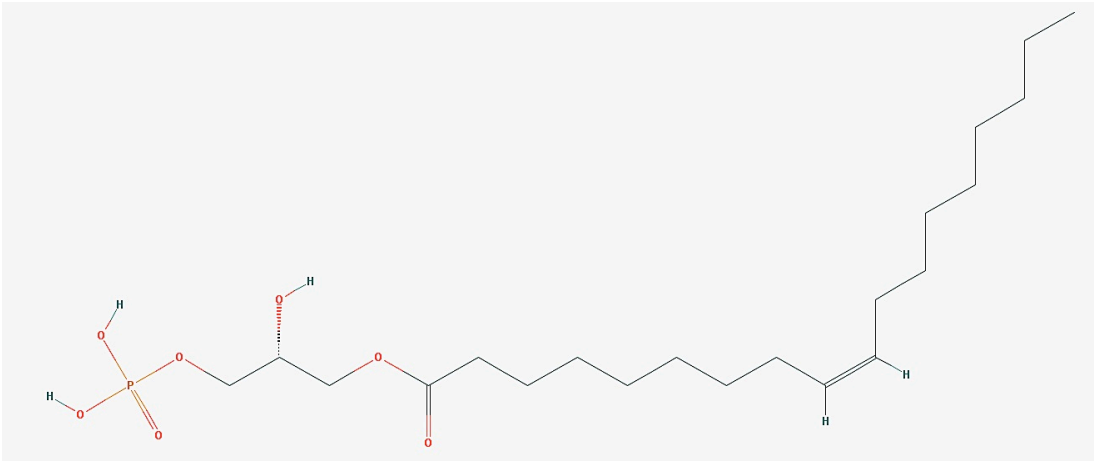

Figure 4: 2D chemical structure of LPA (lysophosphatidic acid) 18:1 (Adapted from the National Center for Biotechnology Information. PubChem Compound Database; CID=5311263)

Lysophosphatidic acid (LPA) is a small bioactive phospholipid that influences many biological processes in development, physiology and pathophysiology. LPA is the simplest phospholipid and is present in all mammalian cell types including blood. In the brain, LPA can effect both neural and non-neural cell types and is a critical signaling molecule for development and functionality of the adult brain (Farooqui & Horrocks, 2006). Structurally, all LPA species have a phosphate head group attached to a glycerol backbone connected to a single aliphatic chain; each species of LPA has a distinct length and saturation of the acyl chain (**Figure** 4). Species with a phosphate monoester and a long chain of more than 12 carbons are biologically active LPA (Lynch & Macdonald, 2002; Lyncha & Macdonald, 2001). Common LPA species in the brain and biological fluids include palmitoyl (16:0), stearoyl (18:0), oleoyl (18:1) and arachidonoyl (20:4) acyl groups; LPA 18:1 is most commonly used in laboratory settings. LPA

acts through activation of 7 G protein-coupled receptors (GPCRs) (LPAR1-6), each of which effects an array of cellular processes.

1.3.1 Lysophosphatidic Acid Metabolism

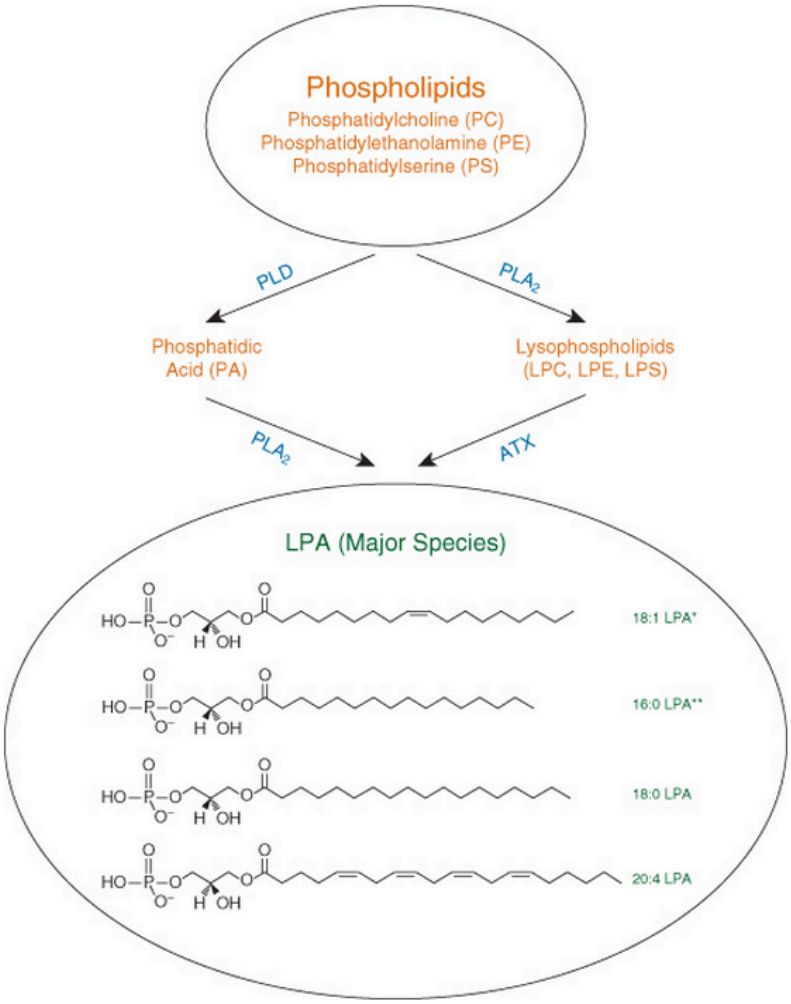

Figure 5: Diagram of LPAs two distinct metabolic pathways. A) a | hydrolysis of phosphatidic acid (PA) by soluble phospholipase A2 (sPLA2) or B) hydrolysis of lysophosphatidylcholine (LPC) by ATX/lyso PLD (adapted from Chun, 2013).

The brain contains the highest concentration of LPA in the body (A K Das & Hajra, 1989). Studies have demonstrated that LPA is produced from activated platelets, adipocytes, and tumor cells (Eichholtz, Jalink, Fahrenfort, & Moolenaar, 1993; Siess et al., 1999). Mechanisms of LPA production neuronal cell types are not well understood; there are two general synthetic pathways that have been identified. 1) Intracellular LPA is produced first by making phosphatidic acid (PA) from phospholipids through diacylglycerol (DAG) and diacylglycerol kinase or phospholipase D (**Figure 5**). Hydrolysis of PAs by phospholipase A1 and A2 (PLA1 and PLA2) then produce. LPA (Aoki, Inoue, & Okudaira, 2008). Intracellular LPA is also be synthesized by monoacylglycerol kinase (MAG-kinase) phosphorlating monoacylglycerol (MAG) and by glycerophosphate acyltransferase (GPAT) acetylating glycerol-3-phosphate (Bektas et al., 2005). Intracellular LPA can be produced by neurons (N Fukushima, Weiner, & Chun, 2000) in the endoplasmic reticulum and the mitochondria (Aoki et al., 2008; Aoki, 2004; Gendaszewska-Darmach, 2008) and at the leading edge of migrating monocytes through calcium independent phospholipase A2 (PLA2) activity (Carnevale & Cathcart, 2001; Mishra, Carnevale, & Cathcart, 2008). Intracellular LPA is transported by fatty acid binding proteins (FABP) as well as gelsolin, which may act to aid in actin assembly within the cell (Pagès, Simon, Valet, & Saulnier-Blache, 2001). 2) Extracellular LPA is produced from phospholipids converted to their lysophospholipid (LP) form and then converted to LPA by lysophospholipase D / autotaxin (ATX) activity (Akira Tokumura et al., 2002; Umezu-Goto et al., 2002). In plasma lysophosphatidylcholine (LPC) is produced from phosphatidylcholine (PC) by PLA1 and lecithin cholesterol acyltransferase (LCAT) activity. In platelets, phospholipids like phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) are converted into their corresponding lysophospholipid forms through secretory phospholipase A2

(sPLA2) or phosphatidylserine-specific phospholipase A1 (PS-PLA1) activity (Aoki et al., 2008) (**Figure** 5). The main extracellular transport mechanism for LPA is by albumin binding, gelsolin is also thought to support circulating LPA (Pagès et al., 2001).

1.3.1.1 Autotaxin

Autotaxin (ATX, also known as lysophospholipase D) is the most well characterized enzyme associated with LPA production. ATX is present in the blood and is highly expressed in the brain especially within the choroid plexus, which leads to high ATX levels in the cerebral spinal fluid (CSF) (Hosogaya et al., 2008; Kanda et al., 2008; Nakamura et al., 2009; Nakasaki et al., 2008). ATX knockout (Enpp2-/-) studies revealed neural tube defects and blood vessel malformation caused mutant mouse pup deaths at embryonic day 9.5 (Koike, Keino-Masu, & Masu, 2010; Tanaka et al., 2006; van Meeteren et al., 2006) whereas ATX heterozygous pups (Enpp2+/-) survived into adulthood with half LPA levels in the plasma as compared to the wild type. These studies demonstrate the importance of ATX signaling for LPA production in development as well as the importance of ATX to maintain LPA levels in plasma. Moreover, ATX is significantly involved in vascular development; ATX induces vascular endothelial growth factor (VEGF) and thus induces endothelial migration and proliferation (H. Lee, Goetzl, & An, 2000; Panetti, Chen, Misenheimer, Getzler, & Mosher, 1997) and matrix remodeling associated with angiogenesis (Siess et al., 1999; Wu, Chen, Lin, Chen, & Lee, 2005). LPA is implicated in many of the processes involving angiogenesis and tissue repair because of ATX's effects on the cell types involved in these processes.

1.3.1.2 Lysophosphatidic Acid Catabolism

Several enzymes involved in LPA metabolism are also involved in catabolism. Dephosporalation of LPA is a major pathway that terminates LPAs' signaling processes. LPA acyltransferase (LPAAT) is a critical enzyme involved in the synthesis of glycerophospholipids by converting LPA back to PA (Aoki, 2004; Pagès et al., 2001). Extracellular LPA is degraded by lipid phosphate phosphatases 1-3 (LPP1-3) and phospholipid phosphatase (PGR-1), which hydrolyses LPA into MAG (Bräuer et al., 2003; Luquain, Sciorra, & Morris, 2003) mediated by ecto-phosphatase activity (Bräuer et al., 2003; Pilquil et al., 2001) and thus LPPs and PGR-1 play a crucial for regulating LPAs signaling.

1.3.2 Lysophosphatidic Acid GPCR Signaling

GPCRs of lysophospholipid's like LPA and sphingosine-1-phosphate (SIP), also referred to endothelial differentiation gene (EDG-), are ubiquitously expressed throughout the body (N Fukushima, Ishii, Contos, Weiner, & Chun, 2001; Hla, Lee, Ancellin, Paik, & Kluk, 2001; Isao Ishii, Fukushima, Ye, & Chun, 2004). Studies suggest that every mammalian cell responds to LPA in one way or another (Anliker & Chun, 2004; Hla et al., 2001). LPA receptors are expressed throughout the brain and many studies have demonstrated the importance of LPA signaling in brain development and function such as proliferation (J. J. Contos, Fukushima, Weiner, Kaushal, & Chun, 2000; Kingsbury, Rehen, Contos, Higgins, & Chun, 2003), cell survival (Kingsbury et al., 2003; Ye, Ishii, Kingsbury, & Chun, 2002), adhesion (Joshua A.

Weiner, Fukushima, Contos, Scherer, & Chun, 2001) and migration (N Fukushima, 2002). LPA acts through six type I, rhodopsin-like, GPCR's (LPAR1-6) and each receptor is highly homologous. LPAR1-6 each have seven transmembrane domains and can couple to one or more of the four heterotrimeric G_α (G12/13, Gq/11, GI/O and Gs) proteins which causes activation of an array of signaling processes and has diverse effects in physiology and pathophysiology. Importantly, the actual pathway evoked by receptor activation and the resulting outcome of the downstream signaling cascades are dependent on many factors such as receptor expression level, developmental stage, cell type, concentration of the LPA ligand and the signaling protein. Therefore, many discrepancies within the literature can be attributed to inconsistencies in these variables. Furthermore, LPA signaling has been emerging as a promising therapeutic target, however many of the limitations in discovery in this field can be attributed to the functional redundancies between LPARs and G-proteins. Many studies utilize LPAR agonists and antagonist, yet because of functional redundancies in LPARs, these drugs have incomplete targeting of LPAs signaling cascade, resulting in inconclusive findings when utilizing these methods to understand the role of LPA in development and disease.

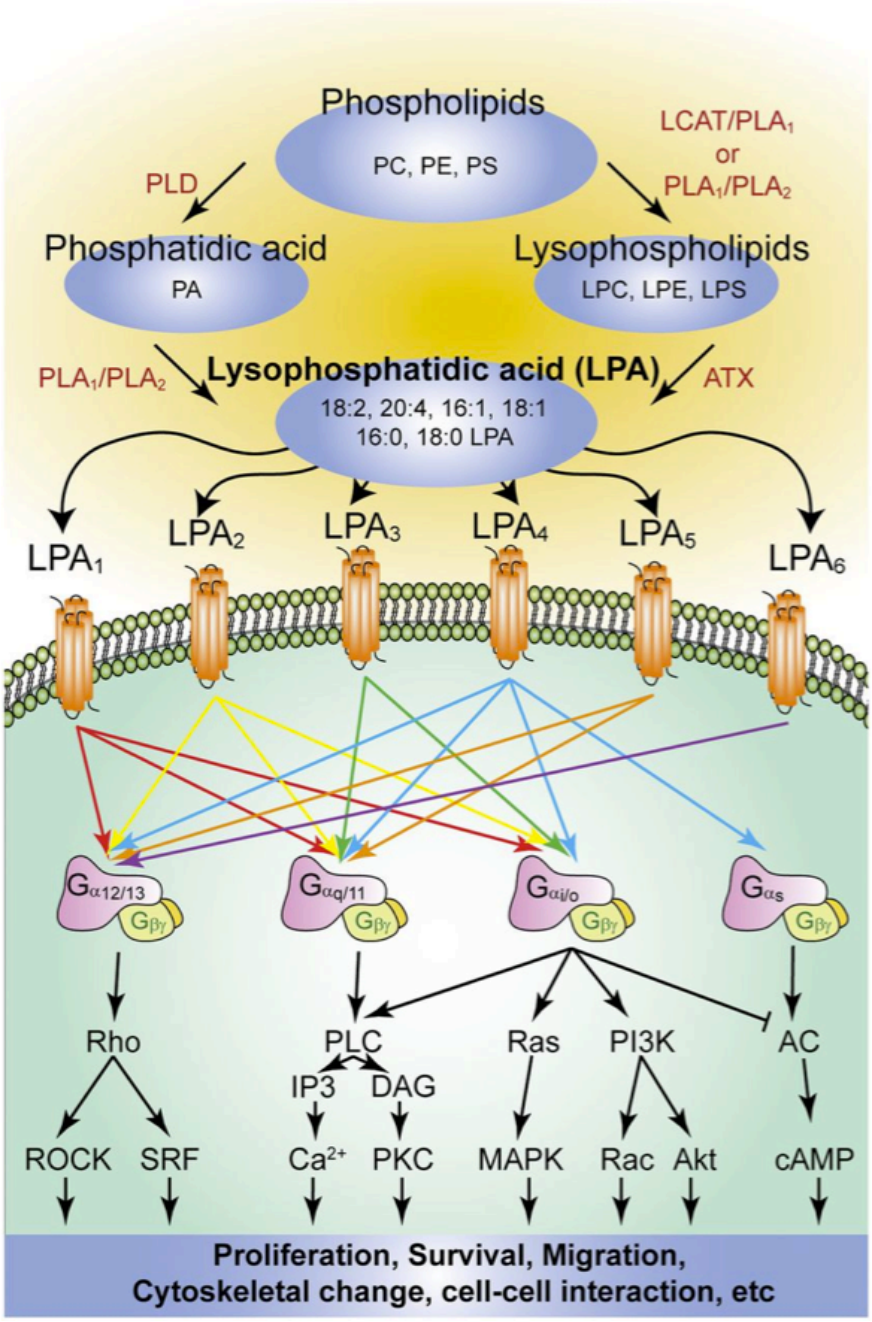

Figure 6: Summary diagram of the major routes of LPA synthesis and the activated signaling pathways via LPAs six known receptors. Enzymes are indicated in red. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PLD, phospholipase D. (adapted from Yung, Stoddard, & Chun, 2014)

1.3.2.1 Lysophosphatidic Acid Receptor 1

LPAR1 (also called EDG-2) was the first receptor identified and hence, the most studied and best characterized receptor of the six. LPAR1 shares 50-60% amino acid sequence with LPAR2 (also called EDG-4) and LPAR3 (also called EDG-7), making these three receptors very similar in structure and function. LPAR1 couples with three G_{α} proteins that signal different downstream cascades like adenylyl cyclase (AC) inhibition, Ca2+ mobilization and Akt activation (N Fukushima et al., 2001; I Ishii, Contos, Fukushima, & Chun, 2000). Through Gi/o protein LPAR1 activation will initiate the RAS/ mitogen-activated protein kinase (MAPK) pathway. With Gq/11 LPAR1 activation leads to activated PLC pathway and with G12/13 LPAR1 activation leads to activated Rho/ROCK pathway (N Fukushima et al., 2001; Hla et al., 2001; Isao Ishii et al., 2004) (**Figure 6**).

LPAR1 is expressed in the human and rodent brain. During CNS development LPAR1 expression is highly regulated spatially and temporally. LPAR1 expression is particularly elevated in the ventricular zones (VZ), which are the neurogenic regions of the developing brain and thus LPAR1 was first discovered to influence differentiation and proliferation of neural progenitor cells (NPC) (J. J. Contos et al., 2000; N. Fukushima, Kimura, & Chun, 1998; Svetlov et al., 2004). Further studies in neurodevelopment using LPAR1 null mice demonstrated that LPAR1 expression is necessary for normal cortical development. LPAR1 null mice demonstrated major defects in sucking behavior, which is attributed to olfactory defects, and thus caused 50% perinatal lethality in these mutants (J. J. Contos et al., 2000). The surviving LPAR1 null mice having significantly smaller bodies, craniofacial dysmorphism, increased death of Schwann cells and other obvious neurodevelopmental defects (Joshua A. Weiner et al., 2001). Other

developmental studies utilizing mouse line with defects in LPAR1 demonstrate during neurodevelopment pups had higher neuronal cell death in both cortical and hippocampal regions (Estivill-Torrús et al., 2008; Matas-Rico et al., 2008). In addition, LPAR1 is present on oligodendrocytes just prior to maturation and colocalizes with myelin basic protein (MBP); LPA is suggested to modulate the maturation process of oligodendrocytes and other myelin-forming glial cells through the Rho-Rho associated protein kinase (ROCK) pathway (Allard et al., 1998; Dawson, Hotchin, Lax, & Rumsby, 2003; Nogaroli et al., 2009; Stankoff et al., 2002; J A Weiner, Hecht, & Chun, 1998; Yu, Lariosa-Willingham, Lin, Webb, & Rao, 2004). These studies strongly support the importance of LPA signaling and signaling through LPAR1 receptors for normal neural development and function in the CNS. Due to the significant role LPAR1 signaling has in development there is significant evidence to support LPAR1s' role diseases with developmental linkage such as in neuropsychiatric disorders (J. J. Contos & Chun, 1998; Matas-Rico et al., 2008), obesity (Dusaulcy et al., 2009) and fibrosis (Pradère et al., 2007; Tager et al., 2008).

Post-mitotically, LPAR1 has the most heterogeneous expression in the CNS (S. An, 1998; James J A Contos, Ishii, & Chun, 2000; Ye, 2008); LPAR1 signaling indirectly and directly effects the function of neurons, astrocytes, oligodendrocytes and neural progenitor cells (Matas-Rico et al., 2008). In the adult brain LPAR1 is highly expressed in the white matter tracts (J A Weiner et al., 1998), which also suggest that myelination is heavily dependent on LPAR1 expression (Miron, Hall, Kennedy, Soliven, & Antel, 2008; Terai et al., 2003). Astrocytes play a significant role in development, physiology and pathophysiology and they express LPAR1-5 on their cell bodies. Studies have shown that within astrocytic cultures primed with LPA there was increased

neurogenesis and this effect was LPAR1 dependent (E Spohr, Dezonne, Rehen, & Gomes, 2011; T. C. S. Spohr et al., 2008). This suggests that LPAR1 expression plays an important role in glial signaling and fate. Studies have also shown that LPAR1 expression is important for neuronal function; LPAR1 null neuronal cultures demonstrated impairments in synaptic transmission and neurotransmitter release (Harrison et al., 2003).

Receptor Tyrosine Kinase – LPAR1 Signaling Complex

LPAR1 signaling can be modulated through complexes like receptor tyrosine kinases (RTKs) and GPCR. RTK-GPCR's form these complexes to regulate common signaling pathways by utilizing components of each receptor to enhance the signal gained by just one receptor acting alone (Pyne & Pyne, 2008). Typically the RTK's will hijack components of the GPCR to enhance signaling. Neurotrophic tyrosine kinase receptor type 1 (TrkA), and LPAR1 are known to form this integrated signaling complex (Miller & Lefkowitz, 2001; Noreen Akhtar Moughal, Waters, Sambi, Pyne, & Pyne, 2004). TrkA typically binds nerve growth factor (NGF) which activated the ERK ½ pathway which is beta-arrestin dependent (Miller & Lefkowitz, 2001; Rakhit, Pyne, & Pyne, 2001) and causes neurite extension. Active LPAR1, in the TrkA-LPAR1 complex, enhances ERK-1/2 response by creating more Gby dimers for TrkA to use. LPA binding to LPAR1 can dissociates this complex (Noreen Akhtar Moughal et al., 2004) and in this way LPA acts to antagonize the effects of NGF on cells and cause neurite retraction as opposed to the opposite effect NGF would have which is neurite extension (Noreen A Moughal et al., 2006).

1.3.2.2 Lysophosphatidic Acid Receptor 2

Unlike LPAR1 expression patterns , LPAR2 (also known as EDG-4) expression is low in the brain fetal and post natal brain development and is highly prevalent in the embryonic brain. LPAR2 null mice are phenotypically normal pre-and postnatal and have similar birth and survival rates as control. However, mice null for both LPAR1 and LPAR2 have an exacerbated phenotype of the LPAR1 null mice A recent study showed that application of LPA ex vivo to E18 mouse brains increased cortical thickness by decreasing cell death, increased terminal mitosis of NPC's; the mouse brain rapidly produced gyri-like folds in the brain - this effect was absent in LPAR1 and LPAR2 null mice (Kingsbury et al., 2003). These studies suggest the importance of both LPAR1 and LPAR2 signaling during development. Similar to LPAR1 signaling, LPAR2 signaling also effects myelin forming cells. LPAR2 activation is generally associated with cell survival and cell migration (Kingsbury et al., 2003; Kuriyama et al., 2014)and in schwann cell cultures LPAR2 activation increases myelin production (Inoue et al., 2004). In the adult brain LPAR2 in interaction with proteoglycan 1 (PRG-1) was found to underlie hippocampal excitatory transmission (Trimbuch et al., 2009).

1.3.2.3 Lysophosphatidic Acid Receptor 3

LPAR3 (also known as EDG-7) couples with Gi/o and Gq but unlike LPAR1 and 2, LPAR3 does not couple to G12/13 and is less sensitive to LPA species with saturated acyl chains(J. J. . Contos & Chun, 2001; Hama et al., 2007; Hama, Bandoh, Kakehi, Aoki, & Arai, 2002) (**Figure** 6). During development LPAR3 is located primarily in the lateral nasal and maxillary process as well as the optic vesicle (Ohuchi et al., 2008). In the human and rodent adult brain LPAR3 is expressed in the hippocampus, amygdala and frontal cortex (Songzhu An et al., 1997; I Ishii et

al., 2000). Similar to LPAR2 null phenotypes, LPAR3 null mice develop normally and have viable litters (Lai et al., 2012). However the most pronounced effect of the LPAR3 null mutant mice are observed in reproductive system of the female nulls having failed embryo implantation (Ye et al., 2005). LPAR3 activation includes PLC activation, Ca2+ mobilization, variable effects on AC and MAPK activation (K. Bandoh et al., 1999; Im et al., 2000; I Ishii et al., 2000). Studies have shown that over expression of LPAR3 in neuroblastoma cells leads to neurite elongation whereas over expression of LPAR1 and 2 lead to neurite retraction and cell rounding (I Ishii et al., 2000).

1.3.2.4 Lysophosphatidic Acid Receptor 4

The amino acid sequence for LPAR4 (also known as P2Y9) is dissimilar to that of the first three receptors discovered (LPAR1-3) (C.-W. Lee, Rivera, Gardell, Dubin, & Chun, 2006a) and was the first receptor identified to involve the Gas protein (C.-W. Lee, Rivera, Dubin, & Chun, 2007). LPAR4 mediates LPA induced CA^{2+} mobilization and cAMP accumulation; studies suggest that this difference is what allows LPAR4 receptor activation to negatively modulate some traditional LPA signaling. LPA is typically a chemoattractant but LPAR4 activation is chemorepulsive thus negatively modulating cell motility (Z. Lee et al., 2008). In addition, many studies have shown that LPAR4 activation causes neurite retraction, cell aggregation and stress fiber formation similar to the other LPARs by activation of the Rho/ROCK pathway (C.-W. Lee et al., 2007; Yanagida, Ishii, Hamano, Noguchi, & Shimizu, 2007). However cell cultures that are LPAR4 deficient exhibit enhanced movement compared to control cultures indicating hypersensitivity to LPA without LPAR4. These studies suggest that LPAR4 may be critical in regulating LPA signaling (Z. Lee et al., 2008). LPAR4 has also been shown to modulate the

differentiation of hippocampal progenitor cells (Rhee et al., 2006). The expression profile of LPAR4 within the developing brain suggests involvement in development (Ohuchi et al., 2008) but adult LPAR null mice develop fairly normally although prenatal survival is decreased (Sumida et al., 2010); more studies are needed to definitively characterize the role of LPAR4 in CNS development.

1.3.2.5 Lysophosphatidic Acid Receptor 5

LPAR5 shares 35% homology with LPAR4 (Kotarsky et al., 2006; C.-W. Lee et al., 2006a) and like LPAR4 the profile of LPAR5 in early mouse brain development is widely expressed, suggesting a role in CNS development (Ohuchi et al., 2008). In addition, LPAR5 also produces neurite retraction and stress fiber formation through Ga12/13 pathway and increases intracellular Ca2+ levels via the Gaq pathway (C.-W. Lee et al., 2006a).

1.3.2.6 Lysophosphatidic Acid Receptor 6

LPAR6 is the most recently discovered receptor previously called P2Y5 (Pasternack et al., 2008). Studies on the effects of LPAR6 activation require higher concentrations of LPA (usually up to 10uM) to show an effect where as LPAR1-5 required nanomolar concentrations (M. Lee et al., 2009). This suggests that LPAR6 activation may be highly involved in pathological states where LPA concentrations are highest. LPAR6 activation resulted in increased intracellular Ca2+ which function through the Gas protein to stimulate cAMP and ERK1/2 (M. Lee et al., 2009). Interestingly, LPAR6 has a higher affinity for 2-acyl-LPA than 1-acyl-LPA (M. Lee et al., 2009).

1.3.3 Lysophosphatidic acid – species specific signaling

Not all LPA species are created equal in terms of biological potency; some LPA species are actually more potent than others. Studies have shown that unsaturated LPA species (i.e. 16:1, 18:1 and 20:4) are more biologically active than saturated LPA species (i.e. 16:0, 18:0, 20:0) and studies suggest that the difference in potency lie in the receptors activated (Koji Bandoh et al., 2000). Studies of smooth muscle cells in vivo and in vitro demonstrated that unsaturated LPA species increased proliferation and dedifferentiation of cells whereas the saturated LPA species did not have this effect (Hayashi et al., 2001; A. Tokumura et al., 1994). Also, unsaturated LPA in a human carcinoma cell line, A431, induced Ca2+ mobilization whereas, the saturated LPA species did not (Jalink et al., 1995). In insect cells transfected with LPARs 1-3 showed different receptors affinities for saturated vs. unsaturated LPA species. While this study showed that LPAR1 and LPAR2 were generally impartial to species saturation, LPAR1 showed very high specificity for unsaturated LPA species 18:1>, 18:2>, 18:3>, 16:1> and 20:1 (Koji Bandoh et al., 2000). These studies highlight the critical need to identify the LPA species that are changing within the brain after trauma in order to better identify the pathways in which LPA is acting and the effects of LPA signaling in the injured brain.

1.3.4 The Cellular Effects of Lysophosphatidic acid in the CNS

Decades of research on the bioactivity of LPA within the CNS have revealed the pleotropic effects of LPA in development, physiology and pathophysiology and often time's studies have revealed major contradictory effects. What results from LPA signaling in the CNS is dependent on many factors like developmental stage, pathology, cell type, concentration of LPA and availability of LPARs. Inconsistencies in experimental conditions between research groups have also lead to contradictory findings and the unknown developmental effects of LPAR channel mutations on adult CNS studies or the inefficacy of LPAR agonist or antagonists to completely modify LPAR signaling also contribute to the array of results and conflicting findings on the effects of LPA in the brain. As a result, unless otherwise specified, the studies discussed herein will focus on the direct role of LPA signaling or the effects of modulating a signaling molecule up- or down-stream of LPA on the cellular responses and brain wide pathologies.

1.3.4.1 LPA in Cell Death & Survival

One of the major effects of CNS pathologies, like brain trauma, is cell death and many studies have focused on neuroprotective measures to enhance recovery after trauma. LPA has been a major therapeutic target for study but many groups have reported opposing effects of LPA signaling. Specifically in rat hippocampal cell cultures and neuronal PC12 cultures LPA has been shown to cause apoptosis and at higher concentrations (1uM) necrosis through activation of Rho, mitochondrial accumlation of reactive oxygen species (ROS) and/or nitric oxide (NO) from an unspecified G-protein activation (Frederick W. Holtsberg et al., 1998; Steiner, Holtsberg, Keller, Mattson, & Steiner, 2000). However, other studies have shown LPA promotes survival in

the same cell type (Fujiwara et al., 2003). Similarly studies of cortical neuroblasts has shown that LPA promotes survival (Kingsbury et al., 2003) whereas other report that LPA signaling enhances cell death of cortical progenitors (Nobuyuki Fukushima, Shano, Moriyama, & Chun, 2007). Studies in immortal rat hippocampal cells (H19-7) suggest that differences in the G-proteins activated may underlie the differential effects that result. They suggest that LPA's pro-survival activity is dependent on LPAR1,2, G_i activation of GSK-3 and LPA's pro-apoptotic effects are due to LPAR1,2 and 4 –G12/13 activation of GSK-3 (Sun et al., 2010). The availability of LPAR4 is what mediates the pro-apoptotic effects of LPA, however why there are differential effects in cell cultures with fairly homologous receptor type expression is yet to be determined.

1.3.4.2 LPA in Proliferation and Differentiation

Many studies in CNS disease also focus on the cues that effect proliferation and differentiation of endogenous progenitors as a way to enhance their supportive effects in and/ or to differentiate and replace dead neurons after brain trauma. Similar to the effects of LPA on cell death and survival, LPA also promotes proliferation and differentiation. Although these effects aren't mutually exclusive, discrepancies on LPAs effects on cell proliferation and differentiation exist. Studies have shown conditions where LPA promotes cell division and others have shown that LPA in fact enhances quiescence; the same discrepancies in the literature are also present for LPAs role in differentiation . The differences between LPAs effects are most prevalent between rodent species. LPA at high concentrations (10uM) inhibit proliferation (Cui & Qiao, 2006) and lower concentrations (up to 1.0uM) promote proliferation of rat cortical NPCs (Hurst et al.,

2008); similar effects were observed in human embryonic stem cells. However, in mouse NPCs high concentrations (10uM) of LPA promote proliferation (Svetlov et al., 2004). The differential effects of LPA species could explain the variance in amounts of LPA and LPARs expressed during development between mouse, rat and human. The effects of LPA on expansion of human embryonic cells are mediated by the Rho/Rock pathway (Dottori, Leung, Turnley, & Pébay, 2008). Select groups have explored the underlying mechanism of neuronal differentiation and shown that LPA stimulates differentiation of neural progenitor cells though the LPAR1-Gi dependent activation (Pitson & Pébay, 2009) while other have shown that LPAR1 and LPAR3 activation of PI3K/AKT as well as the Rho/Rock pathway inhibits neuronal differentiation (Dottori, Leung, Turnley, & Pébay, 2008). More research is needed to understand the effects of LPA signaling on expansion and differentiation of progenitor cells in order to effectively modulate LPA signaling effects on neurogenesis as a therapeutic for degenerative disease.

1.3.4.3 LPA - A potent neuromodulator

Studies have shown LPA to be a potent neuromodulator with neurotransmitter-like effects on early cortical neuroblasts and mature neuronal populations. A compelling study using whole-cell patch clamping on embryonic day 11 cortical neuroblast cultures demonstrates that cortical progenitors selectively responded to LPA and not L-glutamate or GABA. With continued growth in culture, up to 12hrs, the majority of cells produced were LPA responsive; some GABA and L-Glutamate responsive cells were also produced and even those cells preferentially depolarized to extracellular LPA even after a short refractory period of GABA or L-Glutamate activation (Adrienne E. Dubin, Bahnson, Weiner, Fukushima, & Chun, 1999). LPA depolarized NPC's through inward chloride current (Postma, Jalink, Hengeveld, Offermanns, & Moolenaar, 2001).

Another study showed LPA signaling also causes an increase in intracellular calcium of rat embryonic hippocampal neurons through glutamate release and NMDA and AMPA/kainate receptors (Frederick W. Holtsberg et al., 1997). These studies provided compelling evidence that LPA acts as a neurotransmitter and made LPA the earliest reported extracellular stimuli that caused conductive changes in cortical neuroblasts; suggesting LPA plays a major role in the physiology of CNS development. Furthermore, in the postnatal brain, LPA enhances synaptic vesicle formation, trafficking and membrane fission through lysophosphatidic acid acyl transferase (LPAAT) enzyme activity (Kooijman, Chupin, de Kruijff, & Burger, 2003; Schmidt et al., 1999). In rat hippocampal CA1 neuronal NMDA channel activity is increased in response to LPA signaling (Elmes, Millns, Smart, Kendall, & Chapman, 2004) and LPA signaling also increases activity in rat spinal chord neurons (Elmes et al., 2004). These further support the importance of LPA signaling in adult brain physiology. Consistently, studies have also shown that LPA is chemorepulsive, caused neurite retraction, cell rounding and modulates neuronal migration (N Fukushima, 2002; Halstead et al., 2010; Hurst et al., 2008; Pébay, Bonder, & Pitson, 2007). Evidence in development and in the postnatal brain provides compelling support that LPA signaling my also modulate behavior of the endogenous progenitor cells of the adult brain and that attenuating LPA signaling after trauma may enhance recovery.

1.3.4.4 LPA and Astrocytes

Astrocytes are a sub-type of glial cells that act as a significant support cell in the CNS. Astrocytes play a significant role in the microenvironment of the brain, they directly influence neuronal behavior and astrocyte activity plays a prominent role in the brains response to injury. Similar to neurons, LPA signaling can modulate the cytoarchetecture and physiological response

of astrocytes. However, LPA signaling does not directly regulate astrocyte migration (Sato et al., 2011). Mouse astrocyte cultures express LPAR1-5[82] and human astrocyte cultures express LPAR1 and LPAR2 (Hoelzinger et al., 2008). Studies in astrocyte cell cultures have consistently found that LPA signaling induces focal adhesion, stress fiber formation and the reversal of stellation mediated by Rho/ROCK pathway activation (Manning & Sontheimer, 1997; Ramakers & Moolenaar, 1998) mediated, at least in part, by LPAR1 and LPAR2 receptors. LPA is also shown to induce a variety of other effects on astrocyte cultures like increased calcium mobilization and proliferation (Jeffrey N. Keller, Steiner, Holtsberg, Mattson, & Steiner, 1997; Manning & Sontheimer, 1997; Shano, Moriyama, Chun, & Fukushima, 2008; Sorensen et al., 2003), increased production of trophic factors, increased reactive oxygen species (ROS) and inhibition of glutamate and glucose uptake (Jeffrey N. Keller, Steiner, Mattson, & Steiner, 1996; Rao et al., 2003). The studies suggest that LPA signaling plays a significant role in modulating astrocyte function.

Astrocyte-mediated effect of LPA on neurons

LPA can directly effect neuronal development and signaling as well as indirectly affects neurons through astrocytes. Co-cultures of cortical neuroblasts and astrocytes primed with LPA or neuroblasts in condition media from astrocytes treated with LPA induced neuronal differentiation and axonal outgrowth of the neuroblasts mediated through the epidermal growth factor (EGF) and MAPK pathways. This effect was lost in astrocytes null for the LPAR1 and LPAR2 receptors (E Spohr et al., 2011; T. C. L. de S. E. Spohr, Dezonne, Rehen, & Gomes, 2014; T. C. S. Spohr et al., 2008). Furthermore LPA causes astrocytes to release ATP which then causes hyperpolarization and reduced spiking of retinal ganglion neurons (Newman, 2003), thus

demonstrating the LPA can modulate neuronal activity through astrocytes. These studies show that LPA can regulate signaling and behavior of the major support cell of the brain and thus has strong effects on neurons in the surrounding environment.

1.3.4.5 LPA and Microglia

Microglia are the macrophages of the CNS; considered the main immune response of the brain. Microglial cells function in the brains immune response, to maintain homeostasis and to promote repair. LPA is an effective regulator of microglial functions through LPAR1,2 or 3 (Fujita, Ma, & Ueda, 2008; Möller, Contos, Musante, Chun, & Ransom, 2001). However, non-EDG receptors have yet to be identified in microglial cell cultures. LPA enhances chemokinetic migration of glial cells through calcium activated K currents (Möller et al., 2001; T Schilling et al., 2002; Tom Schilling, Stock, Schwab, & Eder, 2004). In human microglial cell cultures LPA is found to stimulate Rho and ERK pathways which are involved in inflammatory signaling pathways (Bernhart et al., 2010).

LPA signaling also enhances proliferation and metabolic activity of murine microglial cultures (Möller et al., 2001) which suggests that LPA may contribute to the immune response of microglial cells. LPA also causes microglial cells to release BDNF and upregulate ATP (Fujita et al., 2008), which suggests that the microglial response to LPA signaling are to secrete supporting factors into the environment and/or decrease neuronal activity. Similar to discrepancies in what's know of LPA signaling in neurons, many studies have also reported contradictory effects of LPA on microglial cells. However. in sciatic nerve injury, a recent study demonstrated that neuropathic pain can be attributed to LPA produced from activated microglial cells (Ma, Nagai, & Ueda, 2010). Although the effects of LPA on microglial cells is not well known its clear that

LPA mediates microglial activation and thus may play a key role in the microglial response to CNS injury.

1.3.4.6 LPA and Blood vessels (Endothelial cells)

LPA signaling is essential for overall CNS development and blood vessel formation is one of the main processed that requires LPA signaling. Autotaxin null (ATX-/-) mutation is a lethal phenotype because of the disruption in blood vessel formation (van Meeteren et al., 2006). In the adult brain, not much is known about the effects of LPA on blood vessels but LPA has been implicating in maintain the structure of blood vessels (Manning & Sontheimer, 1997; Schulze, Smales, Rubin, & Staddon, 1997) and its been shown that an increase in LPA enhances permeability of blood vessels(Gan, Yin, Peng, & Wang, 2008; On, Savant, Toews, & Miller, 2013; Schulze et al., 1997). The effects of LPA on endothelial cells and blood-brain barrier (BBB) maintenance suggests that LPA signaling is involved in angiogenesis and maintaining blood vessel structure. Furthermore, since most CNS injuries involve BBB dysfunction, these studies also suggest that LPA may mediate the pathological effects of brain injury and contribute to a disrupted BBB after injury.

1.3.4.7 LPA and progenitor cells

There is limited literature on the effects of LPA on these adult neurogenic regions. In fact, to date, there are only two papers that explore the role of LPA signaling on adult neurogenesis in vivo. Both of those papers explore the effects of LPAR1 signaling on hippocampal neurogenesis of the SGZ in LPAR1 null mice. Studies in adult mutant LPAR1 (maLPAR1-) mice showed reduced neurogenesis, less brain-derived neurotrophic factor (BDNF), and increased stress

factors like nerve growth factor (NGF) and insulin-like growth factor after exposure to enriched environment and exercise as compared to control mice (Castilla-Ortega et al., 2011; Fujita et al., 2008). This study suggests that LPA signaling may be crucial for regulating learning, memory, and how the brain processes stress. In the spinal cord, LPA signaling enhances migration of neuroblasts (Hirakawa et al., 2007) ; this study implies that an increase in LPA signaling within the brain after injury could be one of the stimulates to direct neuroblasts to the cortex. Furthermore, a later study in the same mouse model, revealed an increase in apoptosis and a decrease in proliferation of adult hippocampal NPC's as well as defects in learning and memory paradigms (F W Holtsberg et al., 1998). This study strongly implies that LPA signaling is crucial for NPC cell survival and signaling for learning and memory in the adult brain. Since its known that NPC activity in not restricted to the SGZ, these compelling studies on hippocampal adult neurogenesis strongly suggest that LPA signaling may also be crucial for SVZ neurogenesis. Although the effects of LPA on adult NPC has not been investigated, its highly probably that LPA will be shown to modulate the survival, proliferation, migration and differentiation of adult-NPCs due to the potent effect of GPCRs on stem cells (Doze & Perez, 2012).

Ex vivo studies suggests that LPA may selectively influence proliferation, differentiation and survival of the NPC niche in the adult brain (Dottori et al., 2008; Frisca, Crombie, Dottori, Goldshmit, & Pébay, 2013; Svetlov et al., 2004) as well as inhibit neurogenesis (Dottori et al., 2008). Although SVZ cells are known to respond to brain trauma by proliferating and migrating toward the injury site, no study has yet identified the effect of LPA on SVZ cell activity. LPAs' role in the adult brain, specifically post-traumatic neurogenesis, is not understood (Jaillard et al., 2005; G Tigyi, Dyer, & Miledi, 1994; Z.-Q. Zheng, Fang, & Qiao, 2004). Mitigating LPA

release shortly after injury may be a key therapeutic mechanism to encourage proliferation, survival, migration and/ or neuronal differentiation of NPCs from the SVZ and cortex. However, in order to identify the efficacy of this approach, the temporal and special changes in LPA need to be characterized and the role of LPA in adult neurogenesis after injury needs to be identified.

1.3.5 Lysophosphatidic Acid and CNS injury

LPA has pleotropic effects in development, physiology, function and pathophysiology; despite this the temporal and special profile of LPA is the adult brain is unknown especially after trauma. As highlighted in the above sections, LPA plays an important role in modulating the function of brain cells, therefore it is likely that LPA signaling has potent effects in injury. Namely, the excitotoxic microenvironment in the injured brain causes cell death, inflammation and morphological changes to cells in and around the damaged region (Harting, Jimenez, Adams, Mercer, & Cox, 2008; Krajewska et al., 2011; Kunz, Dirnagl, & Mergenthaler, 2010; Martin et al., 1998; Palmer et al., 1993). A toxic microenvironment is known to decrease survival of NPCs (Molcanyi et al., 2007; Richardson et al., 2010) except in cases with growth factor interventions(Bentz et al., 2007; X. Chen et al., 2002; Longhi et al., 2005). Lysophosphatidic acid (LPA) is a neuronal signaling molecule synthesized by three pathways, the main of which is autotaxin (ATX). LPA and ATX are suggested to significantly contribute to the toxic microenvironment of the injured brain (a E. Dubin et al., 1999; Frugier et al., 2011; Goldshmit et al., 2012; J N Keller et al., 1996; T. C. S. Spohr et al., 2008; Steiner, Urso, Klein, & Steiner, 2002). LPA and its receptors are enhanced in the injured brain (Frugier et al., 2011; Goldshmit, Munro, Leong, Pébay, & Turnley, 2010; Radeff-Huang, Seasholtz, Matteo, & Brown, 2004) and its probable that the concentrations of LPA increase in the CNS after trauma may be due to a

lack in blood-brain barrier integrity (Eichholtz et al., 1993; G Tigyi et al., 1994) or astrocytes releasing LPA around the injured region(L. Ma, Uchida, et al., 2010; Savaskan et al., 2007). Moreover, LPA, alone, has been shown to cause glial-scar-like formations in naïve cortex(Sorensen et al., 2003), induce neurite retraction and cause cell death in neuronal cultures (N Fukushima et al., 2000; Jalink et al., 1994; Saito, 1997; Sayas, 1999). Lastly, blocking LPA after spinal chord injury (SCI) significantly improves injury outcomes(Goldshmit et al., 2012). Although the role of LPA in injury pathogenesis is not well understood, the following sections will expand on the current understanding of LPAs effects on the cellular sequel of TBI

1.3.5.1 LPA increases after CNS injury.

Under physiological conditions LPA levels are low. In the systemic fluids, serum levels of LPA are between ~0.1-1μM(Baker, Desiderio, Miller, Tolley, & Tigyi, 2001) and CSF levels of LPA in human is ~0.025μM/mL and in mouse ~0.40μM/mL (Crack et al., 2014). Modulation of basal LPA levels in the systemic fluid occurs through auto inhibition of Autotaxin (L. A. van Meeteren et al., 2005). Basal LPA levels in the adult brain vary by location, the brain stem and the midbrain have the highest levels of LPA, thalamus has lower levels and the cortex and cerebellum have the lowest concentrations of LPA (S. H. Lee, Raboune, Walker, & Bradshaw, 2010). Overall, reports of LPA levels in the adult brain are no higher than 80ng/mL (Aaltonen, Laitinen, & Lehtonen, 2010; Arun K. Das & Hajra, 1989; Morishige et al., 2010; Sugiura et al., 1999). This suggests that LPA expression is highly modulated and regulated in the normal adult brain.

However, pathology disrupts the regulation of LPA in the CNS. Many studies have shown an acute increase in LPA within the systemic fluids and within the brain in both human and animal

models of CNS injury. After cerebral hemorrhage LPA levels in the CSF increase to 3μM in the rat (G. Tigyi et al., 1995). LPA is also increased in the mouse CSF after spinal chord injury (L. Ma, Uchida, et al., 2010). Studies in TBI have shown that in the mouse CCI model and in human TBI patients CSF levels of LPA increase at least 2-fold within 24 hours of injury (Crack et al., 2014). Within the rat brain after stroke its been shown that autotaxin levels increase in astrocytes neighboring the contusion (Savaskan et al., 2007), which strongly suggest the presence of LPA around the lesion. This local and acute pulse of LPA suggests that injury enhances LPA production and expression and because LPA is a potent neuromodulator this also implies that this 'pulse' may contribute to the pathophysiological effects of brain injury.

1.3.5.2 LPA enhances BBB permeability

An open BBB is consistent with moderate to severe TBI and this effect of injury causes blood leakage into brain tissue and even systemic fluids. One of the most well defined mechanisms to stimulate LPA production is in fact after platelet aggregation. This suggests that LPA in the injured brain is locally enhanced during blood clotting (Eichholtz et al., 1993; Gerrard & Robinson, 1989; Mauco, Chap, Simon, & Douste-Blazy, 1978; G. Tigyi et al., 1995). Furthermore, autotaxin, one of the main enzymes that produce LPA, is localized to the blood. Thus, this suggests that a BBB breach and subsequent blood clotting in the brain after trauma mainly contribute to the local increase in LPA in the brain after injury. However, even in the absence of injury, LPA alone can disrupt the structure and permeability of the BBB (G. Tigyi et al., 1995). In cultures of brain-derived endothelial cells, LPA was found to increase tight-junction permeability (Schulze et al., 1997) and in vivo studies have also demonstrated that enhancing LPA in the brain rapidly disrupts the BBB structure and increases permeability (Gan

et al., 2008; On et al., 2013). These studies suggest that the LPA pulse after injury has an additive effect in trauma, that increased BBB permeability enhances LPA levels, which in turn enhances BBB permeability in the CNS, effectively exacerbating the detrimental results of brain trauma. Although the effects of LPA on the BBB are evident it is still unknown how much of the local blood-derived LPA directly contributes to the pathogenesis of TBI.

1.3.5.3 LPA a pro-inflammatory cytokine

Inflammation is a well-characterized secondary injury after TBI, which consist of a complex cascade of processes cultivating reactive astrocytes, microglial aggregation, and scar formation. The glial scar formation provides protection of the entire brain from the damaged region but also inhibits axonal sprouting and new functional connections from forming in the region where gliosis has occurred. Studies in astrocyte cell cultures has shown that LPA increases astrocyte proliferation and outgrowth of processes (Ramakers & Moolenaar, 1998; Sato et al., 2011; Sensenbrenner, Devilliers, Bock, & Porte, 1980). A compelling study has demonstrated the direct role of LPA in forming the cortical glial scar in vivo very reminiscent of the scar formed after TBI. In this study, a high concentration of LPA was directly injected into the cortex of a naive mouse brain and within hours reactive astrocytes and a glial-scar like structure forming was observed in the location of LPA injection (Sorensen et al., 2003). This study suggests that the local increase in LPA after injury may directly enhance the formation of the glial scar after injury. This study was also confirmed in a model of SCI in adult zebra fish that LPA application to the transected spinal chord enhanced glial cell proliferation compared to zebrafish treated with normal spinal chord fluids (Goldshmit et al., 2012). Further studies have demonstrated that this formation is dependent on LPAR1 activity (Shano et al., 2008).

LPA also modulates microglial function and activity, and is known as a potent mediator of proinflammatory cytokines(Fourcade et al., 1995; Z. Zhang, Liu, & Meier, 2006). An increase in inflammatory cytokine levels in the brain after trauma has been correlated with poor outcomes after injury. In a model of SCI in the mouse, LPA injection increased microglial activation around the injury site compared to microglial activation in injured mice injected with artificial CSF as the control (Goldshmit et al., 2012). LPA also increased Interleukin 6 (IL-6) inflammatory cytokine (Crack et al., 2014), which is considered to be one of the main divers for the inflammatory response after TBI(Yang, Gangidine, Pritts, Goodman, & Lentsch, 2013). These study strongly suggest that LPA signaling directly modulates the inflammatory processes after injury and that modulating the LPA pulse could effectively attenuate the inflammatory response and improve recovery after injury.

1.3.5.4 LPA is neurotoxic.

One of the strongest sources of LPA in the injured brain is from the injury site where there is the largest amount of blood coagulation due to LPA being released by activated platelets. However, neurons and astrocytes are also though to be a potential source of LPA in the brain due to detection of LPA in neuronal cultures (N Fukushima et al., 2000) and studies have also shown that LPA itself can signal cells to produce and release LPA (Eder, Sasagawa, Mao, Aoki, & Mills, 2000). This implies a potential feed forward mechanism of LPA production and signaling is likely to occur and heighten its toxic effects of LPA signaling in the injured brain. Enhanced LPA signaling perpetuates the excitotoxic microenvironment of the injured brain by modulating calcium and glutamate uptake in both neurons and astrocytes (Y.-J. Chang et al., 2007; a E. Dubin et al., 1999; Frederick W. Holtsberg et al., 1997; Jeffrey N. Keller et al., 1997; Steiner et

al., 2002). This calcium rich environment leads to necrosis and apoptosis of neurons. A study in rat hippocampal cell cultures demonstrated that addition of 0.1-1μM LPA in the extracellular fluid caused a rapid and persistent increase in intracellular Ca2+; and this process was mediated by voltage gated Ca2+ channels (Frederick W. Holtsberg et al., 1997). The concentrations of LPA used to demonstrate these effects were within the appropriate concentration range detected in the CSF of injured rats (3μM) (G. Tigyi et al., 1995) and therefore its likely that neurons in vivo, especially near the injury site, will respond to these concentration. It was subsequently found that the neuronal response to 0.1-1μM LPA caused structural modifications consistent with necrosis and eventually enhanced LDH (F W Holtsberg et al., 1998)(J. J. a Contos et al., 2002). This response was exclusive to LPA in comparison to hippocampal cultures treated with similar phospholipids. This strongly suggests that LPA, at high concentrations, is directly involved in enhancing neuronal death by increasing intracellular Ca2+.

LPA intensifies the excitotoxic environment by effecting Ca2+ gradients and inhibiting glutamate uptake in astrocytes. Astrocytes exposed to 0.1-1μM LPA release Ca2+ into the extracellular space and decrease glutamate uptake(J N Keller et al., 1996; Steiner et al., 2000). Neurons rely heavy on astrocyte functionality to sequester toxic neurotransmitter signaling, like Ca2+, and glutamate. However, LPA causes a polar shift in transmitter gradients by both neurons and astrocytes; thereby increasing intracellular Ca2+ in neurons and extracellular Ca2+ and glutamate in the neuronal microenvironment. This worsens the toxic environment of the injured brain and subsequently enhances neuronal death. Therefore, the potent effects of LPA on Ca2+ gradients effectively enhances neuronal apoptosis and likely contributes to limited functional recovery after injury. In effect, decreasing the LPA pulse in the brain after injury is an attractive therapeutic mechanism to attenuate the toxic effects of LPA.

1.3.5.5 LPA and axonal injury

Axonal damage after TBI is one of the primary effects of injury that underlies functional impairments persisting in trauma patients. Axonal injury of long projected neurons occurs through retrograde degradation of axons and then programmed cell death. Erk-1 and RhoA signaling pathways mediated by LPA have been found to underlie this process (H. Zhang, Wang, Sun, Hall, & Yun, 2007). The signaling cascades that occur from the initial injury to cell death happens within a matter of days. The time course of these processes corresponds with LPA signaling after injury. Although, not much is known about the role of LPA in trauma, including its role within axonal injury, several studies in the field of neuropathic pain has identified LPA signaling potentiates symptoms. Studies have also shown that demyelination, one of the initial effects of axonal injury is mediated through LPAR1 signaling cascades (Makoto Inoue et al., 2004). LPA signaling also causes morphological rearrangements of the cytoskeleton, which include growth cone collapse (N. Fukushima et al., 1998; N Fukushima, 2002). Therefore attenuating LPA singling after TBI could prohibit further degradation of axons and thus subsequent cell loss.

1.3.5.6 LPA receptor modulation after TBI

LPA and its receptors are tightly regulated in the normal adult brain, however after CNS injury LPAR expression changes occur proximal and distal to the injury site. These changes occur within the neuronal and astrocyte populations. In the normal human adult CNS LPAR1-3 expression is low; LPAR1 is expressed along fibers of mylenated axons, LPAR2 and 3 are faintly expressed within the ependymal layer of the ventricular zones. After injury, LPAR1 expression increases in the white matter and also among reactive astrocytes; LPAR2 and 3

prominently increased in the ependymal layers and in reactive astrocytes near the injury site, LPAR3 also increased in the neuronal population near the injury site(Goldshmit et al., 2010). These studies were further supported in animal models of TBI, in the normal adult mouse LPAR1 and 2 are weakly expressed by astrocytes in the spinal cord and their levels increased following injury (Frugier et al., 2011; Goldshmit et al., 2010). These studies suggest that not only is LPA expression changing after injury but that down stream signaling molecules activating or activated by LPARs might also be changing as a result of injury. The Rho-ROCK cascade, downstream of LPAR1-3 is a key pathway involved in the pathogenesis of trauma through LPA signaling (Frisca et al., 2013; Svoboda, Moessner, Field, & Acevedo, 2004). These studies identify the prolific role of LPA receptor signaling injury pathology.

1.4 Anti-Lysophosphatidic Acid as a Therapeutic intervention

LPA has pleiotropic effects in the adult nervous system and physiological levels of LPA can be beneficial to maintain overall functionality of the CNS. However, its also abundantly clear that deregulation of LPA signaling causes harmful effects like neurodegeneration, tumorgenesis and neuropathic pain. Although the temporal and spatial profile of LPA in disease states is unknown, in the CSF LPA levels increase increases acutely under pathological conditions. Through this and many other findings, LPA is recognized as a target for many CNS diseases and many studies aim to directly reduce LPA signaling by blocking enzymes and lipids that produce LPA such as autotaxin (ATX) or by blocking activation of its receptors. One of the main pathways of LPA metabolism is in serum is by ATX and ATX inhibitor PF-8380 was used to test the therapeutic potential of blocking LPA production and was proven successful in a model of inflammation

(Gierse et al., 2010). However, PF-8380 does not target intracellular produced LPA, rendering an incomplete reduction of LPA in many disease states with an ATX inhibitor. Furthermore, ATX is necessary for normal embryonic development and therefore blocking ATX in the human may be toxic (Federico et al., 2012). LPAs other metabolic precursors also have high functional importance in the CNS and thus blocking these lipids are likely to have toxic effects. Therefore, blocking the upstream precursors to LPA will not only incompletely prevent LPA production but will likely have toxic effects.

Other studies have aimed to target LPARs in order to attenuate the effects of LPA signaling in disease. LPARs have functional redundancies in the down stream pathways activated by each receptor and a single cell can express multiple LPARs. Therefore, using an LPAR antagonist will prove difficult in determining the specific molecular pathway involved in a particular disease state. Furthermore, when a single receptor type is blocked the ligand will be forced to use another receptor subtype, which may or may not have the same downstream activators as the targeted receptor subtype. The functional redundancies in LPAR down-stream cascades complicate methods to identify ley receptors involved in pathological conditions. Another caveat of using LPAR blockers as a therapeutic intervention is that LPA receptor expression changes occur in many disease states like neurotrauma. In the rodent, LPAR1-3 expression changes after trauma both proximal and distal to the injury site(Goldshmit et al., 2010), and these changes aren't static but they change over time after trauma. Receptor changes are also evident in human neurotrauma where LPAR1-2 expression is up-regulated (Frugier et al., 2011) and these changes also fluctuate over time. Therefore, the dynamic expression of LPARs in trauma pathology convolutes receptor-specific effects of LPA signaling with LPAR antagonist under pathological conditions. Due to the pleiotropic effects of LPA in the adult CNS, its multiple metabolic

pathways, the functional redundancies in its receptors and dynamic receptor expression profiles in disease; the utility of traditional enzymatic or receptor blockers are ineffective or even toxic for clinical use. Therefore the most promising intervention to reduce the toxic LPA *pulse* in disease is to block the ligand itself.

1.4.1 Anti-LPA

Anti-LPA (B3) is a novel monoclonal antibody that targets and sequesters extracellular LPA. Anti-LPA has a high affinity for all biologically relevant LPA species with no cross reactivity with other lipid species(Crack et al., 2014). The terminal half-life of anti-LPA in the mouse through I.V. injection is 227 hours (Lpath). The permeability of anti-LPA into the injured brain may be enhanced after injury when the BBB is disrupted. This antibody has been proven successful in reducing early secondary injury markers; protecting neurons from death at early time points and enhancing functional recover in a SCI model (Crack et al., 2014; Goldshmit et al., 2012). This suggests that anti-LPA may be a successful therapeutic for CNS trauma. However the crucial window for optimal intervention is not understood and this is because the temporal spatial profile of the LPA *pulse* in the brain after trauma is unknown. Furthermore because of this gap in knowledge, the long-term effects of this LPA *pulse* are also unclear. The LPA *pulse* may be specifically targeting axons and enhancing the effects of injury through axonal pathologies or perhaps LPAs excitotoxic signaling cascade is inhibiting endogenous neural progenitors from migrating and surviving in the injury site. The role of LPA in cortical trauma remains unknown because a change LPA expression in the brain after trauma has yet to be characterized. In addition, the time course and spatial distribution of the probable increase in LPA in the brain has not been identified. Lastly, there are many studies that have shown neural

protective measure is SCI's however the dynamic environment of the injured brain has proven more challenging. To better address this challenge, the effects of Anti-LPA treatment on outcomes after cortical trauma should be identified. The studies herein will identify the temporal and spatial profile of LPA after injury, its long-term effects on secondary injury outcomes and functional recovery in cortical trauma.

1.4.2 MALDI Imaging Mass Spectrometry (MALDI-IMS)

Matrix-assisted laser desorption/ionization (MALDI) Imaging Mass Spectrometry (IMS) (MALDI-IMS) is a useful tool to detect the distribution and localization of an array of lipids and their various species within an intact tissue sample using a histology driven approach. MALDI Fourier transform ion cyclotron resonance (FT-ICR) IMS (MALDI-FTICR-IMS) is used to produce high-resolution images that represent the density of a particular lipid on tissue sections. The matrix for MALDI is made specific to the application; for lipids, 2,5-dihydroxybenzoic acid (DHB) was applied to the brain slice, then within the MS the laser triggers ionization and desorption of the sample and the tissue. Those molecules are then protonated and analyzed. Like a typical mass spectrometer, MALDI plots the relative amount of ions within a sample and plots that signal as a function of the mass to charge ration (m/z), this is called a spectra. However, with MALDI, multiple spectrum are acquired within a single tissue allowing for a visual representation of the distribution of lipids on a sample. Multiple samples were performed on the same run allowing for quantification of signal changes across treatment groups or time points after injury.

1.5 Scope of Dissertation:

Despite decades of research there is still no clinically-proven treatment for TBI (Narayan et al., 2002). The natural response of the brain to injury suggests an early molecular signal may be involved in the acute and chronic secondary-injuries that lead to limited functional recovery. LPA increases acutely after injury but there is very limited understanding of the early, local changes of LPA expression in the brain after trauma and how those changes effect function. Yet, LPA's involvement is suggested in studies of the early pathogenesis of clinical and experimental trauma (Frugier et al., 2011; Goldshmit et al., 2012; Hosogaya et al., 2008; Savaskan et al., 2007). Many studies have also demonstrated that LPA, alone, can activate an array of injury responses like excitotoxicity, inflammation, gliosis, and necrosis[4] (Greve & Zink, 2009; Prins, Greco, Alexander, & Giza, 2013; Werner & Engelhard, 2007). However, the regional and temporal specificity of these changes in the injured brain is unknown. Furthermore, the effects of decreasing the LPA pulse functional recovery after TBI is unknown. Filling this gap in knowledge is a critical step in order to understand the overall effects of LPA in brain injury progression and to identify critical periods for optimal intervention for recovery.

LPA has long been identified as a key bioactive molecule in many disease states including neurotrauma. LPA signaling is known to cause neurite retraction of NPCs (Frisca et al., 2013; Jalink et al., 1994; Kranenburg et al., 1999), necrosis of progenitor and mature neurons (a E. Dubin et al., 1999; F W Holtsberg et al., 1998; Ye et al., 2002), astrogliosis in uninjured cortex (T. C. S. Spohr et al., 2008; Steiner et al., 2002), as well as cell death at high concentrations (F W Holtsberg et al., 1998). Therefore, LPA is a potential therapeutic target in neurodegenerative disease states. Traditional efforts for therapeutic intervention in disease utilize up- or down-

stream blocking methods to attenuate LPA signaling. However, because LPA has many precursors, LPA receptors have many functional redundancies and LPA signaling manifests in many different cellular responses; traditional efforts to block LPA signaling have ineffective, if not toxic, effects as a therapeutic. Though, a novel monoclonal antibody (anti-LPA) against extracellular LPA has proven effective in models of spinal chord injury. Anti-LPA was shown effective in reducing early cell loss in cortical trauma and improving function after SCI. However the long term effects of this drug after cortical trauma is still unknown. The long-term efficacy of anti-LPA to promote sustained recovery after trauma is essential to establish usefulness as a TBI therapeutic.

Many studies in the neurotrauma field have focused on exogenous methods and molecules to enhance recovery after trauma although the NPC response from the SVZ is a promising mechanism to enhance repair. However this response, alone, is insufficient to promote full motor and cognitive recovery after injury. The limiting factor contributing to the insufficiency of the SVZ to promote recovery though neurogenesis is not understood, but is likely related to the failure of neuroblasts to survive in the injured brain and or integrate into functional networks (Arvidsson et al., 2002a; Molcanyi et al., 2007; Richardson et al., 2010; Tajiri et al., 2013; Theus, Ricard, Bethea, & Liebl, 2010). There is still a significant gap in knowledge on how the brain's endogenous response to injury can be manipulated to enhance repair. Understanding the early signaling molecules needed to enhance the NPC population, direct those newly born cells to the injury site and promote their survival at the site of injury is crucial to utilize this stem cell-like population to enhance recovery after injury. Despite strong evidence of LPAs role as an effective neuromodulator that orchestrates the NPC response after injury the role of LPA is

modulating progenitor cells in the adult brain after injury has yet to be characterized. High LPA levels are likely a major factor influencing neuroblast behavior in injured brain. Because LPA is such a potent modulator of neuronal cell types and is robustly expressed throughout the brain, LPA may be that signal limiting the migration, survival and or integration of NPCs.

Despite the plethora of effects of LPA in the brain, no study has investigated the profile of LPA in early time points after injury, or characterized the effects of blocking LPA on markers of degeneration and adult neurogenesis after cortical trauma. Based on the above evidence we postulate that **reducing the initial increase in LPA after injury will improve injury outcomes, reduce markers of neurodegeneration and enhance neurogenesis**. The studies herein aim to fill this gap in knowledge by using a well-established model of TBI in the mouse: the controlled cortical impact (CCI) model and an LPA-blocker. These studies aim to (1) characterize the temporal-spatial profile of LPA metabolism within the injured brain at early time points in order to identify targetable window of enhanced LPA release in the injured brain, (2) to attenuate that release with anti-LPA and explore the effects on axonal injury, neural degeneration, inflammation and functional recovery. Lastly, we aim to (3) determine if this treatment affects the brains endogenous neural progenitor response to injury. Taken together, the studies herein represent the first to address the role of LPA in the brains initial response to cortical injury and repair using a novel LPA blocker. The authors' hope is that the techniques and findings contained in this collection will progress the field of traumatic brain injury therapeutics by providing insight into the potent role of phospholipids in brain disease states as well as propose the usefulness of anti-LPA as an effective trauma therapeutic.

Aim 1: To identify the spatial-temporal profile of LPA in the injured brain and correlate changes in LPA metabolism with early markers of degeneration.

Aim 2: To determine if Anti-LPA treatment prevents major pathologies of injury and improves behavioral outcomes.

Aim 3: To determine if anti-LPA treatment affects the endogenous neurogenic response to injury

2. Chapter 2 - Detailed Materials and Methods

2.1 Animals: C57Bl/6 mice and Sprague Dawley rats were purchased from Charles River Laboratories and housed under NIH guidelines. All experiments were conducted in accordance with the University of California, Los Angeles, (UCLA) Chancellor's Animal Research Committee and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Adult male mice and rats at least 3 months of age were used in all experiments.

2.2 Controlled Cortical Impact (CCI) Injury: All animals were anesthetized with Isofluorane and positioned within a stereotaxic frame. Following a longitudinal skin incision, a 3mm diameter craniotomy was made centered at 0.5mm posterior to bregma and 1mm lateral to the midline in mice. Cortical injury was performed with a flat, 3mm diameter metal tip attached to the CCI device, , at 20psi (rat) or 15psi (mouse) and to a depth of 2.0 mm (rat) or 0.6m (mouse) below the dura. The skull flaps were replaced and silicone elastomer Kimik-Cast^{TM} before suturing the wound closed.

2.3 MALDI:

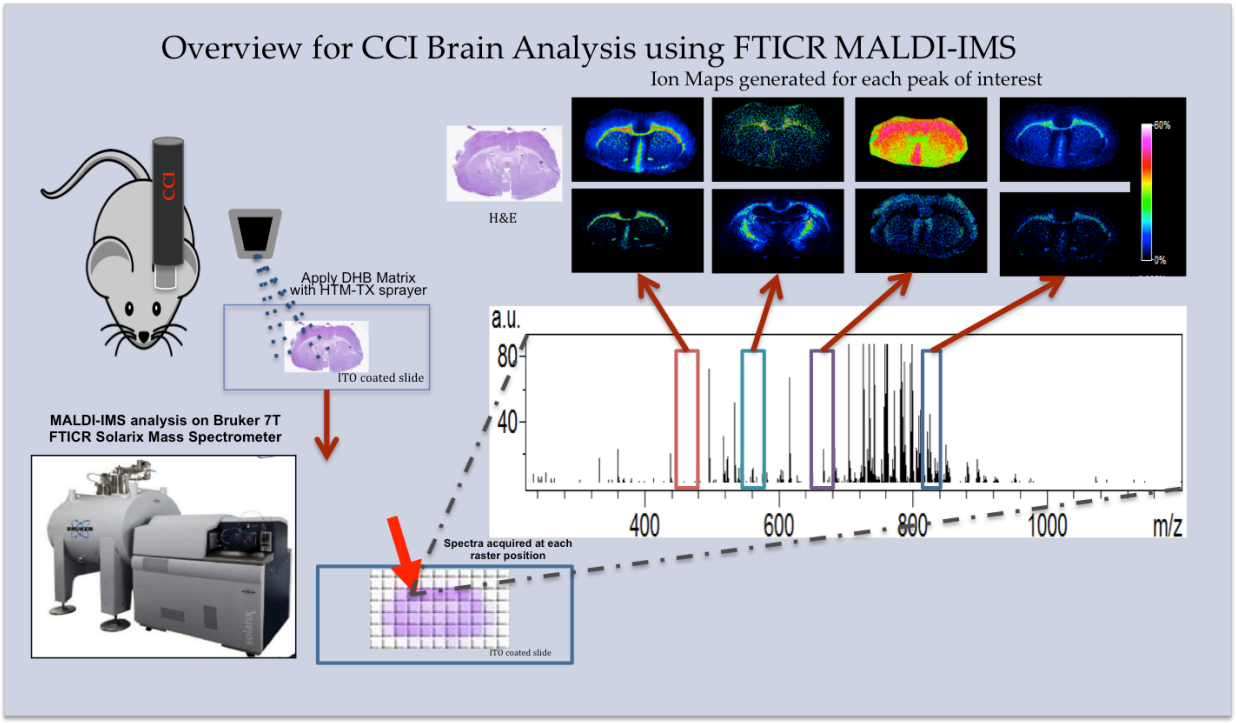

Figure 7: Overview Diagram of MALDI-IMS analysis.

2.3.1 Spectra Acquisition: Brain tissue was harvested from 3-month-old Sprague Dawley rat's: uninjured sham, and at 1 and 3 hours after CCI. The rats were deeply anesthetized with pentobarbital (50mg/kg), PBS perfused and frozen brains were sectioned at 12um and mounted on ITO coated slides. For coronal sections (n=3/ coordinate) uninjured control, 1 and 3 HPI samples were performed on the same slide and for sagittal section uninjured control and 3 HPI were run on the same slide allowing for quantification of signal changes across time points after injury Frozen slides were desiccated at room temperature for 20 min and an HTX-TM (HTX technologies) sprayer was used to add 2,5-dihydroxybenzoic acid (DHB, Sigma) to the slides. .

DHB was made at 30 mg/ml in 50% MeOH and .01% TFA. With a velocity of 950 mm/min at 30mg/mL DHB 1.684 mg/cm2 was added. Spectra were acquired across the entire tissue section on a Solarix dual source 7T FTICR mass spectrometer (Bruker Daltonics) to detect the lipid species of interest (m/z 150−1000) with a SmartBeam II laser operating at 1000 Hz, a laser spot size of 25 μm, and a raster width of 60-80 μm for general profiling for high resolution images. For each laser spot, 400 spectra were collected. Images of differentially expressed lipids were generated using FlexImaging 4.1 software (Bruker Daltonics). Following MS analysis, data was loaded into FlexImaging Software focusing on the m/z range of 150−1000 and reduced to 1 ICR Reduction Noise thresholds (**Figure 7**). All MALDI was normalized using root means square.

2.3.2 Spectra Intensity Measurements: Hematoxylin and eosin stain (H&E) stain was obtained from all adjacent sections analyzed in MALDI. All regions of interest (ROIs) were drawn in image J, based on H&E anatomy (pericontusion ROIs were determined by H&E anatomy and HEME location). ROIs were overlaid for each section (n=3 rats/ time point/ lipid spectra obtained). Spectrum intensity was determined for all ROI in Image J. All data is normalized to the uninjured control values for each slide. Normalized pixel-intensity units (n.i.u) were obtained for each lipid detected as ((spectra values for injured group - spectra values for uninjured control)/ spectra values for uninjured control). Pixel intensity maps for white matter correlation of Beta-APP and spectra were obtained in Image J with the integrated density across ROI.

2.4 Anti-LPA treatment: To test the effects of Anti-LPA treatment on outcomes, mice were randomly assigned a treatment group. 2 hours post injury mice were re-anesthetized with isoflurane, received an intravenous injection, through the penile vein of either treated with Lpathmab-m (Anti-LPA) or the isotype matched vehicle control (IgG) at 25 mg/Kg.

2.5 5-Chloro-2'-deoxyuridine (CldU) labeling:

2.5.1 Acute cell labeling paradigm: To label cells that were actively proliferating on the day of euthanasia (1, 3, or 7 days post-injury (DPI)), 5-chloro-2'-deoxyuridine (CldU, Sigma C6891; 42.5 mg/kg) was administered via intraperitoneal injection (i.p.) every 4 hours over the course of 12 hours (3 injections total); mice were euthanized 4 hours after the last injection

2.5.2 Cell Fate labeling paradigm: To track the fate of cells dividing at 1,3 and 7DPI, CldU (42.5 mg/kg) was administered via i..p injection every day, for 7 days, starting at 1DPI (7 injections total). Mice were euthanized at 14 and 28DPI. CLDU was prepared at a concentration of 5.0 mg/ml in sterile saline.

2.6 Tissue Fixation, Brain Sectioning & Immunohistochemistry: Mice were deeply anesthetized with pentobarbital (50mg/kg) and perfused/fixed with .9% PBS followed by 4% paraformaldehyde, Brains were incubated in 30% sucrose until tissue density equilibrates with sucrose solution, flash frozen with 2-methalbutane at -30. 20 μm coronal sections were cut and standard, multi-label fluorescent immunohistochemistry was performed using the following antibodies: rabbit anti-βAPP (1:100, Invitrogen #51-2700;); rat anti-BrdU (specific for CldU, 1:250, Accurate Chemicals, Westbury, NY, Clone BU 1/75), rabbit anti-DCX (1:500, Abcam,

Cambridge UK), Fluoro-Jade B (0.0004%., Histo-Chem, Jefferson Arkansas, 1FJB); goat anti-Iba1 (1:250, Abcam Cambridge UK ab5076); human anti-LPA (1:250, Lpath Inc, San Diego, CA LT3211). (See table below antibody list). Alexa-fluorophore-conjugated secondary probes (Molecular Probes, Carlsbad, CA) with fluorescent emission peaks at 633, 595 and 488nm (far-red, red & green) were used. For Beta-APP, CldU and Iba1 staining antigen-retrieval was performed, by first boiling the sections in 10mM sodium citrate for 2 minutes and incubating the slides in the same solution for 30 minutes thereafter. Permeablization was performedwith all antibodies except anti-LPA by incubating slides in 0.5% TritonX in TBS for 60 minuets. For some cases, Avidin-Biotin Complex (ABC) method was used too amplify the signal of the primary antibody with a biotinylated secondary antibody, incubation in ABC complex and then 3,3'-Diaminobenzidine (DAB).

1° Antibody	Host	Concentration	Source	Notes
CldU (anti-BrdU; Clone BU 1/75)	Rat	1:250	Accurate Chemicals, Westbury, NY (OBT-0030)	
βAPP	Rabbit	1:100	Invitrogen, Carlsbad, CA (51-2700)	*Avidin/Biotin complex with DAB in chapter 4 only
DCX	Rabbit	1:500	Abcam, Cambridge, UK (AB18723)	
Fluoro-Jade B	-	0.000004%	Histo-Chem, Jefferson Arkansas (1FJB)	
Iba1	Goat	1:250	Abcam Cambridge UK (AB5076)	*Avidin/Biotin complex with DAB
LPA	Human	1:250	Lpath Inc, San Diego, CA (LT3211)	*Avidin/Biotin complex with DAB

2.7 Stereology:

The estimated total number of double-labeled cells was determined by epifluorescence microscopy using unbiased stereology cell counts with the optical fractionator method, as implemented by StereoInvestigator software (MicroBrightfield, Williston, VT, USA) in at least 5, 20 μm sections, 240 um apart and within the ipsilateral SVZ and ipsilateral cortical grey matter region beginning rostrally at the genu of the corpus callosum. Counting regions were grossly defined under DAPI nuclear stain immunofluorescence by contouring an area extending from the junction of ventricle and dorsolateral SVZ to 100 μm medially, 100 μm ventrally and 175 μm laterally. Labeled cells in the SVZ were counted at 63x magnification using a grid size of 82 x 35 μm with a counting frame of 27 x 27 μm and were reported as estimated cell populations. Labeled cells in the cortex were counted at 40x magnification using a grid size of 380 x 380 μm with a counting frame of 130 x 130 μm and were reported as estimated cell populations. All cells were identified initially with Dapi+ nuclear labeling; only cell types co-labeled with Dapi were counted. Gundersen coefficient of error (m=1) ≤ 0.05 was used to indicate appropriate amount of sections and cells were counted for each animal (Gundersen & Jensen, 1987; Korbo et al., 1990).

2.8 Contusion Analysis: Twelve sections from each mouse, located throughout the contusion area (240μm apart) were Nissl-stained, digitally scanned and analyzed for contusion volume analysis using previous published methods (Chen et al, 2003). Contusion cortical grey mater areas were then manually outlined using ImageJ software and normalized to contralateral contusion grey matter for each section.

2.8.1 MALDI Overlap Map: HEME spectra from rat sections at the epicenter of the injury (bregma -1.34) digitally co-registered by affine transformations (Jenkinson and Smith 2001). HEME areas were then manually outlined before images were merged by summation, rescaled and colorized to represent the degree of lesion overlap.

2.9 Grid-walk:

Mice were tested on a raised, 1.1 cm wire with 20cm long and 35cm wide grid size as described by Baskin ((Baskin, Dietrich, & Green, 2003)) for 3 days consecutive days prior to injury. All behavioral tests were videotaped for offline analysis. Right-limb faults (the future affected limb) were scored as "1" by a missed foot placement on the grid resulting in limb placement below the grid to a distance of approximately the full length of the limb. Half-faults (scored as "0.5") were used to describe foot-slips when only the half, or less than half of the limb was placed below the grid. Scores were calculated from a total of 50 steps for each front- and hind limb. Behavioral testing was performed 3 days after, and then weekly from days 7, for 10 weeks thereafter CCI on spontaneously walking the length of the grid. Scores were summed for each animal; trials were averaged for each treatment group/testing day

2.10 Statistical Analysis:

For each quantitative analysis including spectrum intensities, stereology, total cell counting, and contusion volume analysis, the mean ± standard errors of at least three independent experiments ($n \geq 3$) were calculated and statistical significance tests (t-test or ANOVA with Tukey's HSD post hoc) were performed using the "R" statistical package (RStudio Inc. 2009-2012). Statistical significance (α) was set at $p \leq 0.01$ for all comparisons.

3. Chapter 3 – Study 1

Aim 1: To identify the spatial-temporal profile of LPA in the injured brain and correlate changes in LPA metabolism with early markers of degeneration.

3.1 Introduction:

Traumatic brain injury (TBI) is a major public health issue; TBI affects millions of people each year and many of those patients never fully regain their mental, physical or emotional capacities. Despite these compelling facts treatments that promote full functional recovery for these patients remain elusive. Failure to find effective treatments for TBI is attributed to the complexity of TBI pathology. The physical impact of injury causes a cascade of cellular responses that ultimately leads to cell death of local brain tissue and distal disruptions in functional areas of the brain like motor, learning, memory and emotion (Agoston, 2015; Algattas & Huang, 2013; Eapen, Allred, O'Rourke, & Cifu, 2015; a S. Morgan, 1989; Rabinowitz & Levin, 2014; Villapol, Byrnes, & Symes, 2014; Werner & Engelhard, 2007; X. Zhang, Chen, Jenkins, Kochanek, & Clark, 2005). The pathophysiological of TBI suggests an early signaling molecule is involved in initiating the cascade of cellular events that leads to functional impairment in trauma (Hasegawa, Suzuki, Sozen, Altay, & Zhang, 2011; Ostrowski, Colohan, & Zhang, 2006; Saatman, Creed, & Raghupathi, 2010; Weber, 2004).

Lysophosphatidic acid (LPA) and metabolites are phospholipids that act as potent cellular messengers (Fernandis & Wenk, 2007) with involvement in early brain injury pathogenesis; like the inflammatory processes (Adibhatla & Hatcher, 2008a) , calcium homeostasis (Fuentes,

Nadal, & McNaughton, 1999; Frederick W. Holtsberg et al., 1997; Ruehr, Zhang, & Dorman, 1997; Underwood et al., 1998) and ultimately cell death (Bratton & Henson, 2005; M.-K. Chang et al., 2004; Kadl, Bochkov, Huber, & Leitinger, 2004). Studies have also demonstrated that early after cortical and spinal chord injury phospholipid expression dynamically changes (Abdullah et al., 2014; Homayoun et al., 1997, 2000; Nakano et al., 2006, 2009). LPA signaling, in particular, causes astrogliosis (Möller et al., 2001; Shano et al., 2008), cell death (F W Holtsberg et al., 1998; Steiner et al., 2000), inflammation (Gierse et al., 2010; Z. Zhang et al., 2006) and neurite retraction. (Jalink, Eichholtz, Postma, van Corven, & Moolenaar, 1993; Gabor Tigyi et al., 1996; Yamazaki, Katoh, & Negishi, 2008). As a result, LPA is indicated as a prominent biomarker for TBI. However, LPA, like other phospholipids are also crucial signaling molecules that support cellular structure and function due to their involvement in an array of pathways (Divecha & Irvine, 1995; Fernandis & Wenk, 2007). Therefore, at physiologically low concentrations LPA is known to be neuroprotective and aid in repair mechanisms (Gotoh, Sano-Maeda, Murofushi, & Murakami-Murofushi, 2012; Panetti et al., 2001; Z.-Q. Zheng, Fang, Zhang, & Qiao, 2005). However, at higher concentrations, like those associated with disease, LPA signaling is associated with the pathologic process of trauma. Studies of CSF and blood levels of LPA have demonstrated an LPA *pulse* occurring within 24 hours of injury (Crack et al., 2014), suggesting that enhanced LPA signaling after trauma contributes to injury pathology. Attenuating LPA signaling with inhibitors of LPA precursors, LPA receptor blocker and LPA antibodies have proven successful in enhancing recovery after spinal chord injury and reducing symptoms of neuropathic pain (Dray, 2008; Goldshmit et al., 2012). However, to successfully reduce the LPA pulse as a trauma therapeutic a comprehensive understanding of the dynamics of this pulse; i.e. where this pulse is originating from, the extent of its effects and the duration, is

needed. With this knowledge we can then identify the brain regions most effected by LPA signaling and identify the critical therapeutic window for blocking LPA that will most effectively reduce pathology and enhance recovery.

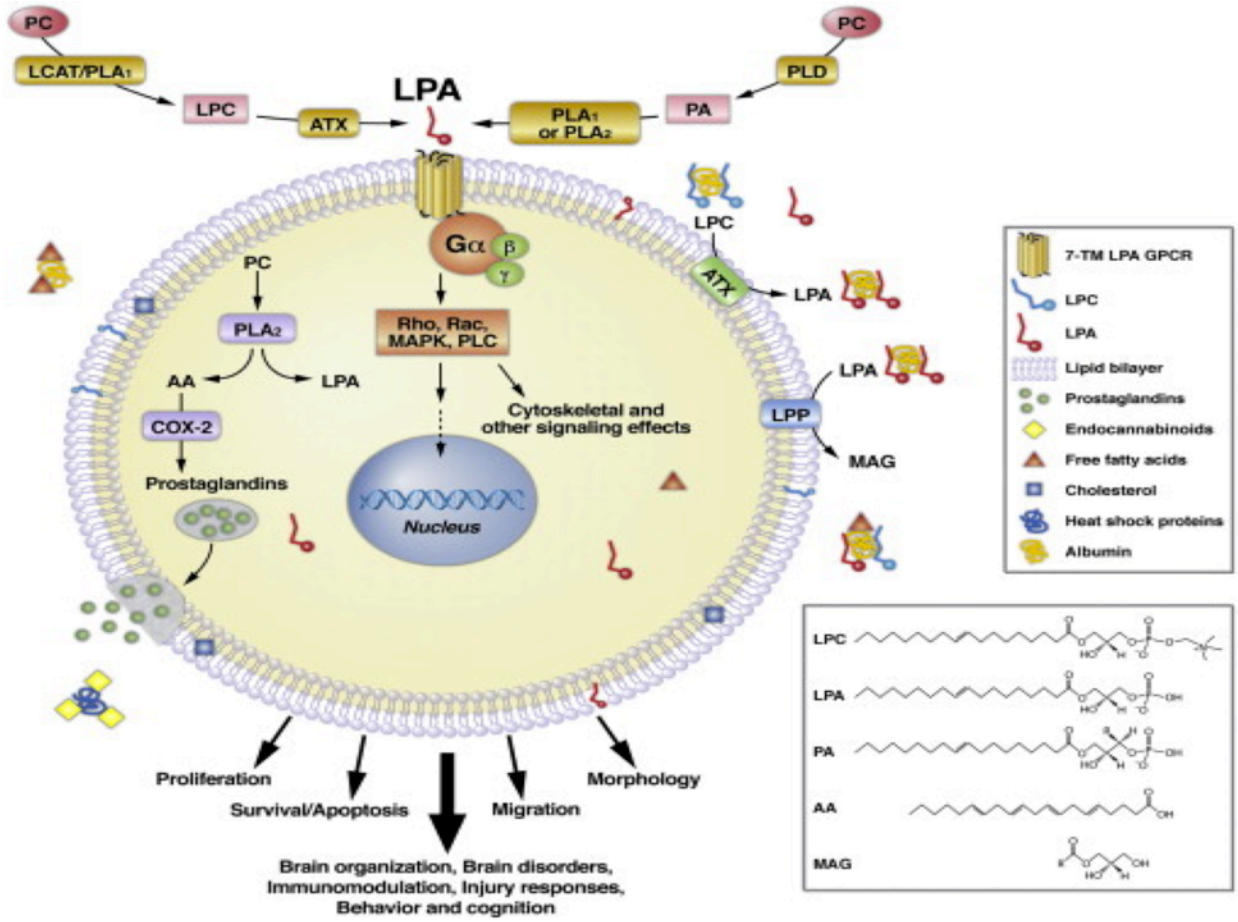

Figure 1: Summary Diagram of metabolic pathways and signaling (adapted from Yung, Stoddard, Mirendil, & Chun, 2015). The synthetic pathways for LPA include conversion of phosphatidylcholine (PC) into lysophosphatidylcholine (LPC) by lecithin-cholesterol acyltransferase (LCAT) and phospholipase A (PLA) 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). Chemical structures are shown to highlight acyl chain composition but do not reflect actual 3-D geometries. Other lipids in the CNS include the endocannabinoid family, fatty acids, cholesterol, and prostaglandins, which are beyond the scope of this Review (Yung, Stoddard, Mirendil, & Chun, 2015).

LPA is unlikely to act alone in trauma; LPAs metabolites may also influence cellular processes after trauma. LPA can be produced by two major pathways; 1) extracellular and 2) intracellular. Studies of serum, plasma, platelets, ovarian cancer and prostate cancer cells comprise the current understanding of LPA metabolism in cells and biological fluids, however cell- and tissue-specific pathways are unknown. LPAs extracellular pathway in serum and plasma is mediated by cleavage of lysophospholipids (LPLs) like lysophosphatidylcholine (LPC), lysophosphatidylserine (LPS) and lysophosphatidylethanolamine (LPE) through Autotaxin activity. In activated platelets these LPLs are produced from phosphatidylserine (PS)-specific PLA_1 (PS-PLA_1). PS-PLA_1 hydrolyzes PS and produces LPS with unsaturated fatty acids. Intracellular LPA is produce through various stimuli, including LPA itself, from the hydrolysis of phosphatidic acid (PA). PA can be generated in two ways 1) by one of the two intracellular phospholipase D (PLD) isozymes (PLD1 or PLD2) converting PC to PA and 2) by the phosphorylation of Diacylglycerol (DAG) by DAG kinase. PA is then converted to LPA by deacylation catalyzed by phospholipase A (PLA)-type enzymes such as PA-specific PLA_{1s} (mPA-PLA1 alpha and mPA-PLA1 beta) (**Figure 1**). mPA-PLA1s are highly expressed in the brain and thus the proposed intracellular pathway suggested for PA and LPA production may hold true for brain tissue (Higgs & Glomset, 1994; Nakajima et al., 2002; Tani, Mizoguchi, Iwamatsu, Hatsuzawa, & Tagaya, 1999). Since the brain is abundant in phospholipids, LPA is likely being produced and released from brain cells. However, the metabolic pathway of LPA in brain cells is unknown and thus current knowledge of intracellular LPA metabolism may not hold true for lipid metabolism after injury. Therefore, to better develop an LPA blocker as a trauma therapeutic its essential to identify the changes in LPA metabolites in the brain that may lead to further enhancement of lipid signaling.

Studies in TBI, spinal cord injury (SCI) and stroke have suggested that enhancement of lipid metabolism, particularly PA, LPC and DAG are associated with the pathogenesis of injury (Adibhatla & Hatcher, 2008b; Billah, Lapetina, & Cuatrecasas, 1981; Demediuk, Faden, Romhanyi, Vink, & McIntosh, 1988; Faden, Chan, & Longar, 1987; Homayoun et al., 2000; Horrocks, Freysz, & Toffano, 1986; J.-G. Lee et al., 2007; Lindsley & Brown, 2012; Nakano et al., 2006, 2009; Wei, Lamb, & Kontos, 1982). Clinical TBI studies have even found a positive correlation between phospholipid concentration in the CSF and patient outcomes(Pasvogel, Miketova, & Moore, 2010). However, the effect of injury on the spatiotemporal of LPAs metabolic changes is unknown. This is important because LPA alone can initiate the production of more LPA in adjacent cells (Aoki et al., 2008; Pagès et al., 2001), creating a feed-forward mechanism of LPA production and signaling in the injured brain. Despite these facts no study has yet identified if the concentration of LPA actually changes within regions distal to the injury site and how those changes are associated with known markers of injury. LPA and its associated phospholipids are essential for LPA metabolic regulation, cellular function and signaling, thus, identifying the spatial-temporal disruption in key lipids will be a crucial step in understanding their effects and roles in the pathogenesis of TBI.

Due to the structural complexity of phospholipids, analysis of their signaling and role in disease states has been neglected in comparison to protein and peptide analysis. However, advances in Matrix-assisted laser desorption/ionization (MALDI) Imaging Mass Spectrometry (IMS) (MALDI-IMS) have simplified the process of obtaining structural information and spatial distribution of molecules in tissue, so that, MALDI-IMS is now viewed as one of the tools of

choice for lipid analysis. In this study we sought to identify LPA and is metabolites spatiotemporal distribution in the brain early after TBI and to determine if those changes correlate with markers of TBI pathology, such as, axonal injury and necrosis, using MALDI IMS, lipidomics and immunohistochemistry (IHC) after unilateral brain injury in the adult rat controlled cortical impact (CCI) model. MALDI IMS analysis was used to identify dynamic changes in LPA and its metabolites at 1 hour and 3 hours after injury. Surprisingly, changes in LPA were observed in brain regions distal from the injury site, which suggests that after injury activated platelets aren't the only determinant of enhanced LPA. Changes in phospholipid concentrations in the white and grey matter of the brain suggest possible cellular metabolic mechanism. Lastly, increases in LPA signaling in the grey and white matter regions were closely associated with markers of neural degeneration and axonal damage. These findings suggest that early changes in phospholipid expression after injury are correlated to longer-term functional impairments associated with axonal injury and cell death.

3.2 Materials & Methods:

Please refer to chapter 2 for detailed methods

Animals: Methods 2.1

Controlled Cortical Impact (CCI) Injury: Methods 2.2

MALDI: Methods 2.3

Tissue Fixation, Brain Sectioning & Immunohistochemistry: Methods 2.6

Contusion Analysis: MALDI Overlap map: Methods 2.8.1

Statistical Analysis: Methods 2.10

3.3 Results:

Injury causes remote changes of lipid metabolism.

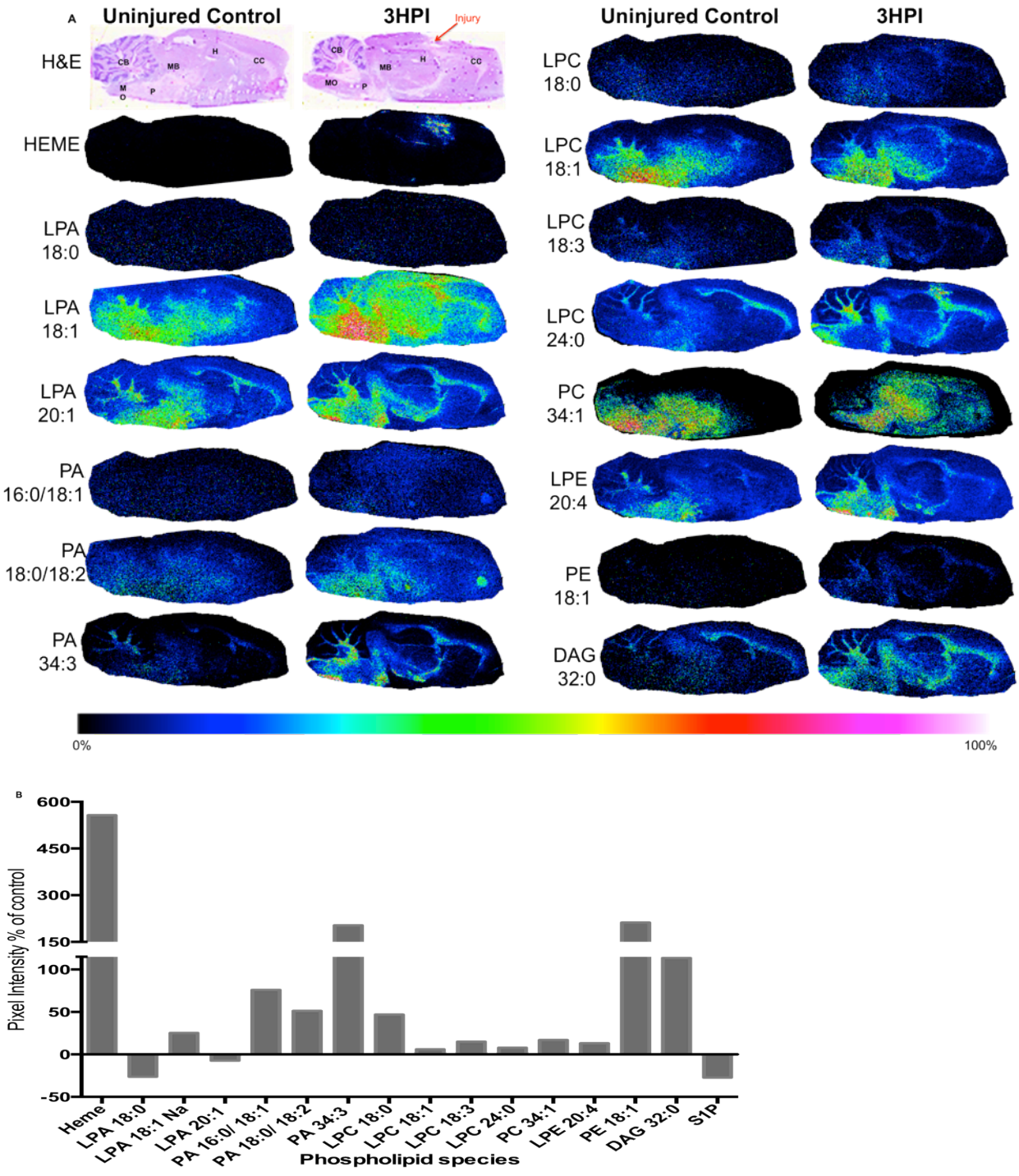

Figure 2: Injury causes metabolic changes in distal regions of the brain. Sagittal sections of uninjured control and 3HPI rats were obtained to demonstrate the brain-wide change in lipids after injury. H&E sections from adjacent sagittal

sections highlight the brain regions: injury site (**red arrow**); corpus colossus (**cc**); midbrain (**mb**); pons (**p**); medulla oblongata (**mo**); hippocampus (**h**); cerebellum (**cb**). Images of select lipids demonstrate the dynamic changes in lipids throughout the brain (**A**). Brain wide pixel intensity measurements were obtained and graphed as percentage change from control (**B**) n=1/group.

Lipid metabolism is essential for cell function however, the brain-wide changes in lipids after injury is not understood. Since lipids are potent signaling molecules, we hypothesize that changes in phospholipid expression, and consequently signaling will occur throughout the brain, but especially within primary injury regions controlling motor, emotion and cognitive function. To identify brain-wide changes in phospholipids, sagittal sections from anesthetic control (uninjured control) and sagittal sections on the ipsilateral hemisphere of 3 hours post-controlled cortical impact (CCI) rats (HPI) were acquired. H & E sections were obtained to determine the location of key brain structures. MALDI spectra were obtained in positive ion mode to detect cell surface and extracellular lipids. The cerebellum (CB) had robust enhancement of LPA 18:1, 20:1 and LPC 24:0 after injury (**Figure 2A**). Robust changes are also observed in the corpus callosum (CC), midbrain (MB), medulla oblongata (MO) and hippocampus (H). Phospholipid signaling is pronounced throughout the white matter tracts of the brain. Pixel intensity measurements determined brain-wide changes in lipid species between uninjured control and 3HPI, with enhancement in LPA species and precursors (**Figure 2B**). Results from sagittal sections suggest that enhancement of LPA and metabolites within the white matter and cerebellum may be associated with impairment of function that these areas modulate.

Injury enhances LPA and metabolites in the injured cortex

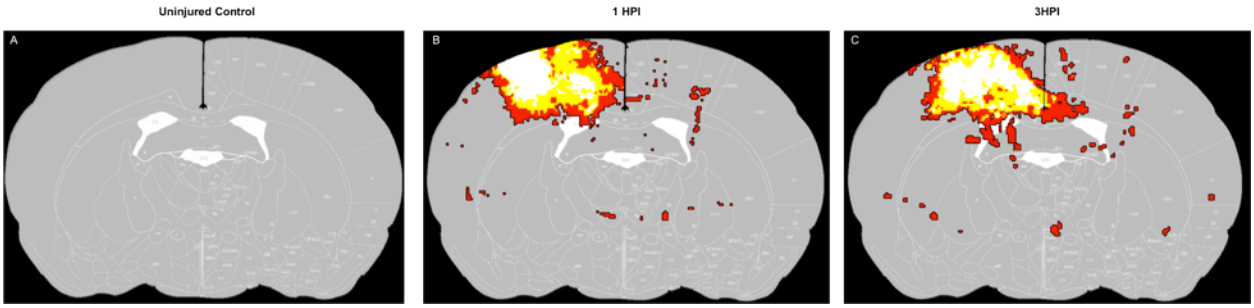

Figure 3: Overlap map of HEME signal indicates distribution of blood in the brain at the injury epicenter. The heme signals (n=3/ group) of the uninjured control (**A**), 1 HPI (**B**) and 3 HPI (**C**) displayed over cortical rat atlas at -1.3mm posterior from bregma.

LPA's primary metabolic pathway in the brain after injury is thought to be through activated platelets from the disrupted blood-brain barrier (Eichholtz et al., 1993). To determine the extent of LPA from blood one must demonstrate the spatial and temporal changes of blood in the brain after injury. To identify these changes, coronal sections through the epicenter of the injury (-1.34 mm posterior from bregma) of 1 and 3 (HPI) rats were acquired. MALDI spectra were obtained in positive ion mode to detect cell surface and cytosolic lipids. The HEME signal overlap map displays the distribution of blood within the brain, and show that within 3 HPI blood is mostly present in the cortical grey matter at the injury site (**Figure 3**) for both 1 (**Figure 3B**) and 3 (**Figure 3C**) HPI. The data obtained from these spectra and pixel intensity measurements of HEME between time periods reveal an enhancement of blood within the brain after injury 1 (9.07 ± 2.07n.i.u) and 3 HPI (13.39 ± 3.74 n.i.u) (**Figure 4D**) as compared to uninjured control.

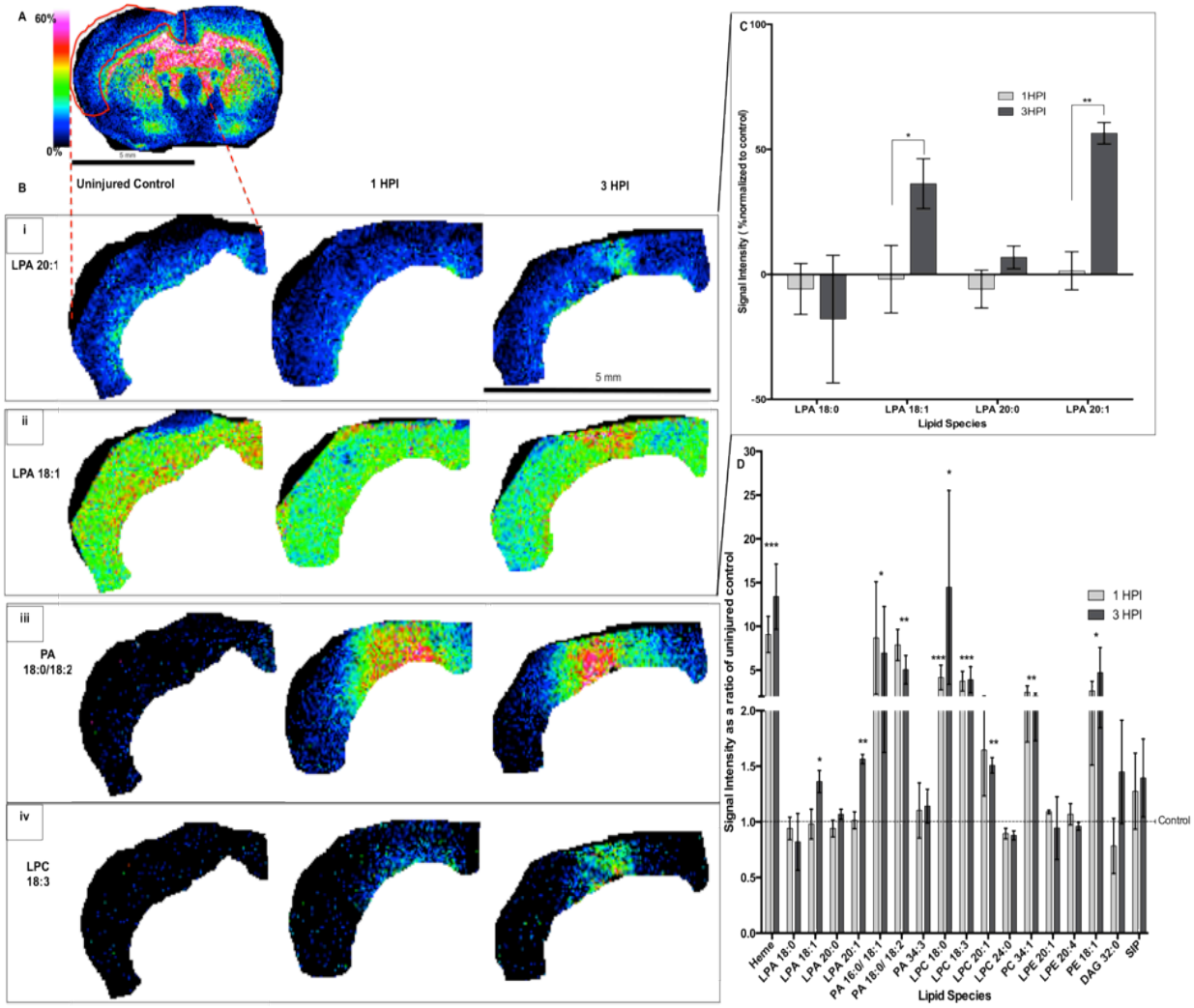

Figure 4. LPA and metabolites are increased in the ipsilateral cortex after injury.

At 1 and 3 hours after CCI rats MS spectra was obtained in positive ion mode from 9 rats (n=3/ time point). Representative image and cortical ROI of MS spectra on cortical section of uninjured control (**A**), and representative MS spectra of cortical ROIs of control, 1 HPI and 3 HPI (**B**) for lipid species LPA 20:1 (**Bi**), LPA 18:1 (**Bii**), PA 18:0/18:2 (**Biii**) and LPC 18:3 (**Biv**). Image J was used to measure pixel intensity of cortical ROIs for each lipid detected, data is represented as a ratio of control pixel intensity and p-values are measured as a difference from the uninjured control pixel intensity value for each lipid (**D**), Pixel intensity changes in LPA species is represented as a percentage change from control and p-values are measured as a difference in pixel intensity between 1HPI and 3HPI (**C**). Results are mean +/- SEM (n-3/group), P-values *P<0.05, **P<0.001, ***P<0.0001 from two-tailed t-test, 95% confidence interval in R. Scale bars: 5mm (**A&B**); 50μM (**Biia-c**).

However, within the time periods demonstrated in this study the blood infiltration is concentrated near the site of injury and is not very diffuse throughout distal brain regions. This suggests that changes in phospholipid expression should be localized to the regions in which the HEME signal is detected. Therefore, pixel intensities for LPA and precursors were obtained from ipsilateral cortical grey matter region of interest (ROI) (**Figure 4A**) and all data was normalized to internal uninjured control values (1). The data identified unsaturated LPA species 18:1 (1.3 ± 0.09) and 20:1 (1.5 ± 0.04) significantly increased in the cortex at 3HPI as compared to uninjured control (**Figure 4C-D**), whereas the saturated LPA species 18:0 and 20:0 did not significantly change from uninjured control. PA, is known as an LPA precursor in the intracellular pathway (**Figure 1)** and PA species 16:0/ 18:1 is significantly increased at 1HPI (8.6 ± 6.41 n.i.u.) as compared to uninjured control (1) whereas PA 18:0/ 18:2 is significantly increased at both 1 (7.8 ±1.78 n.i.u) and 3 HPI (5.0 ± 1.63 n.i.u) as compared to uninjured control (**Figure 4B-D and Table 1**). LPC is known as the extracellular precursor to LPA (**Figure 1);** for saturated LPC species 18:0 has significant enhancement at 1 HPI (4.1 ± 1.40 n.i.u) whereas LPC 24:0 is significantly reduced at both 1 (0.8 ±0.04 n.i.u) and 3HPI (0.8 ± 0.041 n.i.u) (**Figure 4D and Table 1**). Unsaturated LPC species 18:3 is significantly enhanced at both time points (3.7 ±1.12; 3.9 ±1.48 respectively n.i.u) and LPC 20:1 are significantly enhanced at 3 HPI (1.5 ±0.06) (**Figure 4B & D and Table 1**). Unsaturated species of PC 34:1 and PE 18:1 is enhanced at both time points after injury as compared to uninjured control (**Figure 4D and Table 1**). DAG is known to be enhanced within the cortex after injury in association with activate microglia (Homayoun et al., 2000; Nakano et al., 2006, 2009) and MS data show that cortical DAG 32:0 is increased at 3HPI, although not significantly, as compared

to uninjured control (**Figure 4D & Table 1**). The data shows that within the ipsilateral cortical grey matter LPA and associated phospholipids increase with injury, and therefore it's likely that lipid signaling has increased in the brain as well (González de San Román et al., 2015). Furthermore, both intracellular and extracellular LPA precursors are up regulated in the ipsilateral cortex, signifying that both pathways for LPA production are occurring in the injured brain. The evidence also shows that the most significant changes are occurring in LPA's unsaturated species 18:1 and 20:1 (**Figure 4C**). Since unsaturated LPA has more potent effects on cellular function than saturated LPA (Koji Bandoh et al., 2000; Hayashi et al., 2001; Yoshida, 2003), enhancement of these species in particular within the injured cortical grey matter is likely to have robust effects on cells.

Peri-contusion cortex has dynamic changes in lipids that are closely associated with markers of necrosis.

Blood is one of the primary sources of LPA, however changes in LPA metabolism in cortical regions not affected by blood infiltration is unknown. Correlation of these changes to markers of cell death is also unknown. Furthermore, LPA signaling enhances blood brain barrier permeability (Gan et al., 2008; On et al., 2013; Schulze et al., 1997), therefore increased LPA metabolisms in the peri-contusonal cortex is likely to be associated with enhanced blood infiltration in the brain and is suggestive of further damage to surrounding tissue.

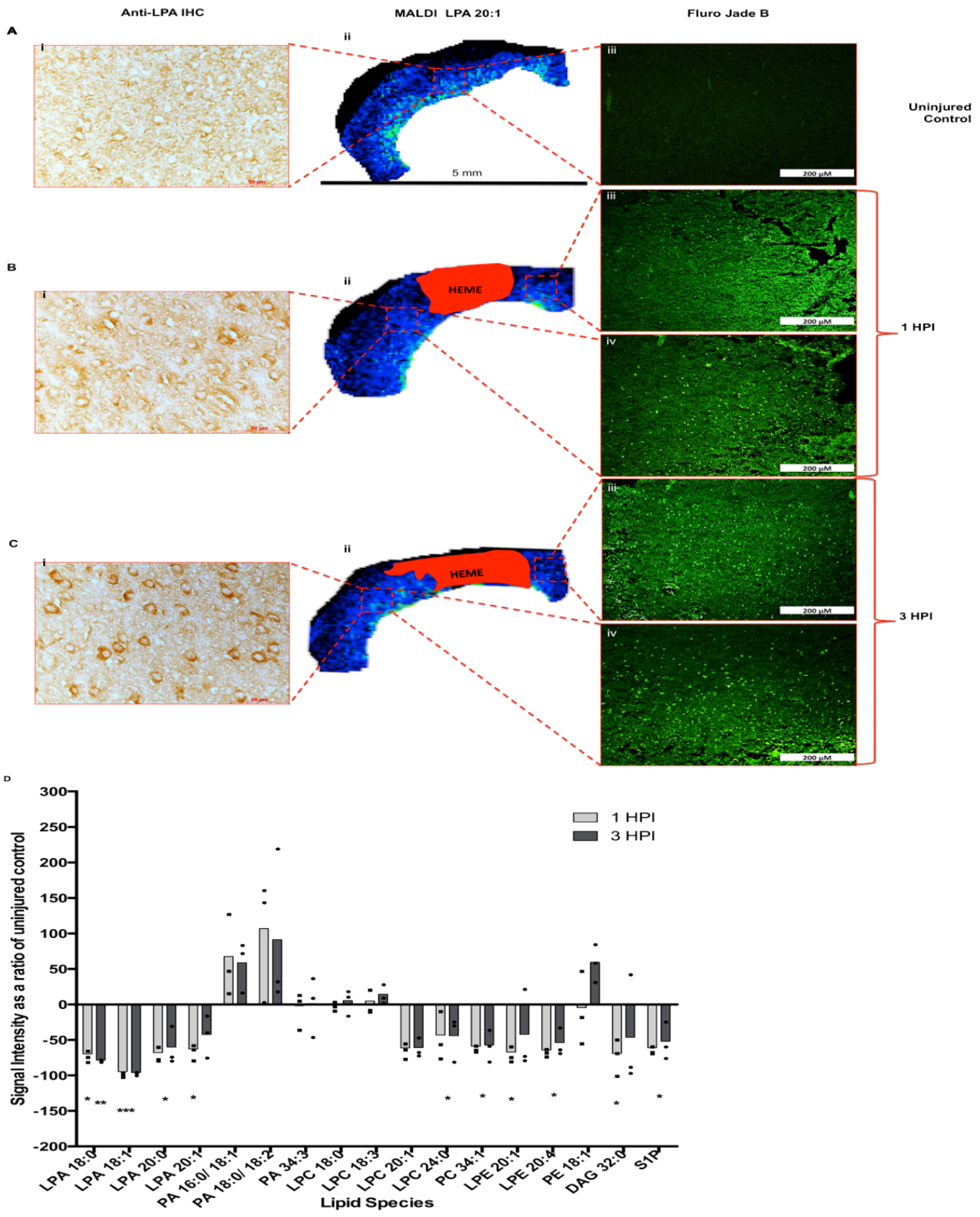

Figure 5: The peri-contusion cortex has reduced LPA species detected in MALDI as compared to control but increase in LPA IHC and strong correlations between markers of necrosis. Pixel intensity measurements of LPA and its metabolites were taken from ipsilateral cortical regions without HEME for uninjured control (**Aii**); 1 HPI (**Bii**); 3 HPI (**Cii**). Standard immunohistochemistry with DAB was used to detect cytoplasmic LPA with a humanized anti-LPA

antibody in uninjured control (**Ai**), 1HPI (**Bi**) and 3HPI (**Ci**) in adjacent sections from the same animals used for MALDI spectra. Markers of necrosis, identified by Fluro-Jade B staining, were detected in same regions of anti-LPA IHC staining as well as LPA spectra for uninjured control (**Aii**); 1 HPI (**Biii-iv**); 3 HPI (**Ciii-iv**). Pixel intensity changes in LPA and its metabolites is represented as a percentage change from control and p-vaues are measured as a difference in pixel intensity between injury time points and uninjured control (**D**). Results are mean +/- SEM (n-3/group), P-values *P<0.05, **P<0.001, ***P<0.0001 from two-tailed t-test, 95% confidence interval in R. Scale bars: 5mm (**Aii**); 200μM (**Aiii, Biii-iv & Ciii-iv**); 50 μM (**Ai**, **Bi** & **Ci**).

To identify changes in LPA metabolism in the peri-contusion cortical grey matter, pixel intensity measurements were obtained from the same cortical regions in the previous experiment (**Figure 4)** but with a modified ROI to omit the region in which HEME signal was present. This analysis showed that all LPA species and many precursors were significantly reduced at both time points after injury (**Figure 5D**). Interestingly, results for LPA precursors, PA species 16:0/18:1, 18:0/18:2 and 34:3, LPC 18:0 and 18:3 species show a robust spread in data, trending toward increased expression as compared to uninjured control (**Figure 5D and Table 1**) suggesting that these suggesting that 1) these species may be a more sensitive marker of injury variability between animals or 2) that intracellular precursors PA may indicate further enhancement of LPA from intracellular stores at later time points. The reduction of LPA in the peri-contusion region and the trending increase in intracellular metabolic precursors suggests the potential for LPA enhancement at later time points, not captured in this study.

MALDi only captures lipids in the extracellular and surface of cells. Therefore, to look at intracellular changes in LPA in the same regions, adjacent brains sections from the same animals used for MALDI analysis were obtained; sections were permeablized (to target intracellular LPA antigens) and stained by standard immunohistochemistry (IHC), using a humanized

antibody for LPA to detect intracellular changes in LPA. Anti-LPA IHC revealed a robust increase in cellular LPA within the peri-contusion cortex at 3 HPI (**Figure 5 Ai, Bi & Ci**). To determine if the enhancement of LPA is associated with markers of necrosis, standard histological staining of Fluro-Jade B (FJB) was used to detect necrotic cells in the same regions measured for MALDI and Anti-LPA IHC. In the uninjured cortex there in little to no FJB staining (**Figure 5Aiiiʃ,** at 1HPI FJB intensity increases (**Figure 5Biii-iv**) and at 3 HPI FJP intensity increases (**Figure 5Ciii-iv**) within the peri-contusion cortex as compared to uninjured control cortical regions. IHC and histology showed an increase in cellular LPA at 3HPI and directly correlated this increase with markers of necrosis in the cortical grey matter without blood.

Intracellular LPA precursor expression precedes expression of neurodegenerative markers in thalamus.

The previous section demonstrated, in the peri-contusion cortical grey matter, that there is a marked reduction in pixel intensities of phospholipids except all of the PA species, PE 18:1 and LPC 18:0 and 18:3, suggesting that these lipids may be sensitive measures of cell stress and thus predictive of further injury to come. Furthermore, studies have shown early changes in cell stress in the thalamus and have correlated this changes with disruption of the thalamocortical pathways after cortical injury (Holschneider, Guo, Wang, Roch, & Scremin, 2013; Pleasant et al., 2011).

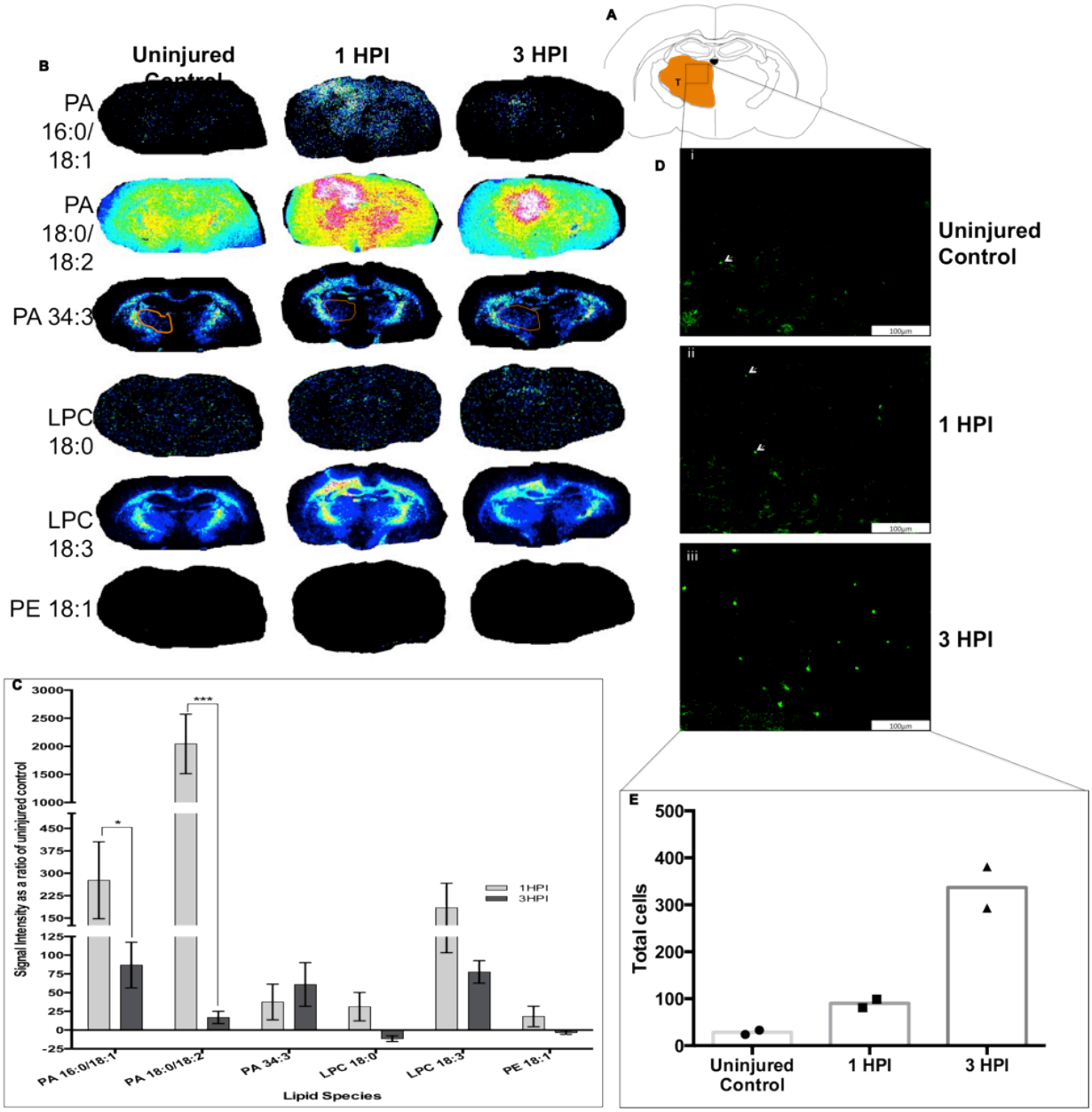

Figure 6 Intracellular LPA precursor expression precedes expression of neurodegenerative markers in thalamus. Pixel intensity measurements of select LPA precursors (**B**) were taken from ipsilateral thalamic regions of interest (**A**) from uninjured control; 1 HPI and 3 HPI. Pixel intensity changes in select LPA precursors (**B**) is represented as a percentage change from control and p-values are measured as a difference in pixel intensity between injury time points for each lipid (n=3/group) (**C**). Standard immunohistochemistry was used to markers of necrosis, identified by Fluor Jade B staining, in adjacent sections in the thalamus of uninjured control (**Di**); 1 HPI (**Dii**); 3 HPI (**Diii**); total thalamic FJB counts for each time point (n=2 rats/ group) (**E**). Results are mean +/- SEM, P-values *P<0.05, **P<0.001, ***P<0.0001 from two-tailed t-test, 95% confidence interval in R. Scale bars 100μM (**D**).

Therefore, to determine if LPA precursors are associated with thalamic pathologies at 1 and 3 HPI, coronal sections at -2.0mm posterior to bregma (from the same animals used in the previous study) were obtained. Pixel intensity measurements were taken from the thalamus ROI (**Figure 6A**) of uninjured control, 1 and 3 HPI for PA 16:0/18:1, 18:0/18:2, 34:3; LPC 18:0, 18:3 and PE 18:1 (**Figure 6B**). MALDI spectra revealed a significantly higher expression of PA 16:0/18:1 and 18:0/18:2 at 1 HPI (276.34 ±128.60 and 2043.14 ±528.75 respectively) compared to 3 HPI (86.8 ± 30.50 n.i.u and 16.7 ± 8.31 n.i.u respectively) (**Figure 6C**). PA 34:3 and LPC 18:3 significantly increased at both time points as compared to uninjured control in the thalamus (**Figure 6C**). LPC 18:0 and PE 18:1 increased at 1 HPI (31.1 ±18.95 n.i.u and 18.1 ±16.61 n.i.u respectively) and decreased at 3 HPI (-11.6 ±3.68 and -3.3 ±2.3 respectively n.i.u) as compared to uninjured control (0) (**Figure 6C**). To determine if there is a correlation at later time points with markers of neural degeneration, standard IHC was used to detect FJB staining in adjacent section and FJB positive cells were counted in uninjured control (**Figure 6Di**), 1 HPI (**Figure 6Dii**) and 3 HPI (**Figure 6Diii**). FJB staining was significantly enhanced in the thalamus at 3HPI (**Figure 6E**). This suggests that the precursors enhanced at 1HPI could possibly be an early marker of cell stress and neural degeneration that occurred at 3HPI.

Changes in white matter lipids are associated with markers for axonal injury

Functional impairment after cortical injury is due in part to diffuse axonal injury (Blumbergs, Jones, & North, 1989; Povlishock, 1992; Rand & Courville, 1946). Axons within the corpus callosum (cc) are particularly vulnerable to processes of axonal degeneration and contralateral motor impairments occur due to disruption of signal processing through the CC and other distal white matter tracts (Huh, Franklin, Widing, & Raghupathi, 2006). Early signs of cell death and markers of axonal injury can predict long-term functional impairments. However, dynamic changes in phospholipid metabolism and expression has not been described in the white matter or associated with know white matter pathologies. To identify white matter changes, lipids pixel intensity measurements of MALDI spectra were obtained from the ipsilateral corpus callosum ROI (**Figure 7C**) for uninjured control, 1 and 3 HPI from coronal sections at the epicenter of the injury (-1.34mm to bregma). There was no significant change in LPA species within the white matter as compared to uninjured control pixel intensities (0) (**Figure 7H**). However, there was significant increase at 1 and 3 HPI of intracellular LPA precursors PA 16:0/18:1 (11.4 ±6.45 and 4.6 ±2.68 respectively n.i.u) 18:0/18:2 (9.6 ±0.199 and 6.7 ±0.50 respectively n.i.u) (**Figure 7A,D, H and Table 1**). There was also a significant enhancement of PC 34:1, PE18:1 and LPC 18:0, 18:3, and 20:1 at both time points as compared to uninjured control (**Figure 7H and Table 1**). Results show that, similar to lipid changes described in the peri-contusional cortex, there was also an enhancement of intracellular LPA precursors at 1HPI in the white matter (**Figure** 7D) suggesting the potential for LPA to be enhanced from intracellular stores in the white matter at later time points after injury. To determine if markers of axonal injury are similarly altered within the corpus callosum, standard IHC with anti-Beta APP as a marker for

axonal injury was used in adjacent sections of the same animals used for the MALDI analysis. Beta-APP staining was enhanced in ipsilateral corpus callosum at both 1 and 3HPI as compared to uninjured control (**Figure 7E-G**).

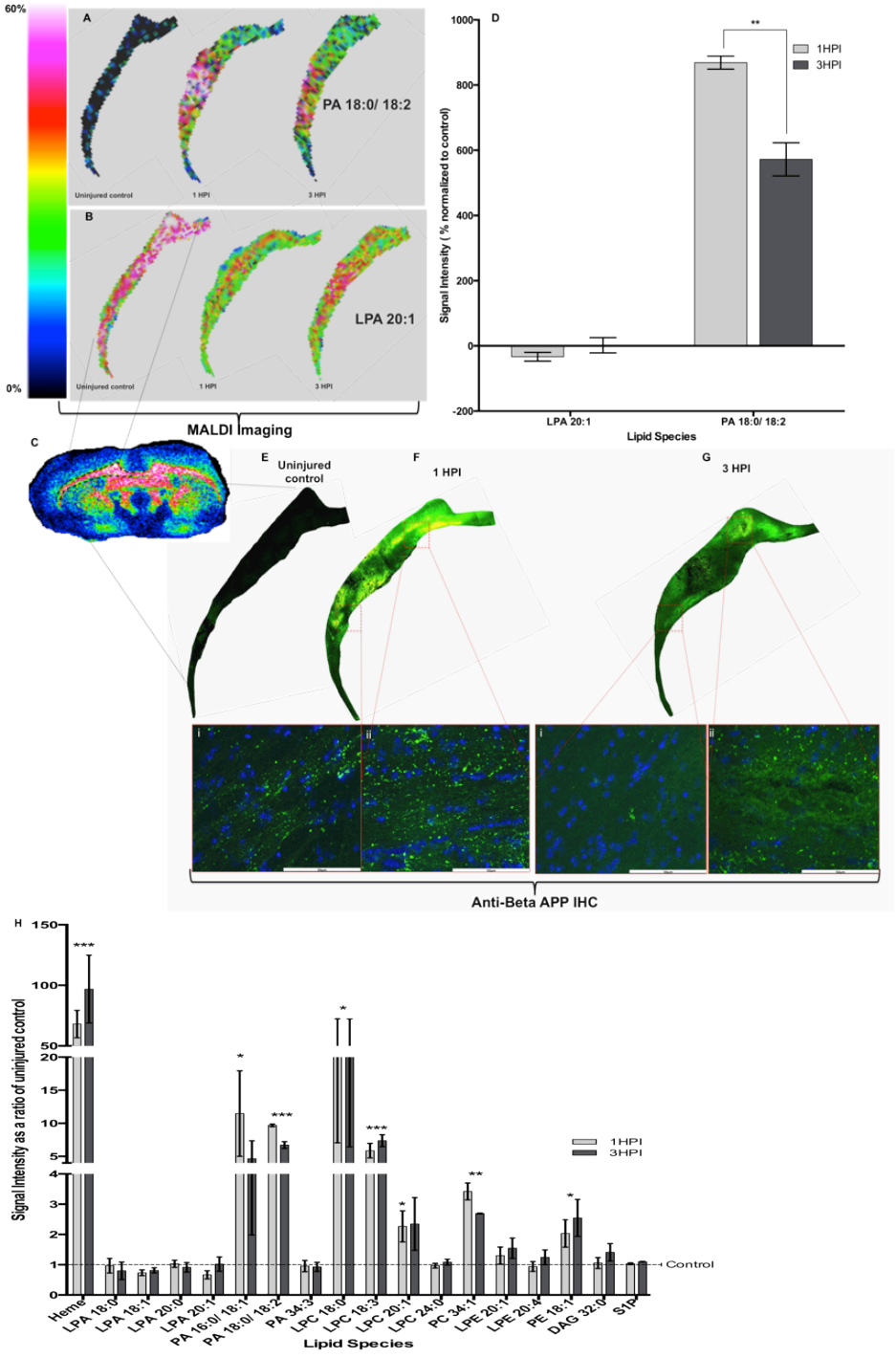

Figure 7 Injury causes a significant enhancement in LPA metabolites in the white matter and LPA changes within the white matter correlate with markers of axonal injury. Pixel intensity measurements were taken for ipsilateral white matter regions (**C**) for LPA and its metabolites. PA 18:0/18:2 (**A**) and LPA 20:1 (**B**) white matters regions from control, 1HPI and 3HPI were selected to demonstrate the acute changes in lipid metabolites within the region. The percentage change in pixel intensity at 1HPI and 3HPI, normalized to uninjured control pixel intensity for select lipids, p-

values are determined as significantly different from control values (**D**). Markers for axonal injury were identified through standard IHC for anti-Beta APP in adjacent section of the same animals used to obtain lipid spectra in MALDI (**E-G**). Insets show extent of axonal injury in the 1HPI (**Fi-ii**) and 3HPI (**Gi-ii**). All lipid species from MALDI in the white matter were graphed and displayed as a ratio of control; p-values demonstrate the significance between pixels after injury to control pixel intensities (**H**). Results are mean +/- SEM (n-3/group), P-values *P<0.05, **P<0.001, ***P<0.0001 from two-tailed t-test, 95% confidence interval in R. Scale bars: 50μM (**Fi-ii** & **Gi-ii**).

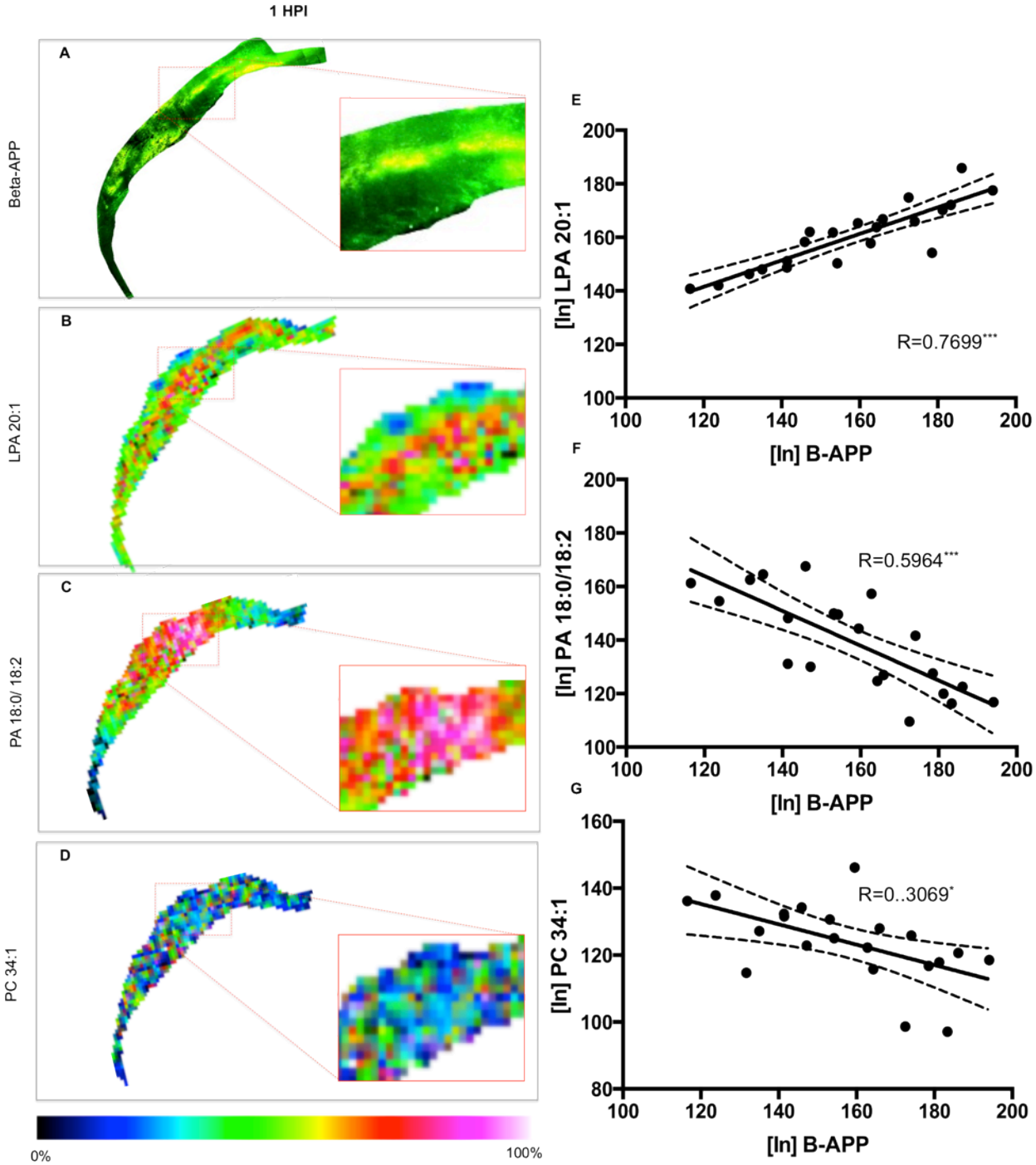

Figure 8. Spatial distribution of axonal injury markers correlate with specific LPA species and precursors. Pixel Intensity maps for LPA 20:1 (**B** & **E**), PA 18:0/18:2 (**C** & **F**) and PC 34:1 (**D** & **G**) against Beta-APPs' (**A**) spatial distribution at 1HPI had significant positive (**E**) and negative (**F** & **G**) correlation coefficients for spatial distribution and intensities of pixels. R-squared values (R=); Pixel n=21 for all correlations; P-values; * ≤ 0.01; ** ≤ 0.001; *** ≤ 0.0001.

Although the changes in LPA species within the CC were not significantly different than uninjured control, analysis revealed distinct changes in spatial distribution of LPA 20:1, PA 18:0/18:2 and PC 34:1 at 1 HPI (**Figure 8**) and a similar distribution of Beta-APP staining. To determine if there is a spatial correlation between phospholipid species LPA 20:1 (**Figure 8B**); PA 18:0/18:2 (**Figure 8C**); PC34:1 (**Figure 8D**); with the spatial distribution of Beta-APP (**Figure 8A**) pixels in the corpus callosum, pixel intensity maps were acquired from the same regions. Pixel intensities intensity distributions were plotted against beta-APP pixel distributions and the data show significant correlations of Beta-APP with these lipids (**Figure 8 E-G**) but not a significant correlation coefficient with other lipids in the white matter (data not shown). This suggests that changes in LPA and metabolites within the white matter are closely associated with markers of axonal injury.

m/z	Phospholipid class	Adduct	+ Ion	Cortex		Peri-Contusion Cortex		White Matter		Saggital (Brain-wide)
				1 HPI	3 HPI	1 HPI	3 HPI	1 HPI	3 HPI	3 HPI
616.16	HEME	na	na	***	***	na	na	***	***	
477.222	LPA	18:0	K+	-	-	*	**	-	(ns)	
459.2482	LPA	18:1	Na+	-	*	***	***	(ns)	(ns)	
590.3426	LPA	20:0	K+	-	-	*	*	-	-	*(n.d.)*
503.29	LPA	20:1	K+	-	**	*	(ns)	(ns)	-	
713.443	PA	16:0/18:1	K+	*	(ns)	(ns)	(ns)	*	(ns)	
739.46	PA	18:0/18:2	K+	**	**	(ns)	(ns)	***	***	
709.49	PA	34:3	K+	(ns)	(ns)	(ns)	-	-	-	
562.32	LPC	18:0	K+	***	*	(ns)	(ns)	*	*	
560.31	LPC	18:1	K+	*(n.d.)*	*(n.d.)*	*(n.d.)*	*(n.d.)*	*(n.d.)*	*(n.d.)*	
540.26	LPC	18:3	Na+	***	***	(ns)	(ns)	***	***	
588.52	LPC	20:1	K+	(ns)	**	(ns)	(ns)	*	(ns)	*(n.d.)*
630.448	LPC	24:0	Na+	(ns)	(ns)	*	*	-	-	
798.5	PC	34:1	K+	**	**	*	*	**	**	
54634	LPE	20:1	K+	(ns)	-	*	(ns)	(ns)	(ns)	*(n.d.)*
524.26	LPE	20:4	Na+	-	(ns)	*	*	-	(ns)	
518.26	PE	18:1	K+	(ns)	*	(ns)	(ns)	*	*	
607.5574	DAG	32:0	K+	(ns)	(ns)	*	(ns)	-	(ns)	
379.3	S1P	na	na	(ns)	(ns)	*	*	-	-	

Table 1 Phospholipid species detected in specified regions of interests. Mass to charge ratio (**m/z**); Phospholipid class: hemoglobin (**HEME**), Lysophosphatidic acid (**LPA**), Phosphatidic acid (**PA**), Lysophosphatidylcholine (**LPC**), Phosphatidylcholine (**PC**), Lysophosphatidylethanolamine (**LPE**), Phosphatidylethanolamine (**PE**), Diacylglycerol (**DAG**) and Sphingosine-1-phosphate (**S1P**). Adducts represented as carbons chain length: number of double bonds; Spectra acquired in positive ion mode, potassium (**K+**) and sodium (**Na+**). For each region of interest (Ipsilateral **cortex**, **peri-**

contusion cortex and ipsilateral **white matter**) N= 3/ group/ time period; P-values for pixel intensities increase (**green**) or decrease (**red**) from control value pixels were determined by bootstrapping the 95% confidence intervals around the median and 2-way Anova for each time period and each ROI in R. P-values; 0.01 < (**ns**) ≤ 0.05; * ≤ 0.01; ** ≤ 0.001; *** ≤ 0.0001. Pixel intensities for a given phospholipid that did not change from control value (p-value > 0.05) are indicated by (-) in grey box and spectra not detected for a given lipid (***n.d.***) indicated the phospholipid species that were not gathered in the MS run. Sagittal, brain-wide, changes are indicated by pixel intensity differences between sagittal ROI of control and sagittal ROI of 3 HPI (n=1/group).

3.4 Discussion

LPA is a candidate biomarker for traumatic brain injury (Crack et al., 2014); however, the direct impact of LPA metabolism on functional areas of the brain remained unexplored until now. The study herein provides a direct correlation between phospholipid changes to markers of neural degeneration and axonal injury in key functional areas of the injured brain.

Phospholipid metabolism as a marker of diaschisis after TBI

Diaschisis occurs in the brain after traumatic brain injury and changes cell metabolism in distal regions, these changes are often associated with long-term functional impairments (Holschneider et al., 2013; Hovda et al., 1995; Nishibe, Barbay, Guggenmos, & Nudo, 2010; Stein & Hoffman, 2003). This study provides early evidence of phospholipid changes in remote regions of the brain after injury with the most pronounced expression in white matter tracts throughout the brain, particularly the cerebellum (**Figure** 2). Studies have demonstrated enhanced phospholipid metabolism causes cell death in variety of neural cells types (Sweetman, Zhang, Peterson, Gopalakrishna, & Sevanian, 1995; Thayer, Perney, & Miller, 1988; Worldcat, Kagan, & Quinn, 2014). Excitotoxicity and death occur in cerebellar Purkinje cell population after CCI due to increased and sustained calcium homeostasis (Igarashi, Potts, & Noble-Haeusslein, 2007; Potts,

Adwanikar, & Noble-Haeusslein, 2009; Weber, 2012) mediated by DAG activating protein kinase C (Berridge, 1987; Blackstone, Supattapone, & Snyder, 1989). Impairment of functional networks in the cerebellum manifests in impaired voluntary motor function (Stein & Hoffman, 2003). Although this study does not directly demonstrate a causative effect of enhanced LPA/PA production with cerebellar cell death and functional impairment, the results provide compelling correlations with enhanced expression of DAG and other phospholipids, that are known to be associated with degeneration, in remote brain regions that are characterized as early responders to cortical injury by enhanced excitotoxicity and apoptosis. These findings therefore suggest that the increase in phospholipid metabolism throughout the brain within 3 hours of injury contributes to the excitotoxicity and that persists throughout the brain at later time periods. Therefore, phospholipid enhancement observed in hippocampus, medulla, pons, corpus callosum, fornix and thalamus regions could be predictive of impairment of the function that these areas provide.

Pons is primarily responsible for sleep and respiration but also posture, therefore enhanced lipids in this region will alter sleep patterning, breathing and posture, which can all contribute to the lethargic symptoms TBI patients experience in early stages (Cantor et al., 2014; Cantor, Gordon, & Gumber, 2013; Zgaljardic et al., 2014). Sensory & motor neurons from the forebrain and midbrain travel through the medulla therefore enhanced lipid signaling in the medulla could cause axonal injury thus disputing those connections and further impairing motor function, sensory function and decision-making. Fiber tracts through the corpus callosum integrate motor, cognitive and sensory performance between both hemispheres. Disruption in one hemisphere leads to impaired motor movements and even sensory relay to the opposing side of the brain and manifests in impaired sensory and motor function on the contralateral side of the body

(Robertson & Jones, 1994; Schaefer, Haaland, & Sainburg, 2009; Smutok et al., 1989; Sugarman, Avni, Nathan, Weisel-Eichler, & Tiran, 2002). Enhanced lipid expression in the corpus callosum in association with markers of axonal injury was observed and suggests that changes lipid in expression within this region could contribute to the reduction and motor and function after injury. Also, enhanced lipid signaling in the hippocampal and fornix regions suggest impairments in learning and memory will persist after injury. Although studies have shown that the entire brain responds to injury (Hovda et al., 1995; Nishibe et al., 2010), studies have failed to demonstrate the profound effect observed in the changes in phospholipids at these early time points after injury. This study provides a profound visual representation of the early changes in lipids after injury on key functional areas of the brain and therefore provides strong correlations between determents in those areas and changes in phospholipid expression. The MALDI technique of identifying the spatial profile of lipids provides a more thorough and unbiased read-out of lipid expression as compared to more traditional biochemical analysis of discrete brain regions.

Bioactive, potent phospholipid species enhanced in the injured brain

LPA alone is known to be a potent neuromodulator (J. W. Choi et al., 2010) in the brain and the potency of LPAs bioactivity is dependent on the species. Unsaturated species are more biologically potent than saturated species; in studies of smooth muscle cells in vivo and in vitro, unsaturated LPA caused increased proliferation and dedifferentiation, where as saturated species did not have this effect (Hayashi et al., 2001; Jalink et al., 1995; a Tokumura et al., 1994; Yoshida, 2003). Despite theses facts, no study had described the role of LPA metabolism in the

injured adult brain, or assessed the signaling effectiveness. This study however demonstrates phospholipid metabolism of potently bioactive species in the ipsilateral cortical grey matter within 3 hours of injury. Studies have shown that LPA 18:1 enhances intracellular Ca2+ through activation of LPA receptors 1 and 2 causing excitotoxicity as well as functional changes and that LPA 18:1 is self propagating, triggering the production of more LPA from intracellular stores (C. W. Lee et al., 2006; L. Ma, Nagai, Chun, & Ueda, 2013; Yano, Ma, Nagai, & Ueda, 2013). The results of our study support those findings and show that the bioactive species of LPA (18:1 and 20:1) are enhanced as well as LPAs intracellular precursors (PA) within 3 hours of injury. This suggests LPA 18:1 and 20:1 that's enhanced in the injured brain will have self-propagating, excitotoxic effects on adjacent cells. Based on evidence that show phospholipid signaling induces apoptosis (Farooqui, Yang, Rosenberger, & Horrocks, 1997; Worldcat et al., 2014), phospholipid metabolism in the brain after injury is likely perpetuating cell death in the immediate area of injury as well as distal throughout the brain. These finding further establishes unsaturated LPA species and its unsaturated metabolites as signaling molecules involved in the progression of TBI pathophysiology.

Intracellular LPA and precursors expression is associated with markers of degeneration in the peri-contusion cortex and thalamus.

The results further demonstrate the self-propagating effect of phospholipid signaling on neural degeneration on cells adjacent to the epicenter of the injury, as expressed by a lack of HEME signal. The results provide a clear correlation between intracellular LPA expression and neural degeneration within the cortical grey matter and MALDI data also show an enhancement of intracellular LPA precursors in the same regions. This suggests that LPA will be further

enhanced at later time points outside of the ones captured in this study and is likely to have further self-propagating effects in surrounding cells. The results also show that LPA precursors were also enhanced in the thalamic regions within 1 hour after injury and PA 16:0/18:1 and PA 18:0/18:2 is significantly higher at 1 HPI compared to 3 HPI; at 3 hours markers of neural degeneration were enhanced in those same regions. This suggests that the acute enhancement of PA at 1HPI, as observed in the thalamus, may correlate with the signaling cascade involved in neurodegeneration. These findings further strengthen the correlation between LPA metabolism and progression of degeneration after injury. This suggests that, contrary to common belief, the LPA *pulse* observed in the systemic fluids after TBI is not confined to just CSF and blood but that injury alone causes a change in lipid gradients within brain tissue. This implies that the observed changes are occurring in addition to those mediated by activated platelets. This study demonstrates, in vivo, that phospholipid changes occur from both the intracellular and extracellular LPA metabolic pathways in brain tissue after injury and that early changes in phospholipid expression is associated with neural degeneration.

Spatial distribution of phospholipids in the white matter is associated with markers of axonal injury

The observed changes in LPA metabolism may be argued to originate from the BBB breakdown, which then initiated a chain response in surrounding cellular regions to produce LPA, and thus LPA metabolism is induced in distal regions. However, if this where the case, we'd expect to see an increase in LPA as time progresses, more LPA closer to the injury site and less LPA in the distal regions like cerebellum and thalamus, or a gradient like profile in the MALDI spectra. However, the data shows distinct expression of phospholipids in white matter tracts throughout

the brain, grey matter regions remote from the site of injury and then may spectra decreased in the peri contusion region. Lastly, and more likely are that the sharp changes in LPA, especially within the white matter regions reveal that LPA after injury is closely associated with the demyelination process of axonal injury. During development LPA is highly expressed along the white matter tracts and as development progresses and axons acquire myelin, LPA signaling decreases (J. a. Weiner et al., 1998). Exogenous LPA also induces demyelination of dorsal root neurons in ex vivo cultures (Makoto Inoue et al., 2004). Cortical injury cues the demyelination process within hours after injury, and this process is observed the corpus callosum as well is distal white matter regions (M. C. Liu et al., 2006; Shi et al., 2015). In addition, LPA receptor signaling mediates the demyelination process (Nagai et al., 2010) and therefore enhanced LPA signaling within the white matter tracts is likely enhancing axonal injury and the demyelination. The study provides supportive evidence of this by demonstrating strong spatial correlation between LPA and precursors to axonal injury markers, suggesting that the LPA in the white matter and the LPA that will be produced will contribute to axonal degeneration mechanisms.

This study further establishes LPA as an early signaling molecule in traumatic brain injury and contributes to the pathophysiology of TBI. This study strongly suggests that LPA plays a significant role in the degeneration process after cortical trauma and provides further support that attenuating LPA signaling within the brain at the observed time frames would most effectively decrease the progression of neurodegeneration and enhance recovery after injury

4. Chapter 4 – Study 2: Effects of Anti-LPA on behavioral outcomes:

Aim 2: To determine if Anti-LPA treatment prevents major pathologies of injury and improves behavioral outcomes.

4.1 Introduction:

Traumatic Brain Injury (TBI) affects millions of people in the US every year. Cortical trauma, in particular, often results in permanent motor and cognitive impairments due to tissue damage at the site of impact and distal disconnections with motor regions of the brain (Bazarian et al., 2007; Gale, Johnson, Bigler, & Blatter, 1995; Holschneider et al., 2013). The initial impact of injury causes secondary effects like, ischemia; edema and activation of biochemical pathways that enhance cell death, inflammation, gliosis and axonal injury (Prins et al., 2013; Tokutomi, 2005; Werner & Engelhard, 2007). As a result, these secondary injuries then cause profound, long-term behavioral deficits and leave many patients partially paralyzed and with cognitive deficits. Currently, there is no FDA approved treatment for brain injury. The ideal treatment would target early molecular signaling that aggravates secondary injuries and leads to the functional impairments experienced by patients.

Lysophosphatidic acid (LPA) is an early molecular signals involved in trauma pathogenesis (Crack et al., 2014; Frisca et al., 2013; Goldshmit et al., 2012, 2010). LPA is a simple phospholipid that's made in plasma and CSF by Autotaxin enzymatic activity, as well as in many cell types through other metabolic pathways (Aoki et al., 2008). LPA has profound effects as a

neuromodulator, with a role in many cellular processes through activation of LPAs G-protein specific receptors (S. An, Goetzl, & Lee, 1998). LPA activity is implicated in TBI because LPA signaling results in pathology associated with secondary injuries both in vivo and in vitro. After injury significantly high levels of LPA have been detected in the CSF and blood of human patients and in experimental models of TBI within 24 hours of injury (Crack et al., 2014). Our previous study showed a significant increase in LPA within the injured cortex within 1 hour of injury in the rat as well as remote changes in LPA metabolism in key functional regions of the brain. Markers of axonal injury and neural degeneration were also observed in association with changes in LPA and associated phospholipids. Therefore, enhanced phospholipids signaling after injury is not just increasing in systemic fluids but also increasing throughout the brain and in association with degeneration in regions of the brain that modulate motor function, coordination, sensory input, and cognition. This provides profound evidence that the presence of LPA metabolism is contributing to the pathogenesis and behavioral deficits associated with trauma. In addition to these finding, other studies have demonstrated LPAs involvement in many aspects of trauma pathology. Studies have shown that LPA acts as a pro-inflammatory signal that is released by activated platelets and modulates microglial cell activity (Eichholtz et al., 1993; Frugier et al., 2011; Z. Zhang et al., 2006). Cell culture studies have demonstrated that high concentrations of LPA induce necrosis of adult hippocampal cells and neurite retraction (F W Holtsberg et al., 1998; Steiner et al., 2000). LPA potently increases blood-brain barrier permeability (On et al., 2013; Schulze et al., 1997) and since LPA is also produced in the blood LPAs concentration and signaling would be exacerbated within the injured brain. A prominent effect of LPA in brain trauma is the glial scar formation and studies have also shown that LPA, alone, causes glial-scar-like recruitment and activation of glial cells (Shano et al., 2008).

Furthermore, high concentrations of LPA cause excitotoxicity and subsequently, cell death by disrupting the calcium/ glutamate gradients in neurons and astrocytes (a E. Dubin et al., 1999; Frederick W. Holtsberg et al., 1997; C. W. Lee et al., 2006). The above studies have clearly demonstrated LPA's potential involvement pathogenesis of trauma and therefore blocking LPA after injury is likely to attenuate these effects and subsequently enhance recovery after injury. Despite this prevailing question, the effects of preventing the LPA pulse on injury outcomes after cortical trauma remains unexplored.

Studies have attempted to block LPA signaling by blocking receptors, inhibiting LPA precursors and by blocking LPAs enzymatic activity. However, due to the functional redundancies of LPAs 6 G-protein coupled receptors, the array of mechanisms in which LPA is produced both in the brain and in systemic fluids these mechanisms have either produces lethal effects or inconclusive results (Estivill-Torrús et al., 2008; Herr & Chun, 2007; Seasholtz et al., 2004). Furthermore, LPA is an essential molecule for cellular structure and function throughout the body, so completely blocking LPAs effects even for a short time after trauma would likely have detrimental effects on the body by preventing the levels of LPA signaling needed to maintain cellular function. The ideal trauma therapeutic that targets LPA signaling would be 1) specific to LPA itself, in order to bypass functional redundancies in receptors and complexities in LPA metabolism.; 2) an acute treatment at a critical window after injury to prevent LPA enhancement at that specified time; 3) a terminal treatment that did not effect the production and signaling of LPA that is physiologically produced in the brain. Our previous study identified enhance expression of LPA intracellular precursors at 1 hour after injury and an increase in expression of LPA throughout the brain at 3 hours after injury. This suggests that the optimal therapeutic

window for treatment with anti-LPA to reduce LPA signaling is between 1 and 3 hours. Anti-LPA (Lpathomab) is a monoclonal antibody against LPA and this antibody was shown to prevent LPA receptor signaling only and not signaling of similar phospholipids (Goldshmit et al., 2012). Once the immune complex between anti-LPA and LPA is formed, anti-LPA becomes inactive and is released by the body through the urine at a half-life of 9.5 days in the blood of mice (data not shown) for the antibody alone. The therapeutic window identified in our previous study and the specificity and terminal action of anti-LPA make an ideal therapeutic mechanism to enhance recovery after injury by blocking LPA signaling.

The effects of anti-LPA on recovery after spinal chord injury (SCI) in the mouse have been described. Mice were treated with anti-LPA 30 minutes after injury and 2x/week for up to 2 weeks. This treatment paradigm improved motor outcomes after injury and the effect of the drug are apt to decrease inflammation, gliosis and cell death around the injury site. Although the pathogenesis of SCI is profoundly different than brain injury, this study provides strong support that blocking LPA after cortical injury will enhance recovery. Therefore, to identify the effects of anti-LPA on recovery after brain trauma the controlled cortical impact (CCI) model was used in the mouse. Mice received a one-time treatment of anti-LPA in the therapeutic window of 2 hours after injury and markers of degeneration and inflammation were assessed between 1 and 28 days after injury and long-term motor impairments were assessed at 70 days after injury. This study identifies the efficacy of anti-LPA in enhancing motor recovery after injury.

4.2 Materials & Methods:

Please refer to chapter 2 for detailed methods

Animals: Methods 2.1

Controlled Cortical Impact (CCI) Injury: Methods 2.2

Anti-LPA treatment: Methods 2.4

Tissue Fixation, Brain Sectioning & Immunohistochemistry: Methods 2.6

Contusion Analysis: Methods 2.8

Grid walk: Methods 2.9

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4.3 Results:

Axonal injury occurs immediately after TBI and injured axons are one of the underlying causes of functional impairments after injury (Hall, Bryant, Cho, & Sullivan, 2008; Johnson, Stewart, & Smith, 2013). Immunoreactivity of beta-amyloid precursor protein (Beta-APP) is a sensitive marker for injured axons due to APP accumulation at the severed axon terminal (Garnett, Blamire, Rajagopalan, Styles, & Cadoux-Hudson, 2000; Kamal, Almenar-Queralt, LeBlanc, Roberts, & Goldstein, 2001; Reichard, Smith, & Graham, 2005). Beta-APP is highly accumulated in the subcortical white matter region after TBI (Lewén, Li, Nilsson, Olsson, & Hillered, 1995; Otsuka, Tomonaga, & Ikeda, 1991; Pierce, Trojanowski, Graham, Smith, & McIntosh, 1996), therefore reducing Beta-APP in this region is likely to improve outcomes. The previous study (see Chapter 3) suggests that LPA and metabolic precursors are enhanced within the corpus callosum in correlation with beta-app expression. However, no study has yet to identify the effects of blocking LPA on Beta-APP accumulation in the sub cortical white mater after TBI. To determine the effect of anti-LPA treatment on Beta-APP accumulation in the subcortical white matter after injury, mice were treated with anti-LPA or the isotype matched vehicle control (IgG) at 2 hours after injury. The mice were then euthanized at 1 day after injury and standard immunohistochemistry was use to stain for Beta-APP in coronal brain sections. Total beta-APP accumulation counts were acquired from the ipsilateral corpus callosum at the epicenter of the injury site (-1.34mm posterior to bregma) for both vehicle (**Figure 1 A & B**) and anti-LPA (**Figure 1 A & C**) treatment groups. There was a significant reduction in beta-APP accumulation in the ipsilateral corpus callosum of Anti-LPA treated mice as compared to the vehicle group (**Figure 1 D**). This suggests that blocking LPA 2 hours after injury reduces axonal degeneration at 1 day after injury.

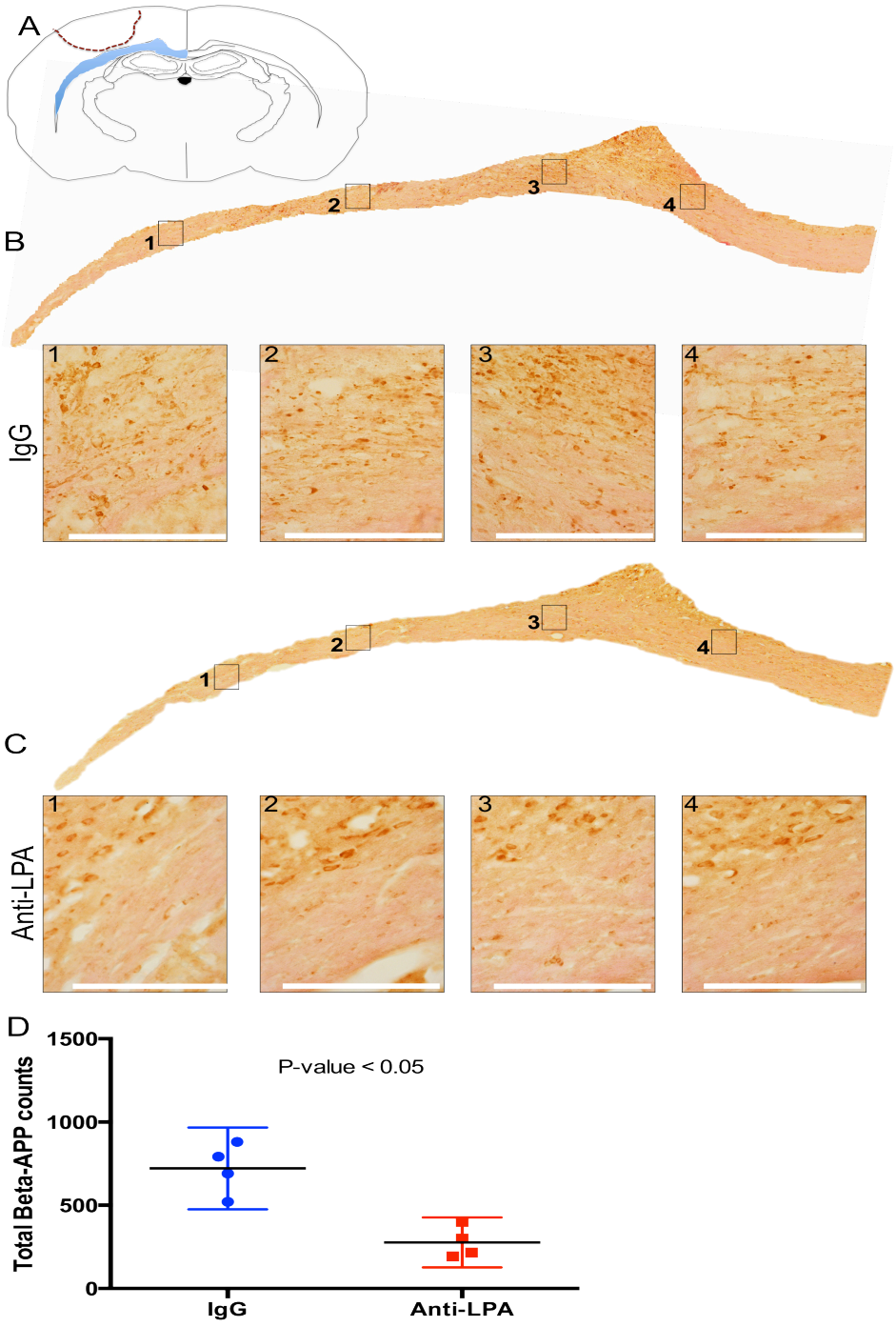

Figure 1: Anti-LPA treatment reduces beta-APP accumulation in the subcortical white matter. Coronal sections from the injury epicenter were obtained at 1 day after injury from anti-LPA and IgG treated mice. Standard immunohistochemistry with DAB amplification was used to detect beta-APP accumulation in the corpus callosum region (**A**) of anti-LPA (**C**) and IgG (**B**) treated mice. Representative high magnification insets were taken from lateral to medial (**B & C 1-4**) corpus callosum for both treatment groups. Total beta-APP accumulation counts from the entire corpus callosum was acquired and displayed as mean and 95% confidence interval error bars (**D**). P-values are measured as difference between treatment groups at each time point and obtained by bootstrapping the means in R. n=4 mice/ treatment group/ time period; scale bars; 40μM (**B1-4 & C1-4**).

In addition to axonal injury, necrosis is a major hallmark of trauma and cell death is thought to be one of the most detrimental secondary injuries resulting from TBI (Anderson, Miller, Fugaccia, & Scheff, 2005; Gao & Chen, 2011; Hutchison et al., 2001; Martin et al., 1998). To this effect many studies have suggested that therapies providing neuroprotective effects after TBI will be most effective in enhancing functional recovery (Do, Md, Md, & Md, 1999; Loane & Faden, 2010). LPA signaling causes cell death of adult hippocampal neurons (F W Holtsberg et al., 1998; Steiner et al., 2000); thus, an increase in LPA expression and LPA metabolite signaling in the brain after injury is likely to enhance cell death. Furthermore, blocking LPA signaling in the brain after TBI should reduce these effects. However, the neuroprotective effects of blocking LPA after cortical trauma are yet to be determined. Fluor Jade B (FJB) is a sensitive marker of neuronal degeneration within 3 days of injury; after CCI FJB accumulation occurs within the cortex with the highest expression near the injury epicenter (Anderson et al., 2005; Gao & Chen, 2011; Hall et al., 2008; Zhou, Chen, Gao, Luo, & Chen, 2012). To identify the neuroprotective potential of Anti-LPA treatment after CCI, mice are injected with Anti-LPA or the isotype matched control (IgG) at 2 hours after injury and euthanized at 3 days after injury. Standard histology was used to detect FJB throughout the ipsilateral cortex (**Figure 2 A-E**) for both anti-LPA (**Figure 2 Aii-Eii**) and vehicle (**Figure 2 Ai-Ei**) treated animals. FJB cell counts results revealed no significant different in FJB expression in the ipsilateral cortical grey matter between treatments groups 3 days after injury. This suggests that a single dose of anti-LPA is insufficient in neuroprotective effects or that cell death is perhaps irreversible.

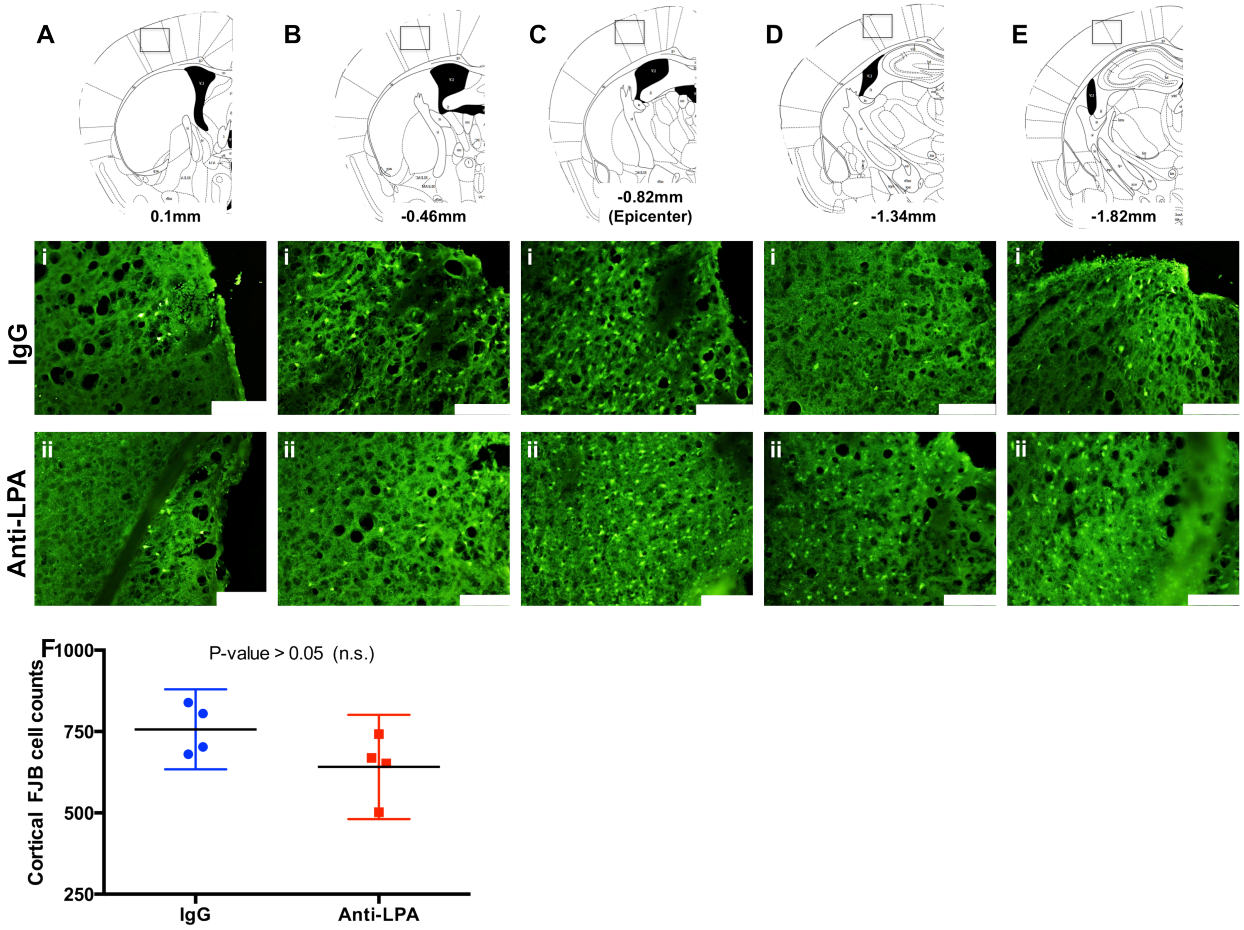

Figure 2 A single anti-LPA dose has no significant effect on neurodegeneration in the cortex at 3 DPI. Coronal sections from both treatment groups were obtained throughout the cortex 3 DPI for anti-LPA (**A-Ei**) and IgG (**A-Eii**) treatment groups. Histological stain for FJB+ cells was performed on cortical sections at 0.1 mm anterior (**A**), -0.46mm (**B**), -0.82 **(C**), -1.34 mm (**D**), and -1.82mm (**E**) posterior to bregma. Total cells in the ipsilateral cortex at these levels A-E were counted for both treatment groups. All data points acquired are displayed as well as the mean and 95% confidence interval (**F**). Scale bars 100μM (**A-Ei-ii).**

Inflammation occurs in the brain within 1 day of injury (Kunz et al., 2010; Nortje & Menon, 2004) and can persist in the brain for several weeks after the initial insult (Acosta et al., 2013; Gentleman et al., 2004). In many degenerative diseases, including trauma, inflammation is identified as a persistent signaling cascade with both beneficial and detrimental effects on cellular function and signaling. Many studies in the TBI field have found that reducing pro-

inflammatory signaling in the injured brain improves injury outcomes (Truettner, Suzuki, & Dietrich, 2005; Yang et al., 2013; Zhuang et al., 2011; Ziebell & Morganti-Kossmann, 2010). LPA acts as a potent pro-inflammatory signaling molecule and LPA metabolism and signaling is associated with activation of microglial cells (Eichholtz et al., 1993; Fourcade et al., 1995; S. Liu, Murph, Panupinthu, & Mills, 2009; Tham, Lin, Rao, Yu, & Webb, 2003; Z. Zhang et al., 2006). Since LPA signaling and metabolisms is enhanced in the brain after injury then LPA signaling is likely associated with enhanced inflammation. Furthermore, blocking LPA after injury is likely to reduce markers of inflammation in the brain. Ionized Calcium-Binding Adapter Molecule 1 positive (Iba1+) cell marker is a sensitive identifier of microglia in the CNS (Chung et al., 2010; Gatson et al., 2013; Ito, Tanaka, Suzuki, Dembo, & Fukuuchi, 2001; Ohsawa, Imai, Sasaki, & Kohsaka, 2004; Y. Tanaka, Matsuwaki, Yamanouchi, & Nishihara, 2013). To determine the effects of Anti-LPA intervention on inflammation after injury, cortical sections from anti-LPA and the vehicle treated mice were obtained at 7 and 28 days after injury. Standard immunohistochemistry was utilized to detect Iba1+ cells around the peri-contusional grey matter cortex (**Figure 3A – c1 and c2 regions**), the ipsilateral subcortical (corpus callosum) white matter regions (**Figure 3 A- 'w' region**) and the anterior nucleus of thalamus (**Figure 3 A- 't' region**) for both anti-LPA (**Figure** 3Bv-viii & Cv-viii) and vehicle (**Figure 3Bi-iv & Ci-iv**) treated groups at 7 (**Figure 3B**) and 28 days after injury (**Figure 3C**). The results revealed no significant change in Iba1+ cell expression in the cortical or thalamic regions. However, there was a significant decrease in Iba1+ cells in the ipsilateral corpus callosum of anti-LPA treated animals at both 7 and 28 DPI. Iba1+ cell counts from the ipsilateral corpus callosum at -1.8 mm posterior to bregma were taken for animals at 7 and 28 DPI and results show a significant reduction in Iba1+ cells in the ipsilateral corpus callosum of anti-LPA treated as compared to the

vehicle treated animals (**Figure 3D**). The data suggests that LPA treatment reduces inflammatory signaling in the subcortical white matter regions and thus a reduction in white matter inflammation may result in improved functional outcomes.

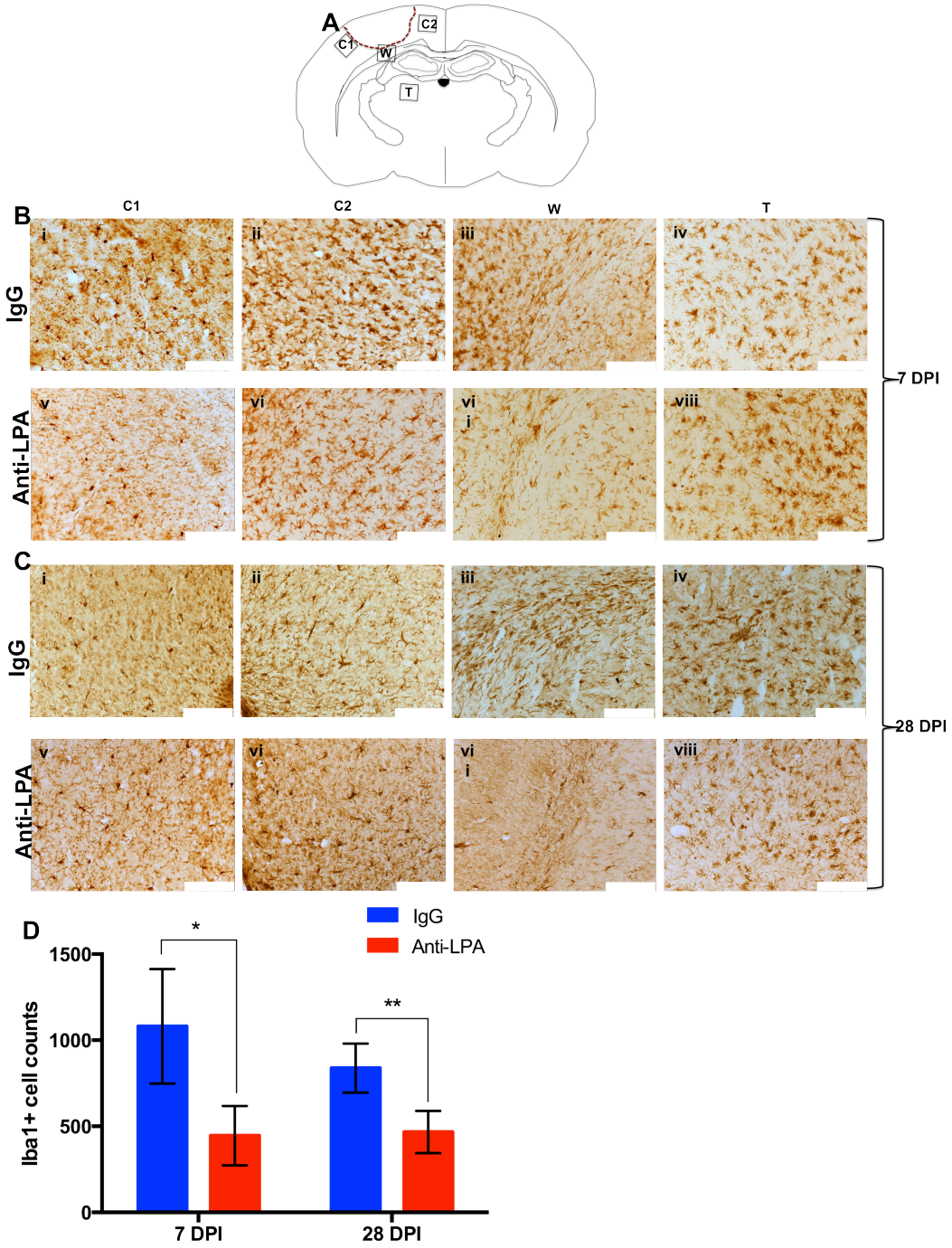

Figure 3; Anti-LPA treatment reduces subcortical white matter inflammation at 7 and 28 DPI.

Coronal sections from -1.8mm from bregma were obtained from animals at both treatment groups. Standard immunohistochemistry with DAB amplification was used to detect Iba1+ cells in the lateral- (c1 inset) and medial- (c2 inset) peri-contusion cortical grey matter, the corpus callosum (w inset) and the anterior nucleus of thalamus (t inset) at 7 (**B**) and 28 (**C**) DPI. Total Iba1+ cells in the ipsilateral corpus callosum were obtained for both treatment groups at 7 and 28 DPI; data is displayed as mean and 95% confidence intervals (**D**). n=4/ mice/ treatment group/ time point; P-values are obtained as the significant difference in mean values between treatment groups at each time point. P-values *P<0.05, **P<0.001, ***P<0.0001 from bootstrapping confidence intervals about the mean in R. Scale bars: 100μM (**B & C**)**.**

The previous results identified a reduction in white matter pathologies at 1 day after injury as determined by beta-APP accumulation and a reduction in white matter inflammation as determined by Iba1 cell density at 7 and 28 DPI. This suggests that anti-LPA treatment reduces white matter degeneration and these effects may manifest in improved functional outcomes. The CCI model produces consistent and measurable sensorimotor deficits (C. E. Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991; Lighthall, 1988; Romine, Gao, & Chen, 2014) and in mouse these deficits persist (Baskin et al., 2003; Onyszchuk et al., 2007). The grid walk behavioral test is one of the best measure of sensorimotor deficits in the CCI model in mice (Baskin et al., 2003; Harris, Mironova, Hovda, & Sutton, 2010; Onyszchuk et al., 2007; Pleasant et al., 2011). To determine the effects of Anti-LPA treatment of sensorimotor function, anesthetic sham mice or mice treated with anti-LPA or vehicle at 2 hours after CCI were utilized. The grid walk behavioral test was performed as a measure of sensorimotor control. Total fault on the front- and hind limbs of the contralateral side of the body was determined out of 50 steps taken on each limb. Faults were characterized as ‘full’ or ‘half’; where the full extension of the limb falls through the grid or half extension of the limb falls through the grid respectively. Behavioral testing was done on 3 separate days prior to injury, at 3 and 7 DPI and 1 time per week thereafter

for 10 weeks post injury for all treatment groups and sham. The results reveal a significant reduction in front-limb faults for anti-LPA treated group as compared to the vehicle group starting at 35 days after injury with significant difference between anti-LPA and the vehicle group groups persisting throughout the testing period (**Figure 4A**). At 49 DPI front-limb deficits of anti-LPA treated mice are not significantly different from sham scores (**Figure 4A**). Hind-limb detriments for the anti-LPA treated groups improve significantly as compared to the vehicle group starting at 28DPI (**Figure 4B**) and persisting through 70 DPI. Hind-limb impairments for the anti-LPA treatment groups at 49 and 63 DPI are not significantly different than impairments in sham group (**Figure 4B**). This suggests that anti-LPA treatment significantly improves sensorimotor function after injury, which is likely to be due to the observed reduction in white matter pathology. Lastly, to determine if anti-LPA treatment had long-term neuroprotective effects injury, ipsilateral brain volumes from anti-LPA and the vehicle group groups were obtained throughout the brain, percentage of ipsilateral cortical volume loss is represented as a percentage of contralateral volumes within each mouse. The results indicate that anti-LPA treatment does not significantly reduce contusion size at 70 DPI (**Figure 5**). This data suggests that the treatment paradigm employed in this study is not neuroprotective in the longer term after trauma.

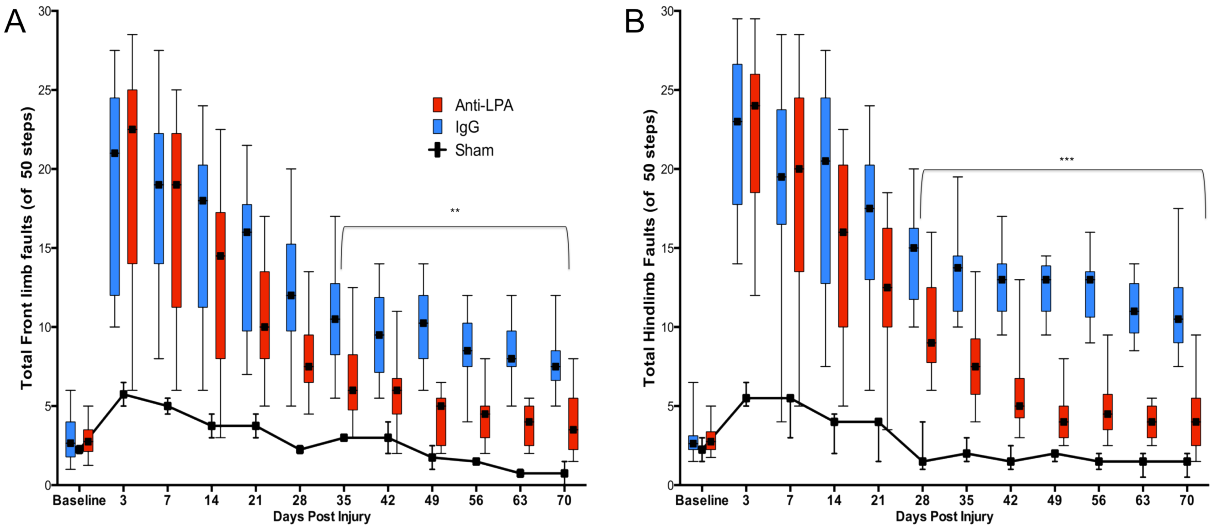

Figure 4; Anti-LPA treatment improves long-term sensorimotor function after CCI. Grid walk task for evaluation of sensorimotor deficits in front- and hind limbs. n=5 for anesthetic sham group (black), n=20 for IgG treatment group (blue) and n=25 for anti-LPA treatment group. Total faults are measured of 50 steps for each front and hind limbs. Data is displayed as median, and 95% confidence intervals. P-values indicate significant difference between anti-LPA and IgG treatment groups and obtained by bootstrapping means around the confidence intervals in R; P-values *P<0.05, **P<0.001, ***P<0.0001

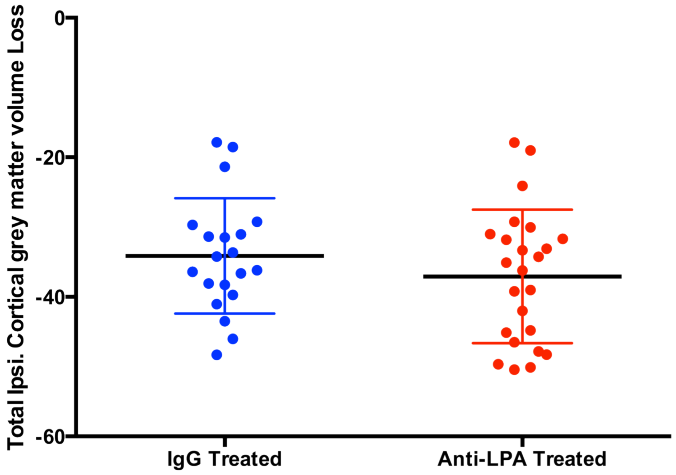

Figure 5; Anti-LPA treatment does not significantly preserve ipsilateral cortical volume at 70 DPI. Ipsilateral cortical contusion volumes were obtained from n=20 IgG treated mice and n=25 anti-LPA treated mice at 70 DPI and values were normalized to the contralateral cortical volume of each mouse. All data points are displayed with means and SE for each treatment group.

4.4 Discussion:

LPA signaling can initiate a cascade of cellular responses that manifest in hallmarks of injury pathology such as inflammation and cell death (J. W. Choi et al., 2010; Steiner et al., 2000). Research efforts in the TBI field have been directed at targeting these and other secondary injuries in order to enhance recovery (Algattas & Huang, 2013; Kumar & Loane, 2012; Narayan et al., 2002; Sheriff & Hinson, 2015). However, the effects of blocking LPAs potent signaling effects in cortical injury outcomes remained unknown until now. The results in this study provide evidence of LPAs role in white mater pathology and sensorimotor function after trauma. The evidence provided herein establishes the efficacy of Anti-LPA treatment, at a critical window of intervention, to improve injury outcomes and function.

Anti-LPA treatment reduces axonal damage and white matter inflammation after TBI.

Axonal injury is one of the initial responses of the brain to a traumatic insult, and diffuse axonal degeneration, characterized by demyelination and axon retraction, occurs overtime (Johnson, Stewart, & Smith, 2013; Povlishock, 1992). Axonal degenerative process leads to functional disconnections of key areas in the brain that modulate sensorimotor function. Clinically, axonal injury results spans concussion, severe disability to vegetative state (Adams et al., 1989; Blumbergs et al., 1995, 1989; Gennarelli et al., 1982; Jafari, Nielson, Graham, & Maxwell, 1998; Povlishock, 1992). Therefore, intensity and diffusion of makers of axonal injury in the white matter within hours of the insult are predictive of future functional impairments (Sandel, Bell, & Michaud, 1998; Shutter, Tong, & Holshouser, 2004). Developmental studies have shown that LPA expression is significantly reduced upon axonal myelination (Stankoff et al., 2002; J. a.

Weiner et al., 1998), an ex-vivo study also demonstrated that LPA induced demyelination of dorsal root within 24 hours(Fujita, Kiguchi, & Ueda, 2007). The above evidence suggests that LPA expression is perhaps associated with the demyelination process of axonal degeneration. Furthermore, studies have demonstrated that LPA signaling causes neurite retractions and is released after nerve injury (Sayas, 1999; Ueda, Matsunaga, Olaposi, & Nagai, 2013). This suggests that enhanced LPA signaling after injury could be associated with the axonal degenerative process. Our previous study demonstrates a strong correlation between LPA and markers of axonal injury in the corpus callosum region. The results herein further support those findings and suggest that blocking LPA with anti-LPA after trauma reduces axonal injury. Furthermore, the significant reduction in axonal injury after treatment suggests that more functional connections could be maintained in the white matter and therefore blocking LPA will enhance preservation of sensorimotor function.

White matter inflammation is also involved in the persistent degenerative process of TBI (Johnson, Stewart, Begbie, et al., 2013). Inflammation persists in the injured brain and studies have shown that microglial activation is enhanced in the corpus callosum at 7 DPI in rodent CCI models (Acosta et al., 2013; Farbood et al., 2015; G. Wang et al., 2013). LPA is established as a pro-inflammatory signaling molecule with a role in an array of disease states (Fourcade et al., 1995; S. Liu et al., 2009; Z. Zhang et al., 2006). LPA is involved is involved in white matter inflammation; studies have also demonstrated that after nerve injury LPA signaling activates microglia and amplifies LPA production and release from microglia (M. Inoue et al., 2008; Jalink et al., 1994, 1993; C.-W. Lee et al., 2007; Ueda et al., 2013). This suggests that LPA signaling in the white matter is a self-amplifying process in which LPA production and signaling initiates both axonal injury and microglial activation, and this further enhances LPA production

and signaling. The results herein further support those findings and suggest that blocking LPA impedes this cycle of LPA signaling in white matter pathology. Anti-LPA treatment reduces LPA signaling in the white matter therefore activating less microglia and reducing white matter inflammation. The results also show that Iba1+ cells are not reduced in the cortex or anterior nucleus of thalamus, suggesting that a single dose of anti-LPA at 2 hours after injury has a greater effect on white matter pathologies than that of grey matter. This also implies that the effects of the initial LPA pulse occurring within 1 and 3 hours of injury has an immediate effect on white matter regions whereas grey matter pathologies may manifest in response to the longer-term, persistent, and diffuse effects of LPA signaling in the brain. Its also possible that grey matter damage is more sever and/or irreversible, whereas white matter damage may be salvageable, and could lead to more functional improvements as compared to saving grey matter. Thus, to decrease inflammation throughout the brain a longer and or repeated treatment design is needed similar to that used to reduce inflammation after SCI (Goldshmit et al., 2012). Despite this, the result provide evidence that blocking the initial pulse of LPA after trauma will reduce inflammation in the white matter, and further establishes LPA as a pro-inflammatory molecule, after injury.

Single dose of anti-LPA at 2 hours after injury does not have significant neuroprotective effects

Cell death is a sensitive effect of injury and studies have shown that mild concussions and even emotional stress can lead to cell death in the brain (C E Dixon et al., 1987; Lucassen et al., 2006; Perez-Polo et al., 2015). Therefore, potent interventions are needed to reduce the sensitive response of the brain to injury that is cell death. Furthermore, LPAs signaling cascade enhances

necrosis is a self-perpetuating cycle of increased intracellular calcium in neurons and reduced calcium and glutamate uptake in astrocytes, perpetuating the excitotoxic microenvironment of cells, which further enhances LPA production in adjacent cells, leading to increased calcium homeostasis and further cell death (F W Holtsberg et al., 1997; Frederick W. Holtsberg et al., 1998; Yano et al., 2013). The results demonstrate the insufficiency of anti-LPA treatment paradigm to provide enough neuroprotection to reduce necrotic markers at 3 days after injury and to preserve cortical tissue at later time points. These results suggest that neuroprotective therapies with anti-LPA will need to target the persistent and self-perpetuating signal of LPA after trauma and not just the initial pulse. The treatment paradigm established in this study was based upon evidence that LPA and metabolites were increased in the brain in association with makers of necrosis at 1 and 3 hours after injury (See chapter 3). Although the results show that the treatment method did not reduce necrosis and improve contusion volume the results do not suggest that anti-LPA lacks neuroprotective properties but that the therapeutic design may be inefficient to establish anti-LPAs neuroprotective role. The one-time dosage of anti-LPA may have been effective enough to reduce axonal injury but did not target the persistent excitotoxic effects of LPA signaling, produced by self-perpetuating LPA production and calcium homeostasis. Therefore, a higher dosage and/ or multiple treatments of anti-LPA is likely needed to have neuroprotective effects after injury.

Anti-LPA treatment improves sensory motor function.

The results herein have demonstrated one does of anti-LPA a 2 hours of injury is sufficient to reduce axonal injury and inflammation in the white matter regions. White matter pathology is directly causative of functional impairments after trauma and thus reducing white matter pathologies should enhance recovery. The results demonstrate significant, long-term improvement in sensorimotor function of mice treated with anti-LPA as compared to the vehicle group. The results demonstrate these effects on both the front- and hind limbs on the contralateral side. This evidence suggest that the treatment paradigm sufficiently attenuated LPA signaling enough to reduce white matter pathologies, and that reducing injury and inflammation in the white matter will have long-term beneficial effects on functional outcomes.

This study demonstrates the efficacy of a single anti-LPA dose, at a critical window to LPA metabolism and expression, to reduce axonal injury, white matter inflammation and enhance sensorimotor function after injury. This study establishes LPA as a promising biomarker for trauma and anti-LPA as a potential therapeutic for traumatic brain injury.

5. Chapter 5 – Study 3: Effects of Anti-LPA treatment on the endogenous neurogenic response to TBI

Aim 3: To determine if blocking LPA after injury affects the endogenous neurogenic response to injury

5.1 Introduction

Traumatic insults to the brain like TBI and stroke stimulate neural progenitor cells (NPCs) of the sub-ventricular zone to divide and migrate toward the site of injury; this phenomena suggests the brain contains the potential for self-repair (Ong, Plane, Parent, & Silverstein, 2005; Parent et al., 2002a; Petraglia, Marky, Walker, Thiyagarajan, & Zlokovic, 2010; Richardson et al., 2007; D. Sun, 2014; Thomsen et al., 2014). However, many TBI patients never fully regain function and studies of SVZ neurogenesis after trauma have shown that NPCs enhanced in the SVZ do not survive, differentiate into neurons or form functional connections near the injury site (Otero et al., 2012; Yi et al., 2013). The excitotoxic environment of the injury site is proposed to underlie the incompetence's of the endogenous progenitor population (D. W. Choi, 1995; Greve & Zink, 2009; A. M. Palmer et al., 1993). In fact, studies have demonstrated that acute excitotoxic signaling, of high calcium and glutamate concentration stimulates NPC proliferation but persistent toxic signaling, as that in trauma, induces death and conversion of NPCs to glial cells (Doze & Perez, 2012; Egeland, Zunszain, & Pariante, 2015; Kempermann, 2006; Lavado & Oliver, 2014; Pontes et al., 2013; Singer et al., 2009; Thomsen et al., 2014; Vicini, 2008; Young, Taylor, & Bordey, 2011). Furthermore, the toxic environment of the injured brain also contributes to the glial scar formation, inflammation, and necrosis; these pathologies further restrict the survival of NPCs in the brain(Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003;

Fuster-Matanzo, Llorens-Martín, Hernández, & Avila, 2013; Kohman & Rhodes, 2013; Silver & Miller, 2004; Tobin et al., 2014; Zhou et al., 2012). Therefore, attenuating the persistent signaling cascade that enhances excitotoxicity in the brain after trauma will likely provide a more supportive environment for NPC to migrate to, survive in, and contribute to functional connections after injury.

Lysophosphatidic acid signaling is enhanced in the brain after injury (chapter 3), LPAs signaling enhances excitotoxicity (Y.-J. Chang et al., 2007; F W Holtsberg et al., 1997; J N Keller et al., 1996; Schousboe & Waagepetersen, 2005; Steiner et al., 2002), is involved in the progression of trauma pathology (Crack et al., 2014; Frugier et al., 2011) and is a potent modulator of progenitor cell signaling and function which can be limited by excitotoxicity (Dottori et al., 2008; Doze & Perez, 2012; Estivill-Torrús et al., 2008; Frisca et al., 2013; N Fukushima et al., 2000; Nobuyuki Fukushima, 2004; Pitson & Pébay, 2009; Yanagida et al., 2007). Increased extracellular glutamate and thus intracellular calcium and caspase and calpain activation are characteristic of an excitotoxic environment (Akhlaq A. Farooqui, Wei-Yi Ong, 2008; Butterfield, 2004; Obrenovitch & Urenjak, 1997). LPA signaling perpetuates excitotoxicity by increasing extracellular glutamate and calcium, increasing calcium uptake in neurons and decreasing glutamate and calcium uptake by astrocytes (A. E. Dubin, Herr, & Chun, 2010; F W Holtsberg et al., 1997; J N Keller et al., 1996). Astrocytes typically act as supportive cells, by maintaining a healthy environment for neurons; however because LPA signaling inhibits astrocytes supportive functions LPA signaling is self-perpetuating excitotoxicity, cell death and degeneration. LPA, itself also acts as a neurotransmitter and activates NMDA receptors which then increases intracellular calcium perpetuating the excitotoxic cascades (a E. Dubin et al.,

1999). Phosphorylation of LPAs metabolites can also cause excitotoxicity and cell death (Adibhatla & Hatcher, 2007; Akhlaq A. Farooqui, Wei-Yi Ong, 2008; Cummings, Parinandi, Wang, Usatyuk, & Natarajan, 2002). These studies provide evidence of the toxic effects of LPA signaling and because excitotoxicity is thought to inhibit NPC survival in the brain, attenuating LPA signaling after injury will likely provide a healthier environment in which NPCs can survive.

In addition to LPAs role in excitotoxicity, LPA potently modulates NPC function during development (N Fukushima et al., 2000; Nobuyuki Fukushima, 2004; McGiffert, Contos, Friedman, & Chun, 2002). Low levels of LPA, similar to that in the uninjured brain, promote neuronal differentiation and proliferation of progenitor cells (Anliker & Chun, 2004; Dottori et al., 2008; Frisca et al., 2013; Kingsbury et al., 2003; Nakanaga, Hama, & Aoki, 2010). However, high concentrations of LPA causes neurite retraction, decreases proliferation and neuronal differentiation of cultured progenitor cells (Dottori et al., 2008; Frisca et al., 2013; Nobuyuki Fukushima, 2004; Gabor Tigyi et al., 1996; Yamazaki et al., 2008). Studies of human embryonic stem cells (hESCs) have demonstrated that a single 10uM dose of LPA inhibits proliferation and neuronal differentiation (Dottori et al., 2008). The evidence suggests that the LPA pulse after trauma is likely inhibiting the expansion and neuronal fate direction of SVZ progenitor cells. Therefore, attenuating LPA signaling in the brain is likely to increase the response of progenitor cells, enhance neuronal differentiation and possible promote migration to the injured cortex.

The previous studies (Chapter 3 and 4) have demonstrated that LPA metabolism and signaling is associated with necrosis, axonal injury, and inflammation. Attenuating LPA signaling, with anti-

LPA, after cortical injury (Chapter 4) and spinal cord injury improves injury outcomes of trauma pathologies and significantly improves motor function (Crack et al., 2014). Anti-LPA treatment improves injury outcomes and reduces hallmarks of trauma pathology likely by reducing LPAs effects in enhancing excitotoxicity of the injured cortex, and thus reducing secondary injury progression. Due to the potent, beneficial effects of blocking LPA signaling in CNS trauma, decreasing LPA signaling should also enhance the survival and differentiation of the endogenous NPCs that respond to injury. Therefore, the studies herein will explore the effects of blocking LPA, with anti-LPA, on the neurogenic response of the SVZ that anti-LPA treatment modulates the neurogenic response in the SVZ and neuroblast survival in the cortex after injury using the controlled cortical impact model of injury in mice.

5.2 Materials & Methods:

Please refer to chapter 2 for detailed methods

Animals: Methods 2.1

Controlled Cortical Impact (CCI) Injury: Methods 2.2

Anti-LPA treatment: Methods 2.4

5-Chloro-2'-deoxyuridine (CldU) labeling: Methods 2.5

Tissue Fixation, Brain Sectioning & Immunohistochemistry: Methods 2.6

Stereology: Methods 2.7

Statistical Analysis: Methods 2.10

5.3 Results:

Anti-LPA treatment acutely enhances the dividing neuroblast population in the SVZ.

Unilateral cortical injury enhances the doublecortin (DCX) cell population in adult SVZ (Altman, 1962; Chirumamilla et al., 2002). Studies have shown that the DCX+ cells may contribute to cortical repair after injury by migrating to the injury site, providing support for resident cortical cells or differentiating into functional neurons (Arvidsson et al., 2002a; Parent, 2003; Saha, Jaber, & Gaillard, 2012). To identify the effects of LPA signaling on proliferation and neuronal fate direction of progenitor cells in the SVZ neurogenesis after injury, mice from both treatment groups received acute CLDU injections prior to euthanasia at 1, 3 and 7 DPI (**Figure 1B**). Triple-label immunohistochemistry (IHC) for dividing cells (CLDU+), neuroblast (DCX+) and total cells (Dapi+) were performed on coronal sections from animals at each time point. Total estimated dorso-lateral SVZ (**Figure 1A**; green rectangle) cell counts of dividing cells; neuroblasts and dividing neuroblasts (double-labeled CLDU+ and DCX+; DNB) were obtained from 5, 20um sections per animal through Stereo Investigator Optical Fractionator probe stereological software. For all cell types there was no significant difference between treatment groups at 1DPI. However, at 3DPI Anti-LPA treatment significantly enhanced the total SVZ density (Dapi+) ($1.8x10^4 \pm 4.0\ x10^2$ vs. $1.0\ x10^4 \pm 1.2x10^3$), total dividing cells (CLDU+) ($1.3\ x10^3 \pm 2.5\ x10^2$ vs. $3.5\ x10^2 \pm 13$), total neuroblasts (DCX+) ($3.0\ x10^3 \pm 1.3x10^2$ vs. $2.0x10^4 \pm 1.5x10^2$) and the dividing neuroblasts (DNB) populations (289 ± 48 vs. 28 ± 7) as compared to the vehicle treated (Anti-LPA, Vehicle, respectively) (**Figure 1 Eii**). Images and insets (**Figure C-D**) demonstrate the marked increase in populations at 3DPI with Anti-LPA treatment.

Though, at 7 DPI there was a paradoxical shift in relative cell densities, where total cell density (Dapi+) ($1.2x10^4 \pm 3.0x10^2$ vs. $2.2x10^4 \pm 4.0x10^2$) and neuroblasts (DCX+) cells numbers ($3.0 x10^3 \pm 56$ vs. $4.3x10^3 \pm 2.3x10^2$) (Anti-LPA, Vehicle, respectively) are significantly reduced for Anti-LPA treated group as compared to vehicle treated group (**Figure 1 Eiii**). There was no significant effect of treatment on the dividing population (CLDU+), however, the dividing neuroblast populations (double-labeled CLDU+ and DCX+; DNB) in the SVZ were still significantly higher for the Anti-LPA treated groups ($6.8x10^2 \pm 23$) as compared to the vehicle group (49 ±1.3) at 7 DPI (**Figure 1 Eiii**). The data suggest that blocking LPA after injury stimulates the SVZ progenitor cells earlier than the vehicle group and directs SVZ progenitor cells toward a pro-neuronal state at early time points.

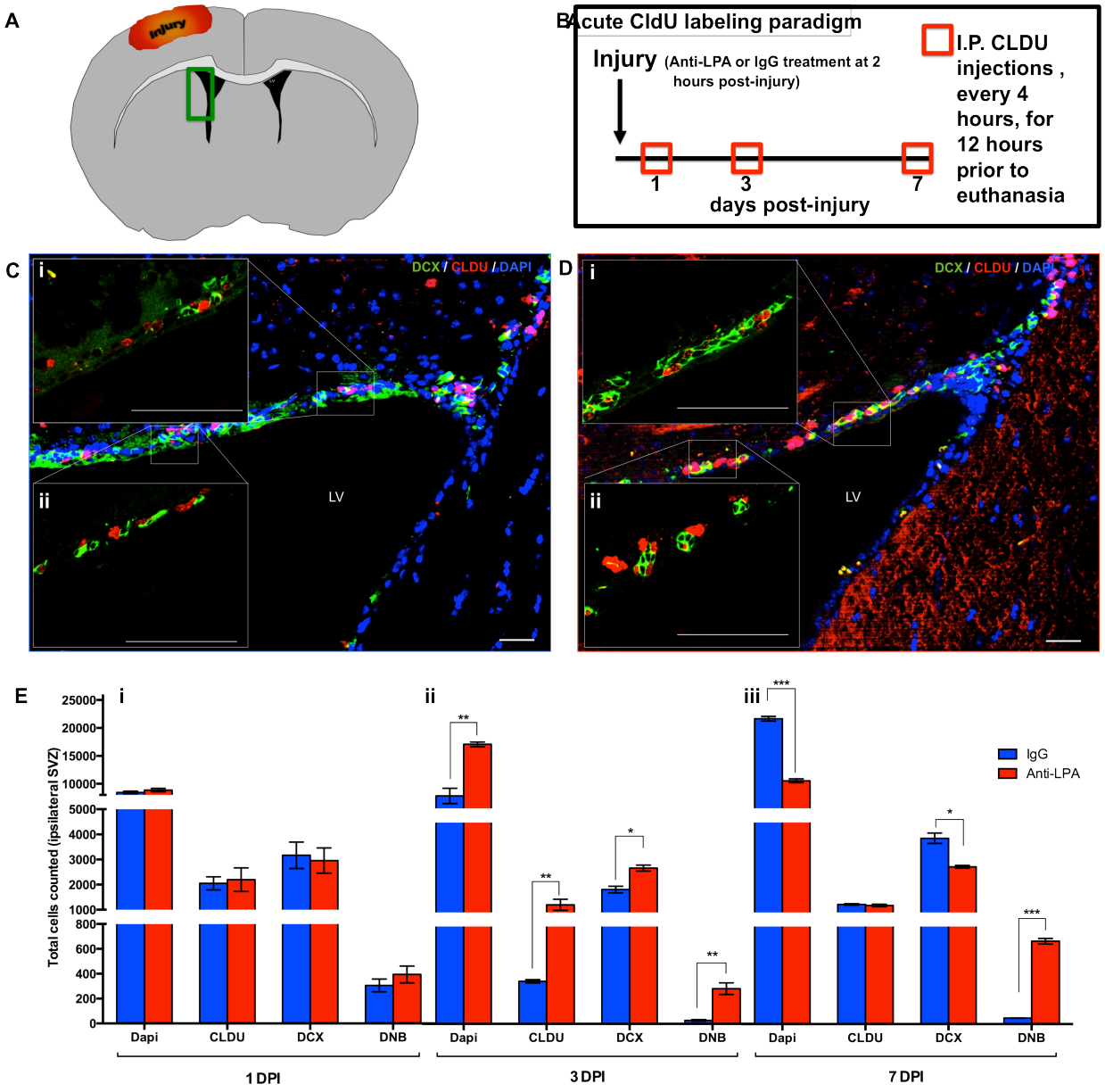

Figure 1: Anti-LPA therapy significantly enhances SVZ neuroblast population within 7 days of injury. C57b6 male mice received (25mg/kg) intravenous (i.v.) injection of anti-LPA or the isotype-matched control, IgG at 2 hours following controlled cortical impact injury (CCI). Actively dividing cells in the dorsolateral SVZ (**A; green box**) were labeled with CLDU (50mg/Kg) every 4 hours for 12 hours, either 1, 3 or 7 days post-injury (DPI) on the day of euthanasia (**B**). Immunostaining of actively dividing cells (anti-5' chloro-2'deoxyuriding; CLDU+), neuroblast (anti-doublecortin; DCX+), dividing neuroblast (double-labeled CLDU+/DCX+) and all cells (Dapi+) from coronal sections were obtained. Stereology using Stereo Investigator software on Zeiss microscope was used to acquire the total cell counts for each cell type. Representative images of n=4 mice/ treatment groups display DCX, CLDU and Dapi distribution throughout the dorsolateral SVZ at 3 DPI (**C & D**).

All data for estimated cell counts are displayed as mean and SEM (**E**). P-values are determined between treatment groups for each cell type, by bootstrapping the means around the 95% confidence intervals in R. P-values *P≤0.01, **P≤0.001, ***P≤0.0001; Scale bars: 40μM (**C & D**).

Anti-LPA treatment reduces the cortical neuroblast populations at early time points.

To identify the role of LPA signaling on the cortical progenitor niche after injury, estimates of all cell counts were performed using stereology on the ipsilateral cortical grey matter (**Figure 2A**) of the same animals in the previous section (**Figure 1**). Representative high-powered images display the relative reduction in peri-contusion cortical grey matter (**Figure 2A**) neuroblast of Anti-LPA treated mice as compared to the vehicle treated mice at 1 DPI (**Figure 2 B-C**). The results demonstrate at 1 DPI there is no difference in total cell density in the peri-contusional cortex between treatment groups and a potent increase in dividing cells for Anti-LPA treatment as compared to the vehicle group ($4.5x10^3$ $\pm 1.3x10^2$ vs. $1.8x10^3$ $\pm 3.3x10^2$) (Anti-LPA, Vehicle, respectively) (**Figure 2Di**). There was also a significant reduction in neuroblasts (43 ± 6 vs. $3.0x10^2$ ±71) and dividing neuroblasts (21 ± 7 vs. 89 ±19) (Anti-LPA, Vehicle, respectively) for the Anti-LPA group as compared to the vehicle (**Figure 2Di**).

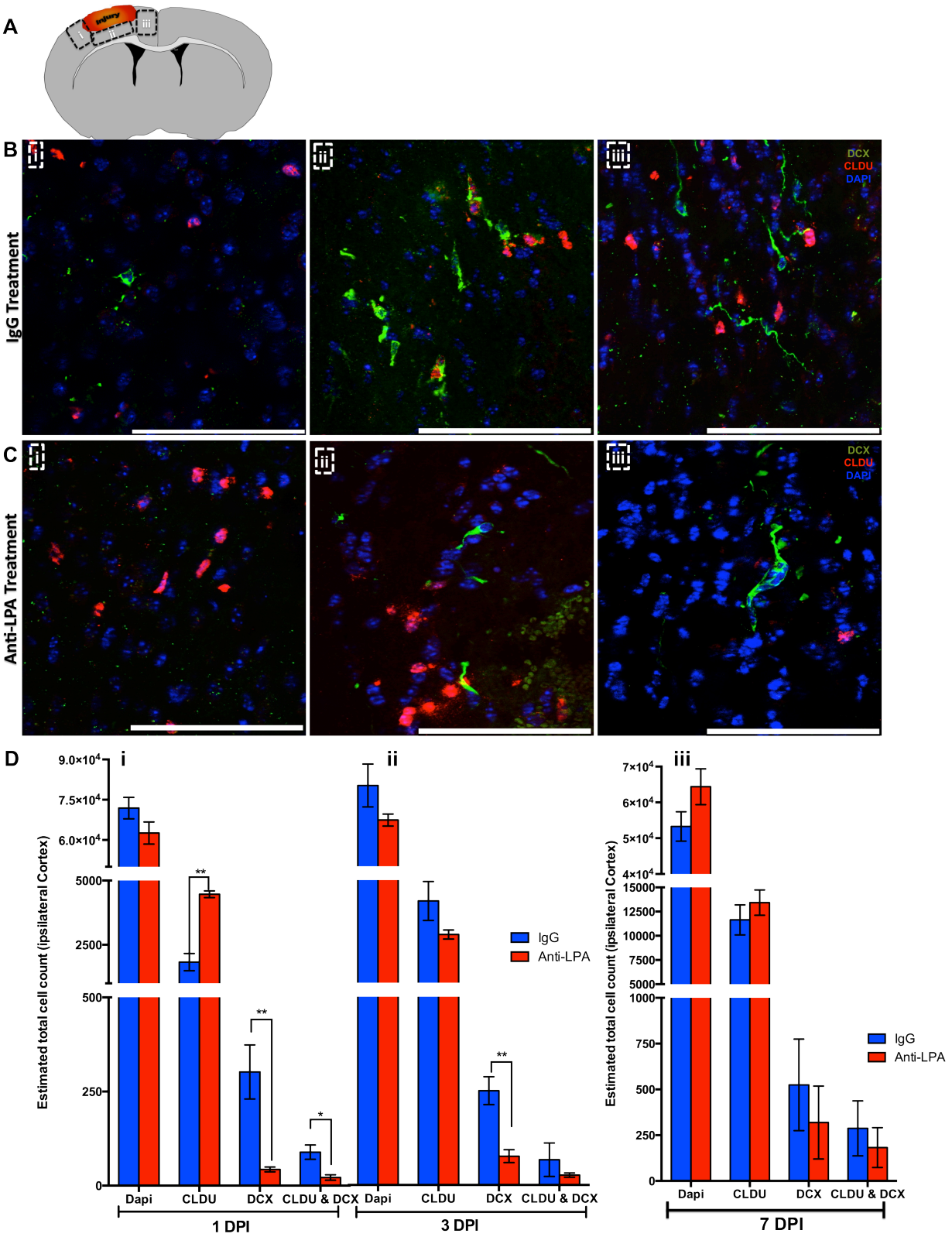

Figure 2: Anti-LPA therapy significantly reduces early cortical neuroblast population within 7 days of injury. CLDU injection paradigm (Figure 1B) was used to measure total cortical cell densities between 1-7

DPI. Stereology using Stereo Investigator software on Zeiss microscope was used to acquire estimated cell counts for total cells (Dapi+), actively dividing cells (CLDU+), neuroblasts (DCX+) and dividing neuroblasts (double-labeled CLDU+/DCX+) in the cortex of mice that received acute CldU injections at 1,3 or 7 DPI from anti-LPA and IgG treated groups. Representative images of n=4 mice/ treatment group were acquired from the peri-contusion cortical grey matter regions (**A**) at 1 DPI for IgG (**Bi-iii**) and Anti-LPA (**Ci-iii**) treatment groups to demonstrate the marked difference in early cortical neuroblast populations within 1 DPI. All data for estimated cell counts are displayed as mean and SEM (**D**). P-values are determined between treatment groups for each cell type, by bootstrapping the means around the 95% confidence intervals in R. P-values *P≤0.01, **P≤0.001, ***P≤0.0001; Scale bars: 40μM (**B & C**).

However, while at 3 DPI (**Figure 2Dii**) there was still significantly less neuroblasts for anti-LPA treatment (77 ± 17) as compared to the vehicle group (251 ± 37) as time progressed (3DPI and 7DPI) there was no effect of treatment for all other cell types (**Figure 2Dii-iii**). These two studies indicate that LPA signaling may have differential role in the SVZ and cortical progenitor cells, that perhaps Anti-LPA treatment is delaying cortical neuroblast migration. The data suggests that within the cortex, LPA signaling after injury may be stimulatory of cortical neurogenesis at early time points, whereas anti-LPA treatment may delay pro-neuronal fate direction of cortical neuroblasts or delay migration of neuroblasts to the cortex from the SVZ. We hypothesize that the delay in neuroblast formation in the cortex may be beneficial to prolong survival of neuroblast in the long term by avoiding the toxic environment of the injured cortex. To test this cortical neuroblast expression at later time-points was assessed.

Early Anti-LPA treatment enhances the cortical dividing neuroblast populations at chronic time points.

To identify the effects of LPA signaling on long-term cortical neuroblast survival; mice from each treatment group received one injection of CLDU per day for 7 days starting at 1 DPI to label dividing cells between 1 and 7 DPI; mice were then euthanized at 14 or 28 days after injury to allow for a 7 or 21 day wash-out period respectively in which the fate of the early dividing cells, within 7 DPI, could be tracked (**Figure 3B**). Stereology as previously described was used to identify total cells, dividing cells, neuroblasts and dividing neuroblasts at 14 and 28 DPI. High power representative images were taken from the peri-contusional cortex (**Figure 3 A; C-D**). For both time periods the data demonstrates a marked increase in neuroblasts and dividing neuroblast populations for the anti-LPA treated compared to the vehicle group with effect of treatment on the total cortical cells or dividing cell populations (**Figure 3 E**). Interestingly, the neuroblast populations in the cortex of the anti-LPA treatment group were observed in the superficial layers of the cortex in migratory chain patterning (**Figure 3Di**), similar to neuroblast migration through the rostral migratory stream (RMS); whereas this patterning was not observed in the cortex of the vehicle group. The data suggests that blocking LPA after injury promotes survival of neuroblasts in the cortex at later time points.

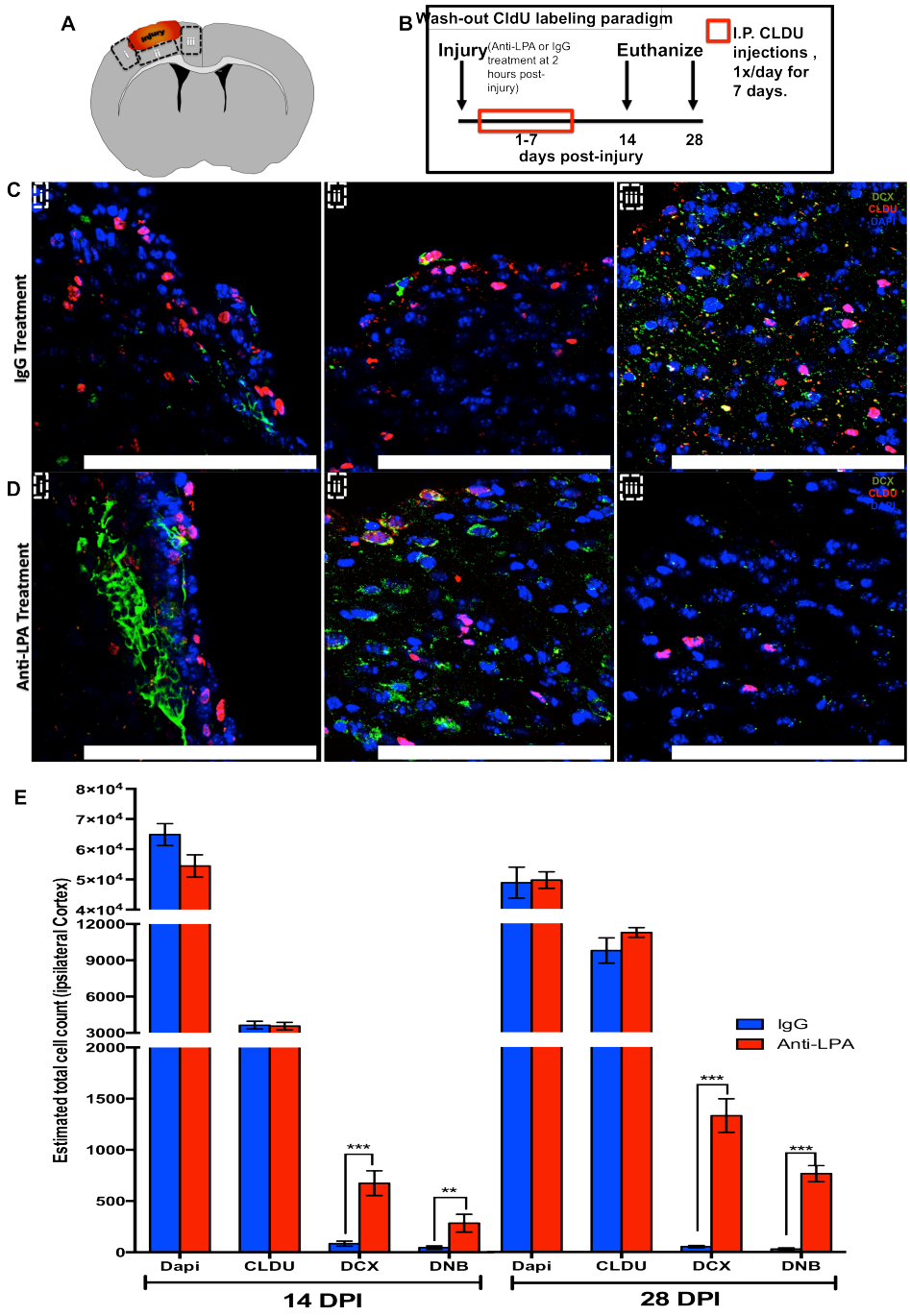

Figure 3: Anti-LPA therapy significantly enhances cortical neuroblast survival at 14 and 28 DPI. Following controlled cortical impact injury (CCI) actively dividing cells were labeled by (50mg/Kg) CLDU injections 1x/ day for 7 consecutive days starting at 1DPI; mice were euthanized at 14 or 28 DPI (**B**). Stereology using Stereo Investigator software on Zeiss microscope was used to acquire estimated cell counts for total cells (Dapi+), actively dividing cells (CLDU+), neuroblasts (DCX+) and dividing neuroblasts (double-labeled CLDU+/DCX+) in the cortex. Representative images of n=4 mice/ treatment group were acquired from

the peri-contusion cortical grey matter regions (**A**) at 28 DPI for IgG (**Ci-iii**) and Anti-LPA (**Di-iii**) treatment groups to demonstrate the marked increase in cortical neuroblasts at 28 DPI. All data for estimated cell counts are displayed as mean and SEM (**E**). P-values are determined between treatment groups for each cell type, by bootstrapping the means around the 95% confidence intervals in R. P-values *P≤0.01, **P≤0.001, ***P≤0.0001; Scale bars: 40μM (**C & D**).

Early Anti-LPA treatment promotes cortical neurogenesis.

The previous experiment suggested that since Anti-LPA treatment significantly enhanced neuroblast survival in the cortex, it may well enhance neuronal differentiation of dividing progenitor cells in the cortex. To determine the effects of blocking LPA after injury on long-term cortical neurogenesis, standard triple-label IHC for total cells, dividing cells, neurons (NeuN+) were performed on the above animals euthanized at 28 DPI. Stereology was performed to identify total cells, dividing cells, neurons and dividing neurons (double-labeled CLDU+/NeuN+) in the cortex. High-powered representative images were taken from the peri-contusional region of the cortex (**Figure 4 A**) for both treatment groups (**Figure 4 C-D**). There was statistical insignificance between treatment groups (p-value= 0.0501; power = 75%), however there is a clear enhancement of cortical neurogenesis for the Anti-LPA treated groups represented by the increased average of total neurons ($1.6x10^4$ $\pm 2.4x10^3$ vs. $1.0x10^4$ $\pm 1.8x10^3$) and dividing neuron ($2.4x10^3 \pm 5.4\ x10^2$ vs. $9.2\ x10^2$ $\pm 1.1x10^2$) for the Anti-LPA treated group compared to the vehicle group (Anti-LPA, Vehicle, respectively) (**Figure 4B**).

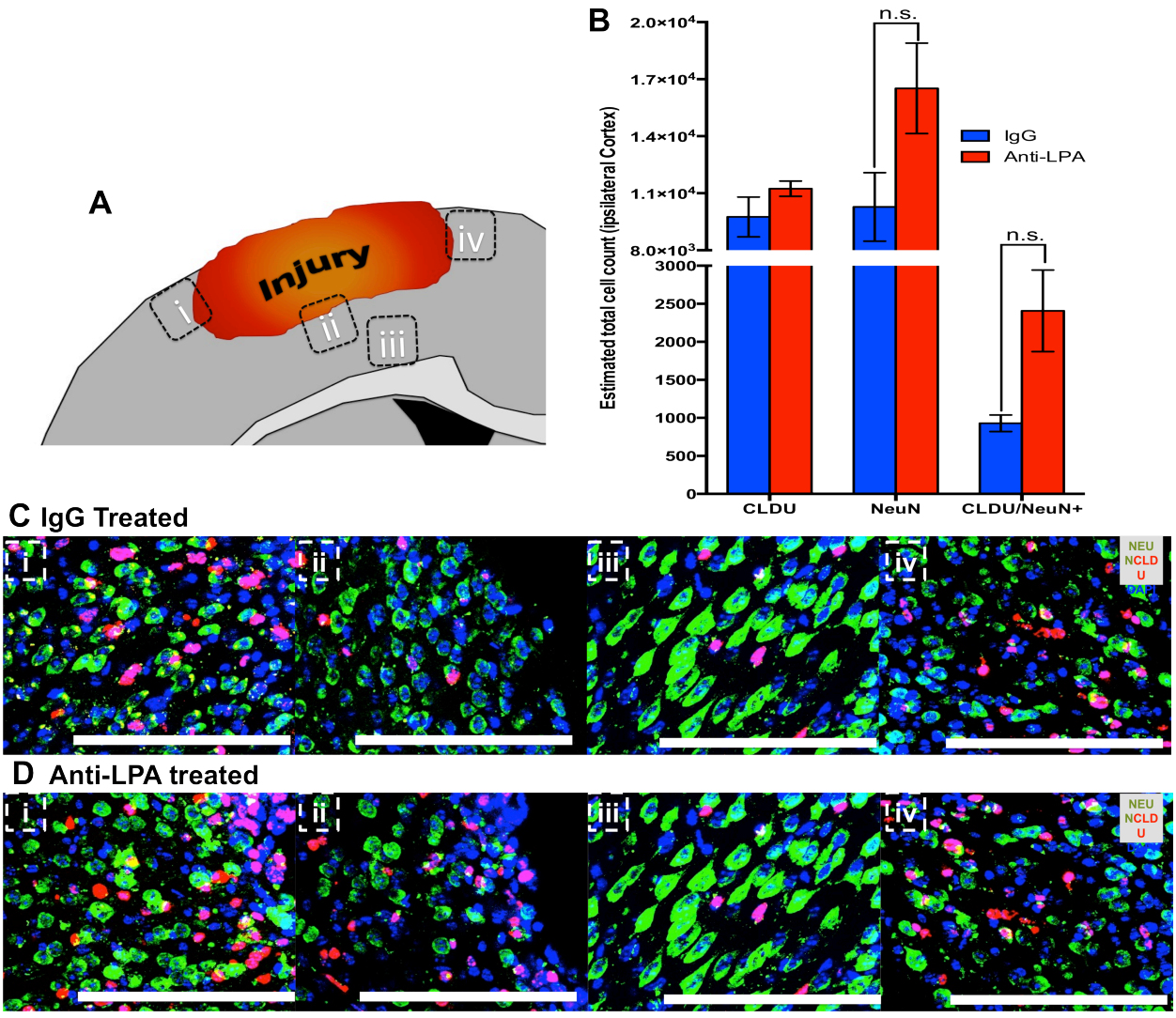

Figure 4: Anti-LPA therapy causes an apparent increase in cortical neurogenesis, though not statistically significant as compared to IgG treatment. Immunostaining of dividing cells (CLDU+) and mature neurons (NeuN+) in the cortex of Anti-LPA (**D**) and IgG (**C**) treated mice at 28 DPI were used to acquire estimated cell counts for total cells (Dapi+), actively dividing cells (CLDU+), neurons (NeuN+) and dividing neurons (double-labeled CLDU+/NeuN+) in the cortex. Representative images of n=4 mice/ treatment group were acquired from the peri-contusion cortical grey matter regions (**A**) at 28 DPI for IgG (**Ci-iv**) and Anti-LPA (**Di-iv**) treatment groups to demonstrate the distribution of neurons and dividing neurons within the cortex at 28 DPI. All data for estimated cell counts are displayed as mean and SEM (**B**). P-values are determined between treatment groups for each cell type, by bootstrapping the means around the 95% confidence intervals in R. Scale bars: 40μM (**C & D**).

SVZ neuroblast populations are significantly reduced with Anti-LPA treatment at 14 and 28 DPI.

To identify the longer-term effects of LPA signaling SVZ neuroblast populations, total cells, dividing cells, neuroblasts and dividing neuroblast populations were estimated from the dorsolateral SVZ (**Figure 5A**) of the same animals used in the previous experiment (**Figure 3 and 4**). Representative images and high-power insets for 28 DPI were attained (**Figure 5 B-C**). At 28 DPI the results revealed a significant reduction in total SVZ density ($7.5x10^3$. ± 5.4 $x10^2$ vs. $1.6x10^4$ ± 8.2 $x10^2$) dividing cells (2.3 $x10^2$ ± 46 vs. $1.4x10^3$ ± 2.2 $x10^2$) and dividing neuroblast (86 ± 1 vs. 2.4 $x10^2$ ± 38) populations for Anti-LPA treatment compared to vehicle (**Figure 5 D**); these trends were also reflected in the 14 DPI time point. The results suggest that reducing the post-injury LPA pulse reduces SVZ progenitor niche activation in the longer term.

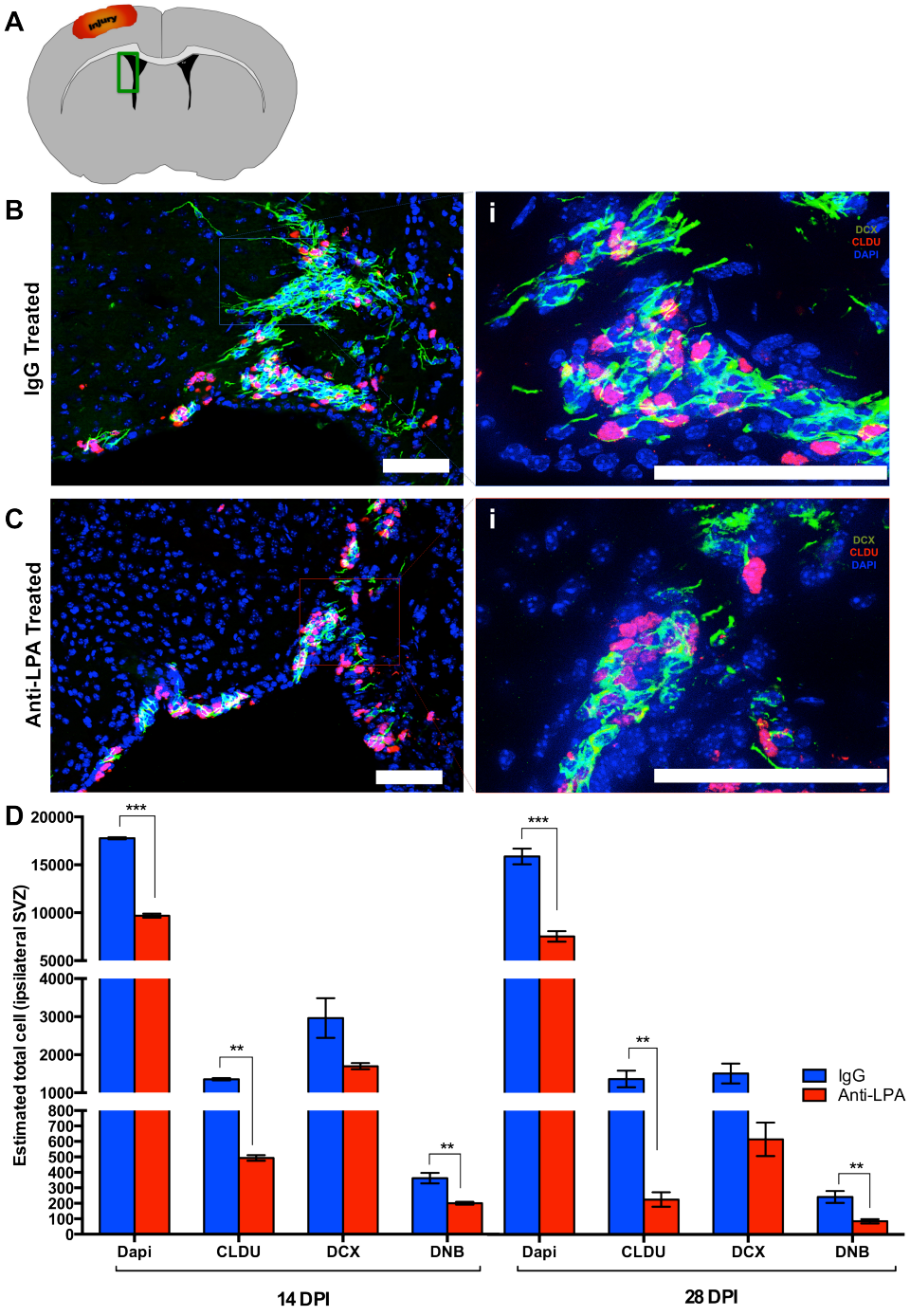

Figure 5: Anti-LPA therapy significantly reduces SVZ dividing neuroblast population at 14 and 28 DPI. CLDU injection paradigm (Figure 3B) was used to measure SVZ cell dividing neuroblast density at 14 and 28 DPI. Stereological techniques were used to acquire estimated cell counts for all SVZ cells (Dapi+), actively dividing cells (CLDU+), neuroblasts (DCX+) and dividing neuroblasts (double-labeled CLDU+/DCX+). Representative images of n=4 mice/ treatment group were acquired from the dorsolateral SVZ (**A; green rectangle**) at 28 DPI for IgG (**B; inset i**) and Anti-LPA (**C; inset i**) treatment groups to demonstrate the

marked reduction in SVZ neuroblast with Anti-LPA treatment at 28 DPI. P-values are determined between treatment groups for each cell type, by bootstrapping the means around the 95% confidence intervals in R. P-values *P≤0.01, **P≤0.001, ***P≤0.0001; Scale bars: 40μM (**B & C**).

Anti-LPA treatment reduces activated microglial populations in the SVZ at 14 and 28 DPI.

Long-term inflammation, identified by activated microglia, in the SVZ enhances neurogenesis of SVZ progenitor cells (Acosta et al., 2013; Deierborg, Roybon, Inacio, Pesic, & Brundin, 2010; Pluchino et al., 2008; Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014; Tepavčević et al., 2011). Furthermore, LPA signaling has been identified as a potent pro-inflammatory signaling molecule(J. W. Choi et al., 2010; S. Liu et al., 2009; Z. Zhang et al., 2006). Ionized calcium-binding adapter molecule 1 (Iba1) is used as a sensitive marker for microglial cells (Ito et al., 2001); and microglial morphology can be used to determine the density of activated microglia (T. Cao, Thomas, Ziebell, Pauly, & Lifshitz, 2012; Shapiro, Perez, Foresti, Arisi, & Ribak, 2009; Streit, Walter, & Pennell, 1999). Activated microglial cells, or cells in the intermediate stages between rest and activated are described to have rounded and swollen cell bodies with thick shrunken processes; where as resting microglial cells have oblong-shape cell bodies and thin, polar processes (**Figure 6**)

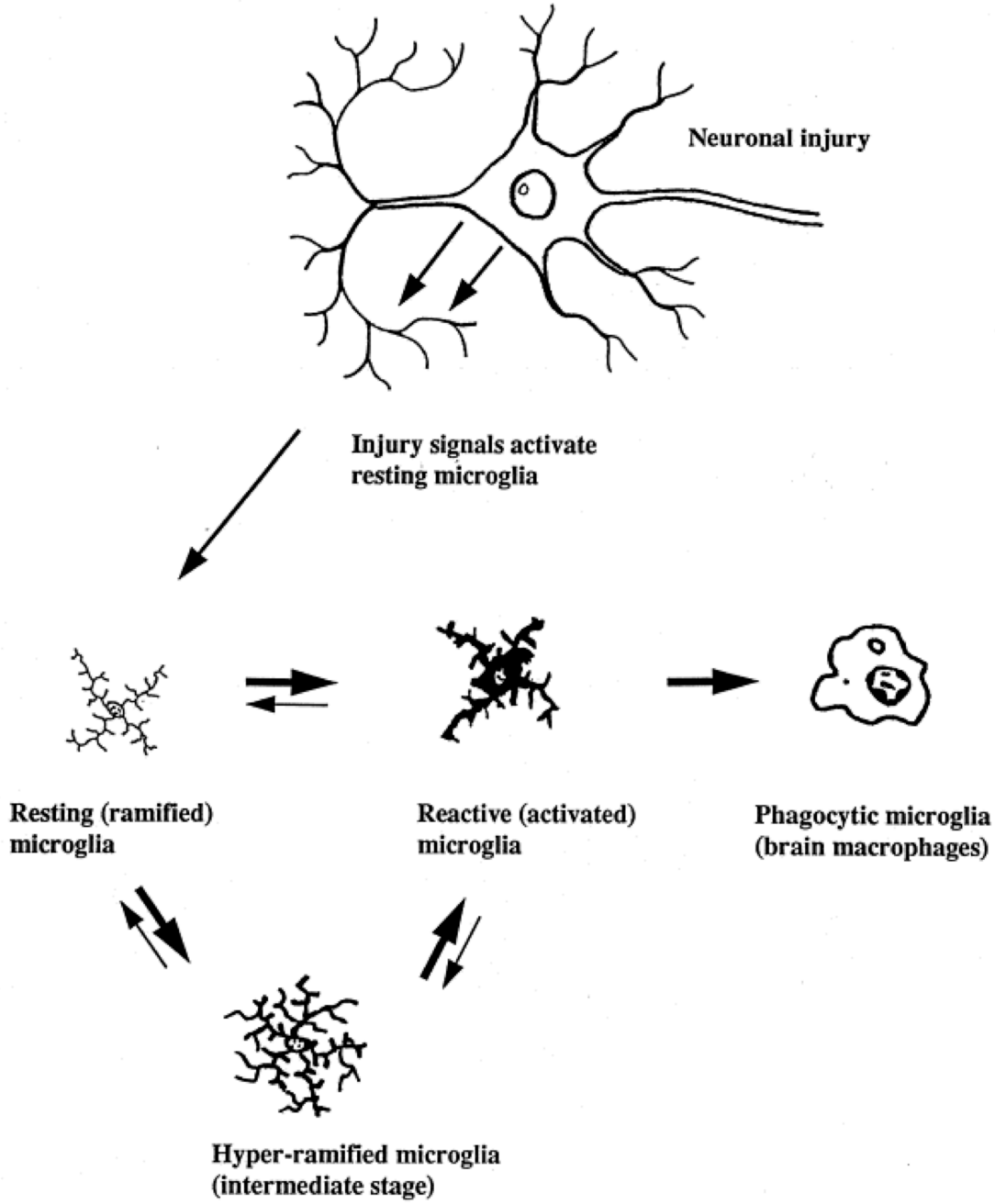

Figure 6: Functional plasticity of microglia. Injured or diseased neurons cause resting microglia to become activated by emitting injury signals (adapted from Streit, 1999). The degree of microglial activation varies with the severity of neuronal injury. The mildest injuries may only cause hyper-ramification of microglia (Wilson and Molliver, 1994), but most types of neuronal damage will cause resting microglia to become reactive microglia. If neurons die, microglia transform into brain macrophages and remove the dead cells. If an injured neuron recovers, hyper-ramified and reactive microglia may revert back to the resting form.

Microglia-derived brain macrophages probably do not revert back to the resting state, but may undergo cell death. (Streit et al., 1999).

Therefore, to determine the effects of LPA signaling on SVZ inflammation, IHC with DAB amplification was used to identify Iba1+ cells in the SVZ at 28 DPI. Morphological characterization of total microglial cells (Iba1+), resting microglia, and activated microglia were determined from the dorsolateral SVZ of both treatment groups (**Figure 7A**). Representative high power images of 4 mice / groups (5 sections/ mouse) demonstrate the marked difference in activated microglia (green star) and resting microglia (white arrows) in the SVZ between treatment groups (**Figure 7 B-C**). Cell counts demonstrate no significant change in total microglia and resting microglia populations between treatment groups, but a significant increase in activated microglia in the vehicle treated groups as compared to Anti-LPA treated group (vehicle: 69 ±6; Anti-LPA: 19 ± 4) (**Figure 7 D**). The data suggest that Anti-LPA intervention has long-term, anti-inflammatory effects in the SVZ and a reduction in long-term SVZ inflammation may explain the accompanying reduction in SVZ neurogenesis with Anti-LPA treatment (**Figure 5**).

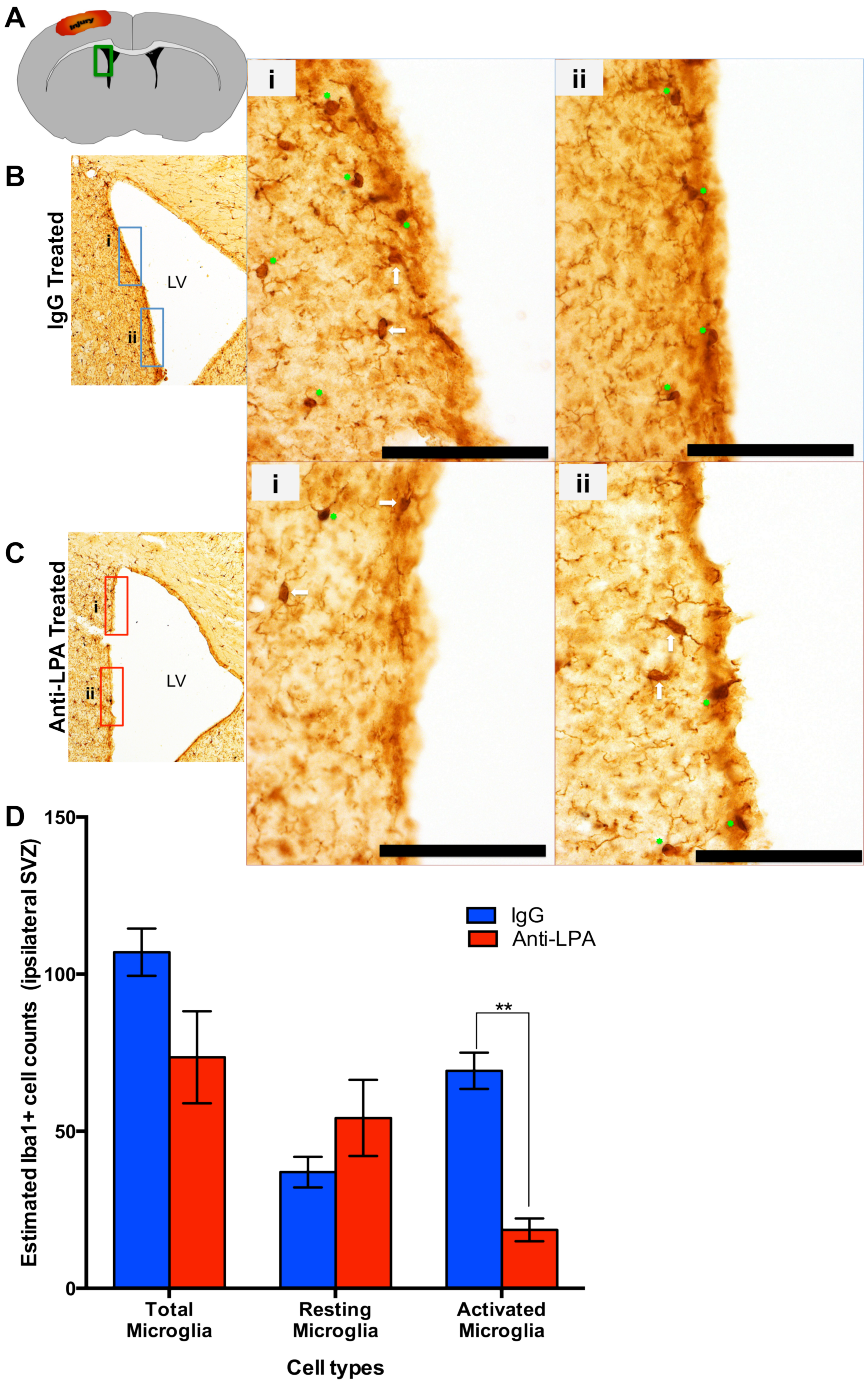

Figure 7: Anti-LPA therapy significantly reduces inflammation in the SVZ at 28 DPI. To determine the effects of Anti-LPA therapy on long-term inflammation of the SVZ, immunostaining of Iba1 with DAB amplification was used on IgG and Anti-LPA treated sections from 28 DPI. Representative images n=4 mice/ treatment group, from the dorsolateral SVZ regions (**A**; green rectangle) were attained from IgG (**B**) and Anti-LPA (**C**) treatment groups. High-power insets (**Bi-ii & Ci-ii**) show activated microglia with rounded cell bodies and short, thick processes (**green stars**) and resting microglia with oblong-shaped cell bodies and thin

processes (**white arrows**). Total cell counts were attained from the dorsolateral SVZ; n=5 sections/ mouse**.** All data for cell counts are displayed as mean and SEM (**E**). P-values are determined between treatment groups for each cell type, by bootstrapping the means around the 95% confidence intervals in R. P-values *P≤0.01, **P≤0.001, ***P≤0.0001; Scale bars: 30μM (**B & C**).

5.4 Discussion:

Several trauma and stroke studies have suggested that the endogenous neurogenic response of the brain after trauma has high therapeutic utility (Kernie & Parent, 2010b; Kreuzberg et al., 2010; Parent et al., 2002a; Richardson et al., 2010; W. Zheng et al., 2011). However the molecular signals that impede the survival of NPCs after injury was unknown. The results of this study provide evidence of LPAs role in NPC cell responses to injury. The results show an apparent enhancement in pro-neuronal progenitor cells and a trend toward enhanced survival and neurogenesis of those progenitor cells in the injured cortex with Anti-LPA treatment. Furthermore, the study provides evidence that inflammation, caused by LPA signaling, may underlie some of the responses of the SVZ. The results of this study further establishes LPAs role in trauma pathology and provides evidence of the long-term, pro-neurogenic effects of blocking LPA signaling on cortical progenitor cells after TBI.

Anti-LPA treatment stimulates neuroblast populations of the SVZ at early time points after injury.

The results demonstrate anti-LPA treatment modifies progenitor cell behavior after injury and directs proliferating NPCs towards a pro-neuronal fate up to 7 days after injury. The results suggest that blocking LPA after injury alters the early fate determination of SVZ neuroblasts.

Interestingly, between 3 and 7 days there is an apparent reversal in relative cell numbers of total SVZ cells with a mirrored reversal in neuroblast populations between treatment groups. At 3 DPI the anti-LPA treated groups have enhanced total cells as well as enhanced neuroblast populations as compared to the vehicle treated and at 7 DPI the vehicle treated groups have more total cells and neuroblasts as compared to Anti-LPA treated mice (**Figure 8**). This suggests that after injury Anti-LPA treatment stimulates SVZ neuroblasts to divide and enhance proneuronal cell types earlier than without Anti-LPA treatment. Studies in human embryonic stem cells (hESC) have demonstrated that LPA signaling decreases neuronal differentiation and that one dose of LPA to neural stem/progenitor cells (NS/PCs) derived from hESCs and human induced pluripotent stem cells (iPSCs) cultures reduced neuronal differentiation and inhibited proliferation of cultures (Dottori et al., 2008; Frisca et al., 2013). The results of this study supportive of the inhibitory role of LPA signaling on progenitor cells and suggests that perhaps, LPA signaling after injury may keep cells in an immature phenotype while blocking LPA signaling, with Anti-LPA, may signal cells towards a more pro-neural, differentiated state. The findings also demonstrate that blocking LPA after injury allows SVZ progenitor cells to respond earlier to injury by proliferating, directing to a pro-neuronal fate and perhaps a significant amount of the SVZ NPC from anti-LPA treated groups have migrated out of the SVZ and headed toward the injury site by 7 DPI.

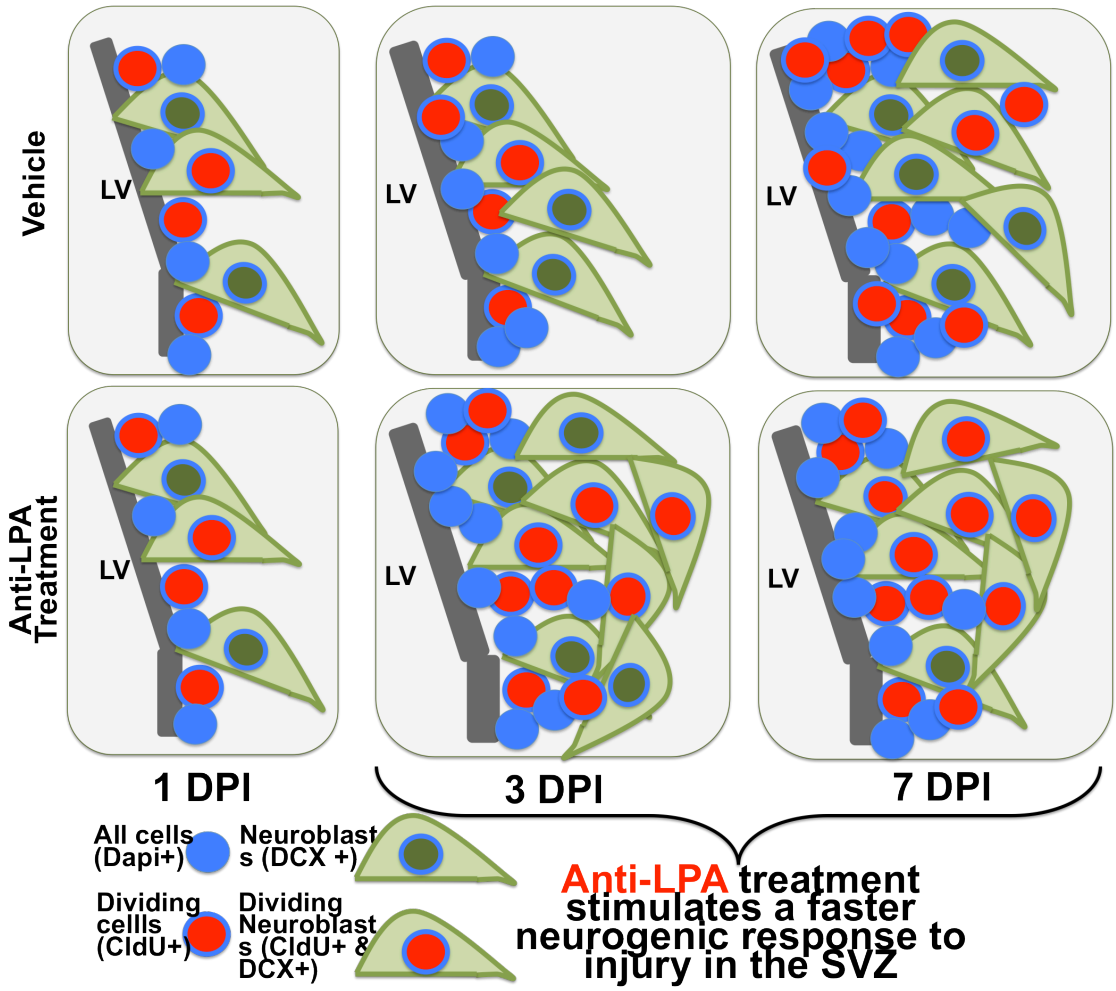

Figure 8 Summary diagram of Anti-LPA treatment stimulating a faster neurogenic response to injury in the SVZ

Anti-LPA treatment enhances cortical neuroblast populations at early time points.

The effects of Anti-LPA intervention in the cortex within 3 days of injury appear to be inhibitory to neuroblast expression while maintaining the actively dividing cell populations in the cortex. Particularly, at 1 and 3 DPI, anti-LPA treatment significantly reduces the neuroblast population in the cortex as compared to the vehicle group (**Figure 9**). The reduction in cortical neuroblasts with anti-LPA treatment suggests that blocking LPA after injury is delaying neuronal maturation of progenitor cells at 1 and 3 DPI and at 7 DPI cortical neuroblast populations for anti-LPA treated animals start to enhance to similar levels as the vehicle group. The findings suggest that the LPA pulse in the brain after injury enhances pro-neuronal progenitor cells at early time points and thus blocking LPA with Anti-LPA delays this early response. Studies of cortical neuroblasts support these findings and show that LPA treatment in the cortex enhances neuronal differentiation (Nobuyuki Fukushima et al., 2007). The findings herein are supportive of the pro-neurogenic effects of LPA signaling in the cortex, at least within early time points. However, the persistent LPA signaling likely perpetuates the excitotoxic environment of the cortex, therefore inhibiting the survival of the cortical neuroblast at later time points.

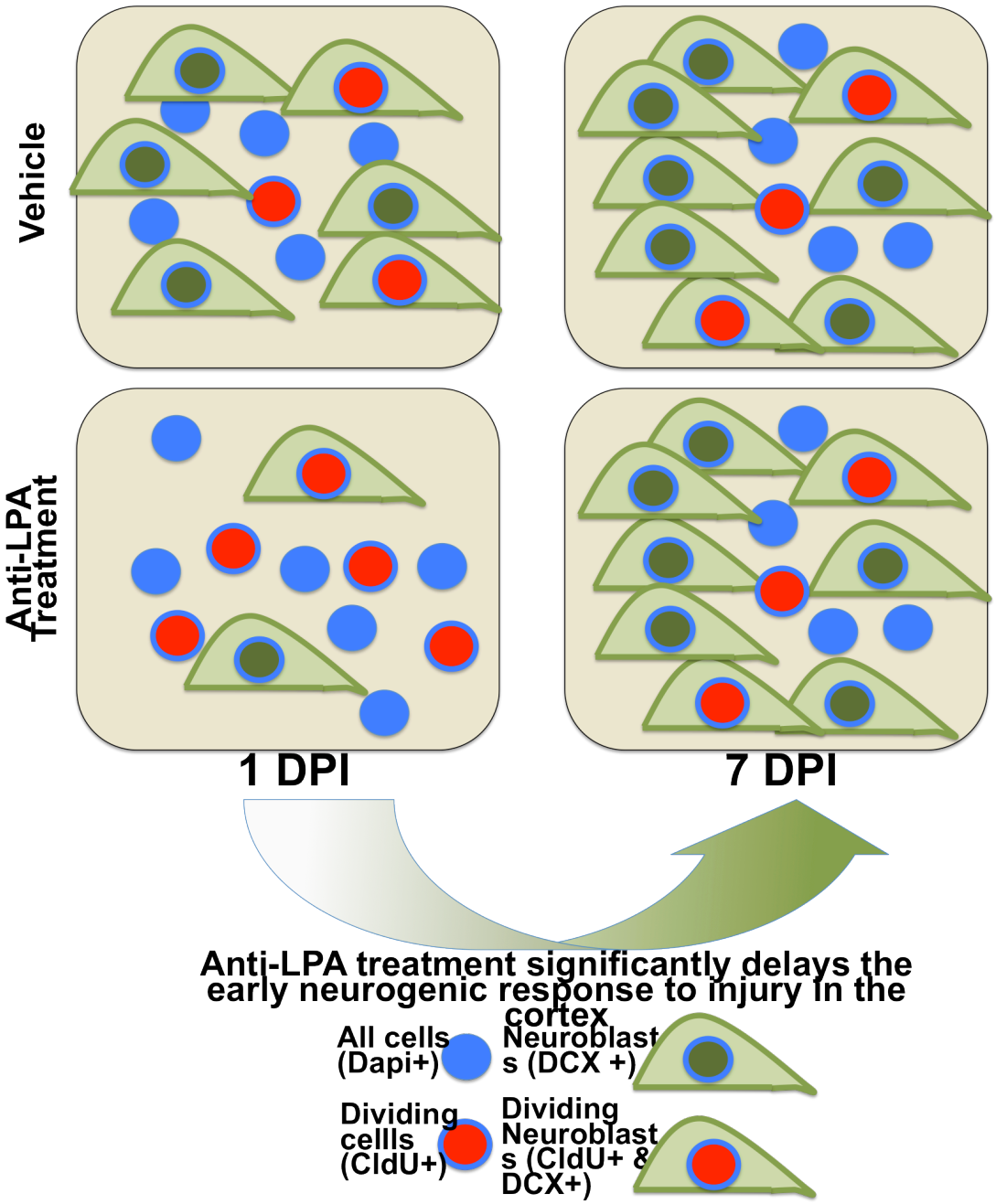

Figure 9: Summary diagram of the delayed neurogenic response in the cortex with Anti-LPA treatment at acute time points.

Anti-LPA treatment enhances the cortical neuroblast populations at later time points

The results of this study demonstrate a drastic reduction in neuroblasts and dividing neuroblast in the cortex of the vehicle treated mice as compared Anti-LPA treatment (**Figure 10**). The effects of LPA signaling on long term neurogenesis has yet to be explored until now and the results show that the LPA pulse after injury is inhibitory to neuroblast expression in the cortex at later time points. Interestingly, neuroblasts in the cortex of anti-LPA treated mice were grouped in chain migration morphology, similar to the migratory patterns of SVZ born cells through the rostral migratory stream that differentiate into functional neurons (James et al., 2011; W. Sun et al., 2010; P Taupin & Gage, 2002). Anti-LPA treatment enhancing the expression of cortical neuroblasts in a similar migratory pattern as that observed under physiological conditions indicates that blocking the LPA pulse increases cortical neuroblast survival and perhaps also increases the likelihood of neuroblasts to perform similar neural replacement functions to that observed under physiological conditions. Although significant difference was not attained between treatment groups for the mature neuronal populations, there is a clear trend toward enhanced overall neurogenesis as characterized by double-labeled CLDU/ NeuN+ cells for the Anti-LPA treated groups. This suggests that Anti-LPA treatment could potentially enhance neurogenesis at later time points not captured in this study.

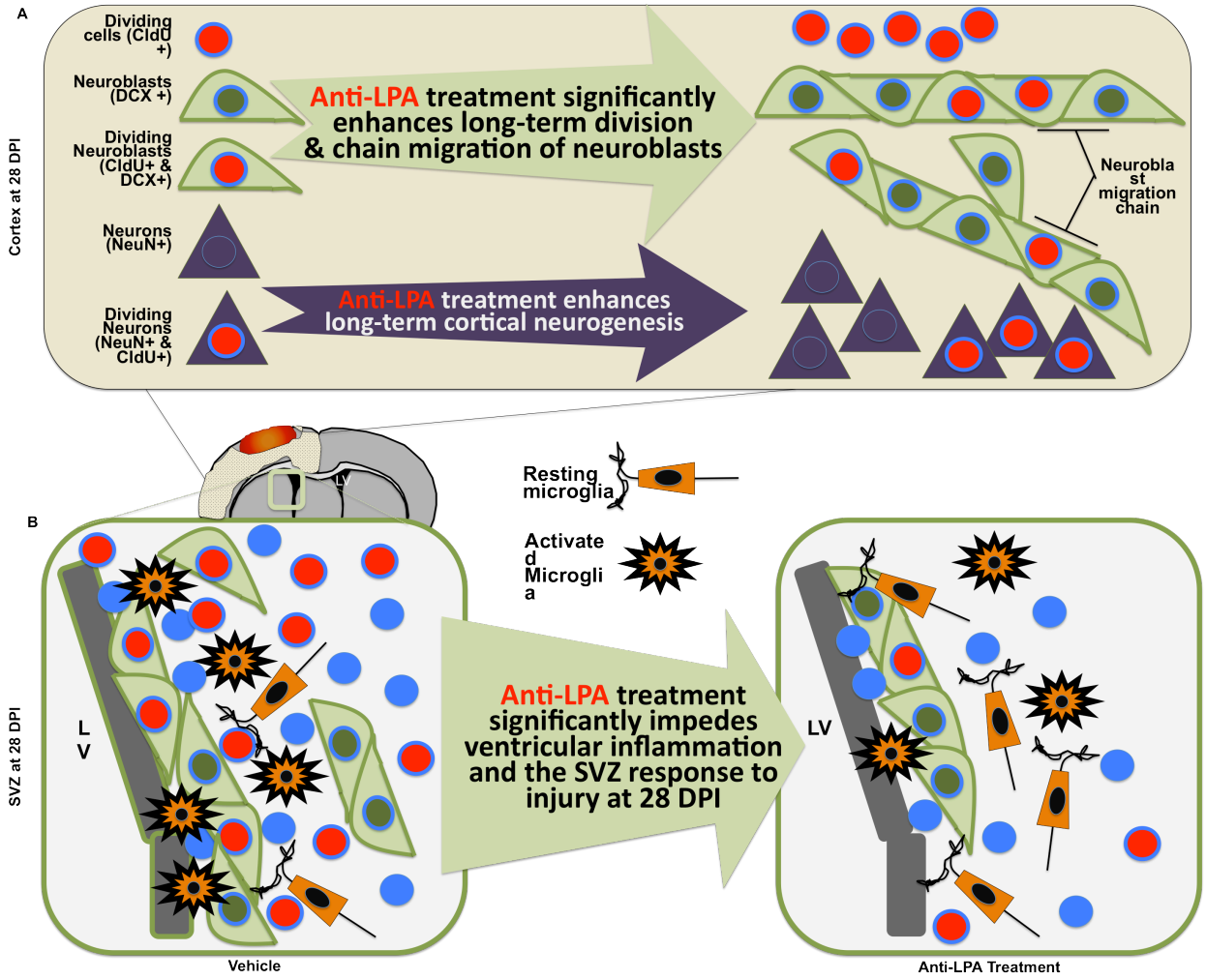

Figure 10: Summary diagram of the pro-neurogenic effects of Anti-LPA treatment in the cortex and anti-inflammatory effects of Anti-LPA treatment in the SVZ at chronic time points.

Anti-LPA treatment reduces SVZ neuroblast and activated microglial populations at chronic time points.

Inflammation has potent effects on progenitor cells and studies have shown that enhanced, long-term inflammation in the SVZ leads to enhanced neurogenesis of SVZ progenitor cells (Acosta et al., 2013; Deierborg et al., 2010; Shigemoto-Mogami et al., 2014). Studies have also demonstrated that LPA signaling is pro-inflammatory (Fourcade et al., 1995; Z. Zhang et al., 2006). The results herein support these findings and suggest that blocking LPA reduces long-term inflammation in the SVZ and consequently, reduces neuroblast activation at later time points after injury (**Figure 10**). The effects of Anti-LPA intervention in the SVZ after TBI are anti-inflammatory. The study provides evidence of the potent effects of LPA signaling on the endogenous neurogenic response to injury and the beneficial effects of blocking the LPA pulse on cortical neuroblast survival after TBI.

6. Chapter 6 - Conclusion & Future Directions

6.1 Conclusion:

The outcomes of traumatic brain injury depend heavily on the pathogenesis of secondary injuries. Complex signaling cascades are involved in the pathology of TBI and thus, mitigating the effects of secondary injuries on function has been a major feat in the neurotrauma research field. One major challenge in developing effective TBI therapeutics is identifying the signaling molecule involved in initiating and perpetuating secondary injury pathogenesis. Another challenge is to efficiently mitigate the effects of that molecule at a crucial window after injury that will aid in enhanced recovery (Menon, 2009; Xiong, Mahmood, & Chopp, 2009). Although several studies have demonstrated that LPA metabolism is increased in the CSF as well as in discrete brain regions after injury; that LPA signaling through GPCRs is enhanced in the brain after injury; and that LPA alone, can initiate secondary injury pathogenesis and even modulate adult progenitor cell function and differentiation (Abdullah et al., 2014; Demediuk et al., 1988; Dottori et al., 2008; Farooqui et al., 1997; Fourcade et al., 1995; Frisca et al., 2013; Frugier et al., 2011; Goldshmit et al., 2012; Pasvogel et al., 2010; Ueda et al., 2013; Yakubu, Liliom, Tigyi, & Leffler, 1997; Z. Zhang et al., 2006); no study has identified the role of LPA on adult TBI and the effects of blocking LPA signaling on outcomes. The work of this dissertation has been aimed towards gaining a more comprehensive understanding of the role of LPA in the adult brain after injury and determining the effects of mitigating LPA signaling on injury outcomes and adult neurogenesis.

Aim 1: To identify the spatial-temporal profile of LPA in the injured brain and correlate changes in phospholipid metabolism to early markers of degeneration.

Study 1 identified spatial and temporal enhancement of LPA metabolism in the brain within 3 hours of injury using MALDI-IMS. The findings demonstrated a significant increase in brain-wide LPA metabolism specifically through the white matter tracts and in the cerebellum at 3 HPI. The study also demonstrated a significant increase in LPA precursors within the peri-contusional cortex, sub-cortical white matter and anterior nucleus of thalamus at 1 HPI in spatial association with markers of neural degeneration and axonal injury. The enhancement of LPA precursors was followed by a spike in LPAs bioactive unsaturated species at 3 HPI at the site of injury and in the peri-contusional cortex in association with markers of neural degeneration. Studies suggests that changes in LPA metabolism after TBI originate from the broken BBB at the injury site, although our findings support this claim we also demonstrated a significant enhancement in LPA metabolism in brain regions distal from the injury site not associated with blood. Based on the spatial distribution of LPA's metabolites with markers of degeneration the data also suggests LPA metabolism is involved in the pathogenesis of injury. Furthermore, these findings suggested the optimal therapeutic window to attenuate LPA signaling would be most effective at 2-hour post-injury, based on the findings that LPA metabolism is initiated within 1 hour after injury and at 3 hours LPAs bioactive species are expressed in the injured brain.

Aim 2: To determine if block LPA with an LPA antibody prevents major secondary hallmarks of injury and improves behavioral outcomes.

In order to more directly examine the therapeutic effects of attenuating LPA signaling at the 2HPI window of intervention, Study 2 determined the effects of a one-time dosage of Anti-LPA on secondary injury outcomes and sensorimotor skills in a CCI mouse model. The results determined Anti-LPA treatment enhanced white matter pathologies but had not significantly affected grey matter secondary injuries. Specifically, the data showed that Anti-LPA treatment reduced axonal injury within 3 days and attenuated white matter inflammation at 14 and 28 days after injury. In addition, Anti-LPA intervention significantly enhanced sensorimotor recovery of both the front and hind limbs through 70 DPI. Despite long-term improvement in function, Anti-LPA treatment had no significant effect on grey matter pathologies: neuronal degeneration at 1 DPI, inflammation at 14 and 28 DPI or contusion volume at 70 DPI. The findings of this study suggested that after cortical injury, targeting grey matter pathology might not produce the most functional improvement perhaps because the grey matter, after cortical trauma, is unsalvageable. However, the data demonstrated that attenuating white matter pathologies might have a more potent effect on improving functional outcomes after injury, perhaps by maintaining functional connections to remaining grey matter regions. Previous in-vitro studies have demonstrated that LPA signaling caused collapse of growth cones, retraction of neuronal projections and demyelination (Fujita et al., 2007; Nobuyuki Fukushima & Ueda, 2013; Jalink et al., 1993; Li, Yang, Selzer, & Hu, 2013; Sayas, 1999; Gabor Tigyi et al., 1996; Ueda & Ueda, 2011). The results of study 2 support these findings and suggests that LPA signaling enhanced after injury has detrimental effects on axons in-vivo, furthermore that attenuating the release of LPA within

2 hours of injury reduces the effects of LPA signaling on white matter axons, thus enhancing recovery perhaps by maintaining functional connection.

Aim 3: To determine if blocking LPA after injury affects the brains endogenous neurogenic response to injury

Therapy that enhances function through axonal protection in lieu of neuronal protection is perhaps unlikely in that functional axons need functional neurons in-vivo in order to survive (Coleman, 2013). The findings of Study 2 suggested that perhaps, Anti-LPA also consolidated functional connections onto a neuronal sub-population that was some how maintained or produced after injury. The question remains as to why or how this neuronal sub-population is maintained. Several studies have suggested that the endogenous progenitor population of the adult SVZ could act as a neural replacement mechanism after injury (Blizzard et al., 2011; Gu et al., 2000; Leung, Coulombe, & Reed, 2007; S S Magavi et al., 2000; D. Sun, 2014; Xiong et al., 2010). Therefore, Study 3 evaluated the effects of Anti-LPA intervention on the endogenous neurogenic response in the SVZ and the expression of progenitor cells in the cortex after injury. The finding demonstrated a paradoxical effect of Anti-LPA treatment on both the SVZ and cortex after injury. Within 7 days of injury Anti-LPA intervention quickly enhanced the SVZ neuroblast population where as therapy reduced cortical neuroblast expression. However, at later time-points, 28 DPI, there was a significant enhancement in cortical neuroblast expression and chain-migration patterning as well as a trending, though not significant, increase in mature neurons. The data suggested that Anti-LPA treatment enhances the number of pro-neuronal progenitor cells in the cortex and perhaps overall neurogenesis at later time points. Interestingly,

Anti-LPA intervention caused a marked reduction of SVZ response at later time point as well as a significant reduction in activated microglia. Since studies have shown that LPA signaling is pro-inflammatory and that long-term SVZ inflammation induces long-term activation of the SVZ progenitor cells (Acosta et al., 2013; Deierborg et al., 2010; L. Ma et al., 2013; Möller et al., 2001; Shigemoto-Mogami et al., 2014; Tham et al., 2003); then the results of Study 3 suggests that attenuating LPA signaling after injury reduces inflammation and consequently the SVZ response. Furthermore, since a prolonged response of the SVZ to neurogenesis is thought to be symptomatic of injury severity (R.L Zhang, & Chopp, 2001; Rui Lan Zhang et al., 2011) this suggests that reducing long-term activation of the SVZ niche is indicative of a less severe injury. The results of Study 3 suggests that LPA signaling has differential effects on the cortical and SVZ progenitor cells and also suggests that blocking LPA after injury enhances cortical neuroblasts and reducing the long-term response of the SVZ to injury perhaps by attenuating SVZ inflammation. Taken together the studies herein suggested that LPA is a potent biomarker for TBI and Anti-LPA is a promising TBI therapeutic.

6.2 Future Directions:

Progression of TBI therapeutics is severely lagging in comparisons to the progresses made in heart disease and cancer therapeutics. Over 30 clinical trials for TBI interventions have failed in part due to the complexities of disease progression, the lack of a known therapeutic window and the unknowns of how early indicators correlate with outcomes (Narayan et al., 2002). Despite this, of the 283 clinical trials for TBI currently active around the globe, only a single study aims to identify biomarkers of TBI and make long-term clinical correlates with outcomes (National Institutes of Health Clinical Center (CC), 2010). Of the interventional TBI clinical trials (n=205), most of them are symptomatic targets and only 7 studies aim to improve overall outcomes after injury. Based on success rate of past TBI therapeutic studies and the complexities of the disease it's likely that most of the current interventional trials will also fail. Furthermore, despite the potent effects of phospholipid metabolism in the brain, not a single clinical trial for TBI is targeting phospholipid signaling. This highlights the urgent need to develop a therapeutic against early biomarkers for TBI aimed at improving overall outcomes after injury. Therefore, although the studies of this dissertation have provided strong evidence that LPA is a biomarker for TBI and Anti-LPA is a promising therapeutic, further studies are needed to establish LPA as a TBI biomarker and improve Anti-LPAs probability of clinical trail success.

Future Aim 1: To characterize the longer-term spatiotemporal profile of LPA metabolism and the effect of Anti-LPA treatment on this profile after injury.

The results of Aim 1 have provided evidence of the increase in LPA metabolism throughout the brain after injury, however longer-term effects of injury on phospholipid metabolism remains elusive. Furthermore, identifying the effects of Anti-LPA on mitigating further increase in phospholipids in the injured brain is unknown. The therapeutic efficacy of Anti-LPA to actually reduce the propagation of LPAs effects after injury is key in order to determine a correlation between enhanced LPA metabolism and the pathogenesis of injury. In addition, a reduction in activation of LPAs GPCRs and down-stream signaling cascades is a likely effect of Anti-LPA intervention and identifying these changes would be an essential step to ultimately determine if Anti-LPA treatment mitigates LPA signaling after injury. To further suggest that LPA signaling is mitigated by Anti-LPA it would be key to detect a reversal of functional impairment at a structural level with anti-LPA treatment. Several studies have demonstrated diaschisis and a significant reduction in functional connectivity of the brain after injury (Bette, Liaghat, Marquez De La Plata, & Diaz-Arrastia, 2012; C. Cao & Slobounov, 2010; Castellanos et al., 2011; Harris, Chen, & Pickard, 2013; Harris et al., 2010; Holschneider et al., 2013; A. M. Mishra et al., 2014; V. L. Morgan & Price, 2004; Nishibe et al., 2010; Turner, McIntosh, & Levine, 2011, 2012). Study 1 also demonstrated a similar distribution of phospholipid metabolism with diaschisis-type changes after injury. Therefore, if Anti-LPA is indeed enhancing recovery its effects will likely enhance functional connectivity after injury. BOLD, fMRI and EEG techniques would be useful in identifying if Anti-LPA intervention actually strengthens functional connectivity after injury. The results of this study would strengthen the correlation between LPA signaling and TBI pathogenesis as well as further establishes Anti-LPA as an effective therapeutic.

Future Aim 2: To determine causation of LPA signaling and secondary injury progression and to identify the optimal dose of Anti-LPA to mitigate the effects of LPA signaling.

Several studies have suggested that LPA signaling is one of the primary signaling molecules involved in initiating secondary injuries related to TBI (Crack et al., 2014; Eichholtz et al., 1993; Frugier et al., 2011; Goldshmit et al., 2010). Studies have also implied that high concentrations of LPA initiate pathology cascades while low levels of LPA maintain normal cell structure and function (Elmes et al., 2004; Gustin, Van Steenbrugge, & Raes, 2008; Frederick W. Holtsberg et al., 1998; Hosogaya et al., 2008; G Tigyi et al., 1994; Z.-Q. Zheng et al., 2004). However no study has actually identified the concentration-dependent effect of LPA signaling on the adult brain in-vivo. Although Study 2 of this dissertation suggests that Anti-LPA improves white matter pathology, no study has yet to draw a causative relationship between LPA signaling and injury pathogenesis. Therefore the question remains; in the adult brain, does LPA signaling alone initiate secondary injury cascades? In order to determine causation, it's crucial to develop a positive control group in which uninjured animals were treated with LPA at concentrations identified in TBI pathology. LPA receptor activation, secondary injury outcomes and function would be assessed in these animals and the results would determine if LPA signaling after injury actually initiated secondary injury cascades. Furthermore, if causation was determined, then those animals could be treated with anti-LPA in order to determine the optimal dose required to mitigate the effects of LPA signaling.

Future Aim 3: To determine the effects of Anti-LPA treatment on learning, memory, decision-making and social behavior.

Since the current research was centered on a sensorimotor injury model, it would be important for future research determine the therapeutic effects of Anti-LPA on memory, balance, cognition and emotion as an indicator of improved quality of life. Among the devastating symptoms of TBI are motor impairments as well as major depressive symptoms, psychosocial impairments and cognitive disabilities (Corrigan, Selassie, & Orman; Dimoska, McDonald, Pell, Tate, & James, 2010; Fann, Hart, & Schomer, 2009; Langlois et al.; Ponsford, Kelly, & Couchman, 2014; Saxton, Younan, & Lah, 2013; Shields, Ownsworth, O'Donovan, & Fleming, 2015; Starkstein & Pahissa, 2014; Wortzel & Arciniegas, 2012). These symptoms are also targets of many TBI clinical trials (Goldstein, Levin, Goldman, Clark, & Altonen, 2001; Hawley & Joseph, 2008; Rapoport, McCauley, Levin, Song, & Feinstein, 2002; Twamley, Jak, Delis, Bondi, & Lohr, 2014; von Steinbüchel et al., 2010). Therefore, identifying the efficacy of Anti-LPA treatment to improve cognition and emotion in addition to motor skills this would position Anti-LPA as one of the most promising TBI therapeutics.

6.3 Closing Remarks

The role of phospholipids in the adult brain is virtually unchartered territory for TBI therapeutics. Albeit, the evidence exists that suggests LPA signaling is involved in initiating secondary injury cascades of trauma. However, a more comprehensive understanding of LPAs involvement in the early events immediately after injury and identifying LPAs direct role in functional outcomes is needed to establish LPA as a TBI biomarker and Anti-LPA as a potent therapeutic. Taken together, the results of this dissertation provide initial evidence that LPA is

involved in trauma pathogenesis and Anti-LPA enhances motor recovery. As the ultimate goal is to provide an effective therapy that will improve overall functional outcomes after TBI, it is hoped that answers to questions posed in this dissertation will help in achieving this goal. This is unquestionably an exciting time in the field of traumatic brain injury therapeutics.

7. Appendix

7.1 Abbreviations

Anti-LPA	(see Lpathomab)
Ara-C	cytosine-ß-D-arabinofuranoside
ATX	autotaxin
B cell	SVZ astrocytic stem cell
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
Beta-APP	beta - amyloid precursor protein
BrdU	5-Bromo-2'-deoxyuridine
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
C cell	SVZ transit-amplifying cell
CC	Corpus callosum
CCI	controlled cortical impact
CldU	5-chloro-2'-deoxyuridine
CNS	central nervous system
CSF	cerebrospinal fluid
CTX	cortex
DAG	Diacylglycerol
DAI	diffuse axonal injury
DCX	doublecortin
DG	dentate gyrus
DHB	2,5-dihydroxybenzoic acid
DNB	dividing neuroblasts/ CLDU+ & DCX+
DPI	days-post injury
E cell	SVZ ependymal cell
EDG	endothelial differentiation gene
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
Enpp2	phosphodiesterase family member
FABP	fatty acid binding protein
FGF	fibroblast growth factor
Fig.	figure
FJB	Flurojade B
FT-ICR	Fourier transform ion cyclotron resonance
GFAP	glial fibrillary acidic protein
GPAT	glycerophosphate acyltransferase
GPCR	G protein-coupled receptor
HEME	hemeoglobin

hESCs	human Embryonic stem cells
HPI	hours post-injury
Iba1	Ionized Calcium-Binding Adapter Molecule 1
IgG	Isotype-matched vehicle control
IMS	Imaging Mass Spectrometry
iPSCs	human induced pluripotent stem cells
IHC	Immunohistochemistry
K^+	Potassium
L.V.	lateral ventricle
LCAT	lecithin cholesterol acyltransferase
LPA	Lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
LPAR	LPA receptor
Lpathomab	Monoclonal mouse antibody against LPA
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPP	lipid phosphate phosphatases
MAG	monoacylglycerol
MALDI	Matrix-assisted laser desorption/ionization
MAP	microtubule-associated protein
M/Z	Mass to charge ratio
n	number of samples
Na^+	Sodium
NeuN	Mature neuronal marker
NPC	neuroprogenitor cell
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PLA1	phospholipase A1
PSA-NCAM	polysialylated neural cell adhesion protein
PS-PLA1	phosphatidylserine-specific phospholipase A1
RMS	Rostral migratory stream
ROI	region of interest
S phase	DNA synthesis phase
S1P	Sphingosine-1-phosphate
SEM	standard error of the mean
SCI	spinal cord injury
sPLA2	secretory phospholipase A2
TBI	Traumatic Brain Injury
Vehicle	(see IgG)
VEGF	vascular endothelial growth factor
VZ	ventricular zone
WM	white matter

8. References

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