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Lysophosphatidic acid receptor-1 and 2 (LPA_1, LPA_2): Novel roles in brain and vascular development

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Siew T. Teo

Committee in charge:

Professor Jerold Chun, Chair
Professor Judith Varner, Co-chair
Professor Joan Heller-Brown
Professor Anirvan Ghosh
Professor Binhai Zheng

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The dissertation of Siew T. Teo is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

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DEDICATION

This dissertation is dedicated with love and gratitude to my parents and my sisters, who have unconditionally provided me with so much love and happiness over the course of my life thus far. I would like to thank my fiancé, Deron Herr, who has supported me mentally and emotionally during the course of my degree. Last, but not least, I would like to dedicate this to the fond memory of my grandfather, who has taught me by example to lead a life of integrity and honesty, and to stay true to what is important.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Figures

Tables

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VITA

February 12, 1983	Born in Singapore
2002-2005	Bachelor of Science, Cell and Molecular Biology University of Michigan, Ann Arbor
2005-2006	Research Assistant, Institute of Molecular and Cell Biology, Singapore
2006-2011	Doctor of Philosophy, Biomedical Sciences University of California, San Diego

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Lysophosphatidic acid receptor-1 and 2 (LPA_1, LPA_2):

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Siew T. Teo

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Professor Jerold Chun, Chair
Professor Judith Varner, Co-Chair

Hypoxic insult during early pregnancy can give rise to neuronal disorders of the newborn, such as autism, schizophrenia and epilepsy. Of these disorders, the underlying similarity is cortical dysplasia, in which the neuronal layers are disrupted due to abnormal neuronal migration. The infliction of hypoxia during the critical period of neuronal development and migration can cause abnormalities which can propagate into these migration defects observed after birth. The purpose of this dissertation is to investigate the effect of hypoxia on early neuronal development, and the role of a certain receptor, lysophosphatidic acid receptor-1 (LPA_1) in this pathological process.

LPA_1 is one of 6 G-protein-coupled-receptors (GPCRs) which are activated by a bioactive lipid, LPA. It is highly enriched in the ventricular zone of the cortex during development, suggesting its importance in neural progenitor cells (NPCs). Previously, LPA treatment of *ex vivo* cortices at embryonic age 13.5, a period of neurogenesis and

early neuronal migration, was reported to induce ectopic displacement of mitotic NPCs. Strikingly, in this study, a similar displacement of mitotic NPCs, together with delayed early post-mitotic neuronal migration and N-cadherin disruption, was observed in cortices exposed to hypoxia, which was obliterated in LPA_1 null cortices. This indicates that LPA_1 is an essential mediator of hypoxia in the developing cortex. Through the use of pharmacological inhibitors, it was demonstrated that hypoxia signals through LPA_1 through Gi and subsequent downstream effectors. Importantly, a novel mechanism of how hypoxia activates LPA_1 was discovered: Hypoxia inhibits GRK2, a GPCR kinase which associates with LPA_1 to cause sequestration, hence promoting LPA_1 activity.

On another note, LPA signaling has been implicated in various stages of vascular development, but blood vessel integrity in these receptor knockout mice have not been investigated in depth. In a separate study, it was observed that LPA_1/LPA_2 double null mice exhibit an increased blood vessel leakage and blood vessel density specifically in the cortex during the period around birth persisting into early postnatal life. Also, these cortical endothelial cells appear to be unhealthy and electron-light. These striking phenotypes could shed light on the role of LPA signaling in blood brain barrier formation in the brain.

I. INTRODUCTION

1.1 Fetal hypoxia and related CNS disorders

Prenatal hypoxia is defined as a lack of adequate oxygen supply to the fetus during development, which can occur during pregnancy due to cord prolapse, cord occlusion, placental infarction and maternal smoking. Perinatal hypoxia (birth asphyxia) occurs when an inadequate supply of oxygen is supplied immediately prior to, during or just after delivery. This form of hypoxic insult may result due to prolonged labor, breech delivery in full-term infants, premature delivery and other complications during labor.

Both forms of hypoxic insult can lead to hypoxic ischemic encephalophathy (HIE) which has been largely associated with various neurological and neuropsychiatric disorders. These include sudden infant death syndrome (SIDS), epilepsy, autism spectrum disorders, schizophrenia and cerebral palsy (Bergamasco, Benna et al. 1984; Cannon, van Erp et al. 2002; Kolevzon, Gross et al. 2007). Perinatal hypoxic-ischemic encephalopathy occurs in one to three per 1000 live full-term births, and 15%–20% of affected newborns will die in the postnatal period, with an additional 25% will develop severe and permanent neuropsychological sequelae (Vannucci and Perlman 1997). The incidence of prenatal hypoxic insult is more difficult to assess, since the prenatal period is much longer than the perinatal period, and hence harder to monitor throughout the whole term. Early hypoxic insults during pregnancy could also lead to abortions, which could undermine the significance of such insults.

HIE is characterized predominantly by neuronal cell death, both apoptotic and necrotic, which manifests as white matter injury (Fatemi, Wilson et al. 2009). Many animal models have been fashioned to imitate such white matter damage, in which the

hypoxic-ischemic insult is afflicted at the age approximating the perinatal period of birth in humans (Painter 1995). Out of these CNS disorders, cerebral palsy is predominated by such white matter damage (Fatemi, Wilson et al. 2009). On the other hand, several other CNS disorders affected by hypoxia, such as epilepsy, autism spectrum disorders and schizophrenia, are delineated by defects in neuronal migration. In a study of 120 patients with epilepsy, a significant subset possessed periventricular heterotopias (d'Orsi, Tinuper et al. 2004). Periventricular heterotopias are clusters of neurons which are found abnormally in the ventricles of the brain, and are due to a defect in neuronal migration during early development of the fetal brain (Sarkisian, Bartley et al. 2008). In a previous study involving patients treated for epilepsy, more than half had focal cortical dysplasia, which is an abnormality where the neurons failed to migrate in the proper formation in utero (Palmini, Andermann et al. 1991). Also, postmortem studies of schizophrenia patients have found an abnormal cytoarchitecture of the entorhinal cortex characterized by layer II neuronal clusters and laminar disorganization, suggesting again a defect in neuronal migration (Arnold 2000). In addition, the model for the autisms is one in which higher-order association areas of the brain that normally connect to the frontal lobe are partially disconnected during development, which is attributed to defects in prenatal events such as neuronal migration and axon pathfinding (Geschwind and Levitt 2007).

Little is known about the pathologies of such neuronal migration defects. Neuronal migration is a very important process in brain development. In the early stages of brain development, new neurons born migrate away from their place of birth and migrate out in an “inside-out” fashion to form a total of six layers in the normal brain

(Gupta, Tsai et al. 2002). It is this choreographed, precise migratory patterns of the new neurons which leads to the proper formation of the brain. Many genetic models have been found with defects in neuronal migration, hence shedding light on the molecular pathways associated with the proper neuronal migration and formation. Yet, unlike the many hypoxic-ischemic animal models to model white matter injury, no model have been effectively developed, to my understanding, which have looked at the role of early prenatal hypoxic insults in such defects in neuronal migration linked to the CNS disorders mentioned.

1.2 Lysophosphatidic acid (LPA) and its receptors

Lysophosphatidic acid (LPA) is a bioactive lipid component of serum that is also produced by many cell types such as activated platelets (Eichholtz, Jalink et al. 1993) and postmitotic neurons in culture (Fukushima, Weiner et al. 2000). LPA stimulates many different cellular activities by activating a family of cognate G protein-coupled receptors. Six mammalian cell-surface LPA receptors have been identified to date and they are designated LPA_1-LPA_6. LPA_1 is the most widely expressed receptor, with high mRNA levels in the brain, colon, small intestine, placenta, and heart, and more modest expression in the pancreas, ovary, and prostate, as well as other loci (Hecht, Weiner et al. 1996). LPA_2 and LPA_3 have more restricted gene expression patterns compared with LPA_1. In mice, LPA_2 gene expression is found to be most abundant in the embryonic brain, kidney, and testis, with low levels found in numerous other organs, while LPA_3 is present in the lung, kidney, and testis (Contos and Chun 2001; Anliker and Chun 2004). In contrast, LPA_4 shows adult gene expression at very low levels in most human tissues, but is expressed significantly in the ovary (Noguchi, Ishii et al. 2003). LPA_5 has broad, low levels of expression in many tissues including the embryonic brain, and is enriched in the small intestine and moderate levels in skin, spleen, stomach, thymus, lung, and liver (Lee, Rivera et al. 2006). The expression of LPA_6 has not been reported to date (Yanagida, Masago et al. 2009). During development, LPA_1 and LPA_2 are expressed in the embryonic brain during neurogenesis, with high levels of LPA_1 present in the cerebral cortical ventricular zone. LPA_3 is expressed during the early postnatal period in the brain (Contos and Chun 2001; Anliker and Chun 2004).

LPA induces a variety of cellular responses in many cell types, including intracellular calcium mobilization, stress fiber formation, cell rounding, neurite retraction, proliferation, and survival (Fukushima, Ishii et al. 2001) (Figure 1.1). The great variety of cellular and biological actions of LPA is explained by the fact that LPA receptors can couple to at least four distinct G protein families defined by their alpha subunits ($G\alpha_i$, $G\alpha_{12/13}$ $G\alpha_{q/11}$, and $G\alpha_s$), which, in turn, feed into multiple effector systems (Mutoh and Chun 2008). LPA activates $G\alpha_{q/11}$ and thereby stimulates phospholipase C (PLC), with subsequent phosphatidylinositol-bisphosphate hydrolysis and generation of multiple second messengers leading to protein kinase C activation and changes in cytosolic calcium. LPA also activates $G\alpha_i$ (van Corven, Groenink et al. 1989), which leads to at least three distinct signaling routes: inhibition of adenylyl cyclase with inhibition of cyclic AMP accumulation; stimulation of the mitogenic RAS–MAPK (mitogen-activated protein kinase) cascade (Kranenburg and Moolenaar 2001); and activation of phosphatidylinositol 3-kinase (PI3K), leading to activation of the downstream RAC GTPase, a key regulator of the cell morphology and motility (Van Leeuwen, Olivo et al. 2003), as well as to activation of the AKT/PKB antiapoptotic pathway (Radeff-Huang, Seasholtz et al. 2004). LPA receptors activate $G\alpha_{12/13}$, leading to activation of the small GTPase RhoA, which drives cytoskeletal contraction and cell rounding (Etienne-Manneville and Hall 2002). In addition, $G\alpha_s$ activation can increase cAMP levels through LPA_4.

There are at least two different metabolic pathways postulated for the production of LPA (Choi, Herr et al. 2010). The first pathway is predominant in serum and plasma,

and involves the conversion of phospholipids (PLs) to lysophospholipids (LPLs) by either phospholipase A1 (PLA_1) and A2 (PLA_2), and subsequent conversion to LPA by lysophospholipase D (lysoPLD) (Figure 1.2). Recent studies have identified autotaxin (ATX) as the predominant lysoPLD (Tokumura, Majima et al. 2002) responsible for most of the LPA present in normal serum. In platelets and certain cancer cells, the second pathway occurs with the conversion of PLs and diacylglycerol (DAG) by phospholipase D (PLD) and diacylglycerol kinase (DGK), respectively, to phosphatidic acids (PAs). The PAs are subsequently deaceylated to LPA by either PLA_1 or PLA_2 (Pebay, Bonder et al. 2007; Aoki, Inoue et al. 2008; Ye 2008). This combined regulation of ligand and receptor distribution, in addition to the diversity of second messenger activation, provides an expansive range of biological roles for LPA signaling in different tissues and developmental stages.

1.3 LPA receptor knockout mice

To date, 4 of the 6 receptors (LPA_{1-4}) have been characterized by gene knockout studies in mice. Since the expression pattern of these LPA receptors is heterogeneous, the phenotype of these knockout mice varies considerably. LPA_1 knockout mice suffer from suckling defects, resulting in a 50% neonatal lethality rate. The impaired suckling defect was attributed to faulty olfaction, possibly due to a defect in olfactory bulb or cortex development. The surviving knockouts exhibit a reduced body size, craniofacial dysmorphism, embryonic frontal cranial hematomas, and increased apoptosis in sciatic nerve Schwann cells, which correlates with the expression pattern of LPA_1 (Contos, Fukushima et al. 2000). The deletion of LPA_2 did not cause any obvious phenotypic defects, with mice born at the expected frequency. Furthermore, $LPA_{1,2}$ double knockout mice did not result in any additional phenotypic abnormalities relative to those observed in LPA_1 single knockout mice, except for an increased frequency of frontal hematomas in the head, which provides evidence for the involvement of these receptors in vascular development. However, $LPA_{1,2}$ double knockout embryonic fibroblasts showed a severe reduction or absence of LPA-induced responses, including phospholipase C activation, calcium mobilization, proliferation, JNK activation, Akt activation, and stress fiber formation. This reduction of LPA responsiveness was less pronounced in LPA_1 or LPA_2 single knockout fibroblasts, indicating redundancy of the LPA_2 receptor with respect to LPA_1 (Contos, Ishii et al. 2002).

The targeted deletion of LPA_3 resulted in a considerably different phenotype characterized by pronounced reproductive defects. Litters born to $LPA_3^{-/-}$ breeder female

mice were less than half those of controls, with no dependence on stud male genotype, indicating defects in female reproduction. LPA_3 knockout mice exhibit delayed embryo implantation and altered embryo spacing, resulting in delayed embryonic development and hypertrophic placentas and embryonic death. This was attributed to a down regulation of cyclooxygenase 2 (COX2) which led to reduced levels of prostaglandins E_2 and I_2, which are essential players in implantation (Ye, Hama et al. 2005).

LPA_4-deficient mice did not exhibit any apparent abnormalities, and were born at the statistically expected Mendelian rate. However, LPA_4-deficient mouse embryonic fibroblasts (MEFs) were found to be hypersensitive to LPA-induced cell migration. LPA_4 deletion caused a potentiation of Akt and Rac activation, implying that LPA_4 negatively regulates the phosphatidylinositol-3-kinase pathway, which is in contrast to activation of this pathway by other LPA receptors. The co-expression of LPA_4 with LPA_1 in B103 neuroblastoma cells attenuated LPA_1-driven migration, indicating functional antagonism between the two LPA receptor subtypes. This inhibitory crosstalk is likely to be essential for ensuring physiologically appropriate responses to LPA (Lee, Cheng et al. 2008).

1.4 LPA in cortical development

Several LPA receptors, notably LPA_1 and LPA_2, are expressed significantly in the embryonic cortex, with LPA_1 exhibiting an enriched expression pattern in the ventricular zone (VZ) (Estivill-Torrus, Llebrez-Zayas et al. 2008). Importantly, LPA signaling has been implicated in cortical neurogenesis. Using an organotypic ex vivo culture system, it was observed that embryonic cortices exposed to exogenous LPA increased in width and produced folds resembling gyri, which was absent in LPA_1LPA_2 double null cortices. This growth was found to be due to reduced cell death and increased terminal mitosis of neural progenitor cells (NPCs), suggesting that LPA signaling contributes to normal neurogenesis (Kingsbury, Rehen et al. 2003).

Surprisingly, the genetic removal of LPA_1 in mice was associated with only minor brain defects (Contos, Fukushima et al. 2000). However, a variant of LPA_1 null mice has shed new light on the *in vivo* significance of LPA signaling in cortical development. The Málaga LPA_1 null mice showed a reduced VZ, increased cortical cell death resulting in subsequent reduction in cortical NPCs and cortical mal-development (Estivill-Torrus, Llebrez-Zayas et al. 2008). This alteration in cortical development was shown to persist well into adulthood, where LPA_1 adult neurons exhibited retarded proliferation, survival and differentiation (Matas-Rico, Garcia-Diaz et al. 2008). LPA signaling in the central nervous system (CNS) is also conserved in other non-mammalian models, where homologs of LPA receptors were reported to be essential for normal cortical actin assembly (Lloyd, Tao et al. 2005). In all, these studies argue strongly for the significance of LPA signaling in cortical development.

Furthermore, several *in vitro* studies have reported the multifaceted effects of LPA signaling in a variety of neuronal cell lines and primary neuronal cultures. LPA signaling is found to influence several aspects, such as morphology, survival, migration. In explant cortical cultures, LPA induces rounding and accumulation of NPCs in the VZ, indicating a biological role for LPA signaling in cortical neurogenesis (Fukushima, Weiner et al. 2000). In primary cultures of young, postmitotic neurons, exogenous LPA treatment induces neurite retraction and formation of F-actin-based retraction fiber caps (Fukushima, Weiner et al. 2002). The fiber caps are reminiscent of the actin retraction fibers formed by neuroblasts from the cortical VZ in a previous study (Fukushima, Weiner et al. 2000), suggesting that LPA influences actin assembly and morphology of early neurons. In support of the role of LPA in neurite retraction induction, neurite retraction and growth cone collapse have been reported in a variety of neuronal cell lines in response to LPA (Jalink, Eichholtz et al. 1993; Tigyi, Fischer et al. 1996; Kozma, Sarner et al. 1997).

In addition, LPA signaling was also found to affect neuronal migration in embryonic cortical explants, implicating LPA signaling in the processes associated with early postmitotic neurons during development (Fukushima, Weiner et al. 2002). In hippocampal neurons, LPA was shown to elicit neuronal apoptosis as well. In a study involving PC6 cells, LPA was reported to induce oxidative stress and subsequent apoptosis (Holtsberg, Steiner et al. 1998). In murine cortical neurons, LPA seemingly invokes a dual, dose-dependent effect on cell death. Low concentrations of LPA served to protect neurons from apoptosis, whereas high concentrations induce apoptosis (Zheng,

Fang et al. 2004). In view of these studies, LPA is a molecule well positioned to influence cortical neurons at the cellular level, as well as structural aspects of cortical development.

1.5 LPA in vascular development

1.5.1 Vasculogenesis

The first definitive study that linked LPA to vascular development was that of the autotaxin (ATX/ Enpp-2)-deficient phenotype. ATX functions as a secreted lysophospholipase D that converts lysophosphatidylcholine (LPC) into LPA (Tokumura, Majima et al. 2002). ATX-deficient mice die at E9.5 with profound vascular defects in the yolk sac and embryo, which coincides with the age of increased ATX and LPA receptor expression in normal embryos (van Meeteren, Ruurs et al. 2006). ATX-deficient mouse embryos were found to possess severe defects, including allantois and neural tube malformations. Importantly, severe vascular defects, such as absence of yolk sac vascularization and the presence of enlarged embryonic blood vessels, were apparent by E9.5. The normal occurrence of blood islands, as well as PECAM-1 (CD31) staining, demonstrated the presence of differentiated endothelial cells from angioblasts, thus suggesting that reduced LPA concentration did not alter the capacity of endothelial progenitor cells to differentiate. ATX heterozygous mice were found to possess half the plasma LPA levels as normal littermates, which implies that ATX is the major LPA-producing enzyme *in vivo* (Tanaka, Okudaira et al. 2006). This strongly suggests that loss of LPA production is responsible for the observed pathological phenotype and that LPA signaling is critical for vasculature development.

Furthermore, $G\alpha_{13}$ knockout mice share a similar phenotype with ATX-deficient mice, with impaired blood vessel formation in both yolk sac and embryo and embryonic lethality at E9.5 that are, interestingly, also associated with significant brain

abnormalities (Offermanns, Mancino et al. 1997). Since LPA is a major upstream activator of $G\alpha_{12/13}$ (Kranenburg, Poland et al. 1999), it is likely that LPA signaling mediates some of the embryonic vascular development processes through this pathway. This model is supported by the observation that individual LPA receptor (LPA_{1-3}) knockouts showed moderate vascular defects with the presence of frontal hematomas in 2.5% of LPA_1 single knockout and 26% of $LPA_{1,2}$ double knockout embryos (Contos, Fukushima et al. 2000; Contos, Ishii et al. 2002). In addition, approximately 40% of $LPA_{1,2}$ double knockouts also showed blood within the lateral ventricles, suggesting that bleeding may have occurred from internal structures such as the choroid plexus (unpublished data). Also, microhematomas were noted on the developing skin and surface of the embryos (unpublished data). The lack of more severe vascular defects might be due to redundancy of the LPA receptors, as well as the continued presence of S1P lysophospholipid receptors that have proven roles in vascular development and integrity (Ozaki, Hla et al. 2003; Hla 2004; Hla, Venkataraman et al. 2008). It is possible that more severe disruptions of vascular development will only occur with the simultaneous deletion of additional LPA receptors and/or in conjunction with S1P receptors.

1.5.2 Angiogenesis

Angiogenesis is a process which involves the proliferation and migration of endothelial cells to form new sprouting blood vessels from existing vasculature. LPA has been implicated in both of these processes. In a study using adult bovine aortic and human umbilical vein endothelial cells, LPA was observed to facilitate closure of

wounded endothelial monolayers of the endothelial cells *in vitro*. This was due to a stimulation of both endothelial cell migration and proliferation by LPA treatment (Lee, Goetzl et al. 2000). In another study, LPA was shown to induce a chemotactic response in bovine pulmonary artery endothelial cells, similar in intensity to that observed with optimal levels of known endothelial cell chemoattractants such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (English, Kovala et al. 1999). Also, LPA was shown to play a chemokinetic role in inducing the migration of several endothelial cell types, including fetal bovine heart endothelial cells and bovine aortic endothelial cells (Panetti, Nowlen et al. 2000). LPA-induced migration of bovine pulmonary artery endothelial cells was found to be mediated by the recruitment of hydrogen peroxide inducible clone 5 (Hic-5), a paxillin family member, to the focal adhesions of the endothelial cells (Avraamides, Bromberg et al. 2007). In EAhy926 human endothelial cells, the induction of migration by LPA was found to be due to an increase in matrix metalloproteinase-2 (MMP-2), a critical player in endothelial cell migration and matrix remodeling during angiogenesis (Wu, Chen et al. 2005). LPA also stimulates a significant increase in the uptake of tritiated thymidine in endothelial cells, demonstrating that LPA can act as a mitogen and proliferation signal for endothelial cells (Panetti, Chen et al. 1997). In addition, LPA was found to evoke the formation of new blood vessels in a chicken chorio-allantoic membrane (CAM) assay, confirming it as an angiogenic compound (Rivera-Lopez, Tucker et al. 2008). In contrast, neither LPA nor ATX was found to be angiogenic in cultured mouse allantois explants. Rather, they were found to *maintain* existing vessels by preventing disassembly of the vessels (Tanaka, Okudaira et al. 2006).

LPA has also been documented to be a major player in pathologic angiogenesis during the development of various cancers. Elevated LPA levels have been detected in the ascites of 98% of ovarian cancer patients, including 90% of patients with stage 1 disease, suggesting that LPA is important for early events in carcinogenesis. Also, ovarian cancer cells have been found to produce LPA, thereby maintaining an LPA-rich microenvironment (Xu, Gaudette et al. 1995; Westermann, Havik et al. 1998; Fang, Schummer et al. 2002). In ovarian cancer cells, LPA was found to upregulate expression of hypoxia-inducible factor-1-alpha (HIF1α), which plays a central role in tumor angiogenesis (Kim, Sengupta et al. 2006). Furthermore, LPA has been reported to induce VEGF-A (VEGF) expression via HIF-1α activation in a variety of cancer cells, including prostate cancer, ovarian cancer, lymphoma, and hepatoma cells (Lee, Park et al. 2006; Park, Jeong et al. 2007; Hu, Mendoza et al. 2008). In ovarian cancer cells, this was inhibited by the knockdown of LPA_2 or LPA_3 receptors (Yu, Murph et al. 2008). VEGF is the predominant regulator of angiogenesis that stimulates vascular endothelial cell growth, survival, and proliferation. It has been shown to promote survival of existing vessels and stimulate new vessel growth (Ferrara 2004). Recently, LPA was found to enhance Sp-1-mediated VEGF transcription by inducing the phosphorylation of Sp-1 and binding to the VEGF promoter via a HIF1α-independent pathway through the $G\alpha_{12/13}$-Rho-ROCK-c-Myc pathway (Song, Wu et al. 2009). These results show that LPA is implicated in tumor angiogenesis.

LPA signaling has also been implicated in endometrium and placenta angiogenesis. An increase in serum lysophospholipase D activity has been noted during

pregnancy in humans, suggesting that the consequential production of LPA might be important in pregnancy (Tokumura, Kanaya et al. 2002). LPA-conditioned medium of endometrical stromal cells was able to stimulate migration, permeability, capillary tube formation, and proliferation of human endometrial microvascular endothelial cells. This was mediated by the production of IL-8, an angiogenic factor, via a $G\alpha_i$ protein-coupled pathway (Chen, Lee et al. 2008). LPA was shown to influence critical steps of endometrium angiogenesis during pregnancy.

The formation of the corpus luteum during ovulation is a period of marked angiogenesis (Chen, Lee et al. 2008). Coincidentally, LPA was found in significant amounts in the follicular fluid of the preovulatory follicle, suggesting a possible role for LPA in ovulation (Tokumura, Miyake et al. 1999). This was demonstrated in a study where the LPA-primed medium of human granulose-lutein cells was found to stimulate processes of angiogenesis, including migration, tube formation, and proliferation of umbilical vein endothelial cells. Both IL-6 and IL-8 secretion was found to be induced by LPA to mediate these processes, via a $G\alpha_i$ protein-coupled, NF-κB-dependent pathway (Chen, Chou et al. 2008).

Since both blood and lymphatic vascular systems are composed of vessels lined by endothelial cells, it is not surprising that LPA plays a role in lymphangiogenesis as well. In a study involving human umbilical endothelial cells, LPA upregulated vascular endothelial growth factor-C (VEGF-C), a critical player in lymphangiogenesis *in vitro,* and subsequent endothelial cell tube formation in Matrigel assays (Lin, Chen et al. 2008). Furthermore, the knockdown of LPA_1 in zebrafish resulted in defects in lymphatic vessel

development, such as the absence of a thoracic duct and edema in the pericardial sac. Taken together, these studies suggest that LPA plays a significant role in the lymphatic vascular system (Lee, Chan et al. 2008).

1.5.3 Pathological vascular responses

LPA has also been implicated in the regulation of pathophysiologic vascular responses. LPA was found to signal through $G\alpha_q$ to promote the growth and migration of VSMCs (Kim, Keys et al. 2006), which are essential for the development of intimal hyperplasia after vascular injury. Mice deficient in LPA_1 and LPA_2 were protected from intimal hyperplasia (Panchatcharam, Miriyala et al. 2008), suggesting that migration induction of SMCs by LPA plays a role in this process. Furthermore, unsaturated LPA was found to stimulate VSMC de-differentiation through the activation of ERK and p38 MAPK, which is a hallmark in the development of atherosclerosis. Thus, naturally occurring unsaturated LPAs may act as atherogenic factors (Hayashi, Takahashi et al. 2001)). This is supported by the fact that LPA-like compounds have been found to accumulate in human atherosclerotic plaques (Siess, Zangl et al. 1999).

LPA may be involved in thrombus formation. LPA promoted human platelet activation by inducing platelet shape change and calcium mobilization. LPA also induced the aggregation of platelets, which is essential in thrombosis (Rother, Brandl et al. 2003). On the other hand, the addition of LPA attenuated aggregation of murine platelets, thereby preventing thrombosis. Intravascular administration of LPA prolonged bleeding time and inhibited thrombosis in mice, as well as a transgenic overexpression of autotaxin (Pamuklar, Federico et al. 2009). Therefore, LPA might play dual roles as a species-

specific modulator of platelet function, thrombosis, and hemostasis. This difference between the actions of LPA on murine and human platelets may be attributed to a difference in expression of LPA receptors, resulting in differential activation of signaling pathways.

LPA causes a dose-dependent increase in the cell death of both HUVECs and porcine cerebral microvascular endothelial cells. Brain explants and retinas exposed to LPA also exhibited diminished vasculature (Brault, Gobeil et al. 2007). When endothelial cell monolayers were incubated with LPA, a loss of confluence due to cellular detachment was noted, which indicated a loss in vascular integrity. Also, a gain in hydraulic permeability in rat mesenteric venules was observed *in vivo* when exposed to LPA, showing endothelial dysfunction and loss of vascular integrity (Wu, Chen et al. 2005; Neidlinger, Larkin et al. 2006). Therefore, LPA may be involved in vascular injury by causing endothelial cell death and vascular degeneration. The topical application of synthetic LPA was also found to induce dose-dependent vasoconstriction in the cerebral circulation of newborn pigs. Since LPA-like bioactive mediators were found to be generated in an intracranial hematoma model (Tigyi, Hong et al. 1995), this suggests that LPA might contribute to the development of posthemorrhagic vasoconstriction.

1.5.4 Blood brain barrier (BBB) formation

The blood brain barrier (BBB) is a structural component specific to the vasculature of the central nervous system, which acts as a selective barrier to the paracellular flux of molecules between the bloodstream and surrounding neural tissue. It is composed of three cellular components: endothelial cells with associated tight

junctions, end feet of astrocytes ensheathing the vessels, and pericytes embedded in the capillary basement membrane (Ballabh, Braun et al. 2004). LPA has been implicated in the maintenance of each of these components.

One of the most distinctive features of the BBB is the presence of tight junctions, which are sites of fusion between the plasma membrane of adjacent endothelial cells. These tight junctions exhibit high electrical resistance and limited transcellular flux, and greatly limit the permeability of the BBB to hydrophilic solutes and ions. In porcine brain endothelial cells, LPA was found to cause both a rapid, reversible and dose-dependent decrease in transcellular electrical resistance (TER) and an increase in the paracellular flux of sucrose, indicating increased tight junction permeability. This was attributed to formation of stress fibers, focal contacts, and focal contact-associated phosphorylation rather than relocalization or phosphorylation of adherens junction- or tight junction-associated proteins (Schulze, Smales et al. 1997; Nitz, Eisenblatter et al. 2003). Currently, there is no evidence of a direct effect of LPA on tight junction proteins.

Astrocytes have been shown to communicate with endothelial cells through calcium (Ca^{2+}) signaling ((Braet, Paemeleire et al. 2001) which has been reported to affect blood brain barrier permeability (Abbott 2000)). LPA signaling may be involved in this interaction since the addition of LPA has been shown to induce a dose-dependent increase in intracellular calcium concentrations in several studies involving rat astrocytes. Higher LPA concentrations (>10μM) usually elicit sustained increases in Ca^{2+} concentrations, whereas lower concentrations stimulate Ca^{2+} transients (Keller, Steiner et al. 1997; Manning and Sontheimer 1997; Tabuchi, Kume et al. 2000). In addition, LPA is

capable of inducing many other responses in astrocytes, which might influence the role of astrocytes in maintaining the BBB. LPA was found to mediate the up-regulation of various immediate early genes and cytokines (Tabuchi, Kume et al. 2000). In addition, LPA has been shown to stimulate lipid peroxidation and the rate of DNA synthesis in astrocytes as well through pertussis toxin sensitive G protein(s). LPA also inhibits the uptake of glutamate and is capable of reversing the stellate morphology of astrocytes induced by cyclic adenosine monophosphate (cAMP) (Keller, Steiner et al. 1997; Manning and Sontheimer 1997). Actomyosin contraction of astrocytes can be stimulated by LPA, which is part of the wound healing process that occurs following the disruption of the BBB (Manning, Rosenfeld et al. 1998). Conditioned medium from LPA-primed astrocytes was found to increase neural differentiation of neural progenitor cells, suggesting that the presence of an LPA-induced, astrocyte-derived soluble factor (de Sampaio, Choi et al. 2008). It is possible that astrocytes could influence endothelial cells in a similar manner via indirect, as well as direct, actions of receptor-mediated LPA signaling.

Although little is known of the effect of LPA on pericytes, a related family of lysophospholipid receptors, sphingosine 1-phosphate receptors ($S1P_{1-5}$), has been reported to play a significant role in vascular maturation propagated by pericytes. Knockout mice deficient in $S1P_1$ die at ~E11-12 because of a failure in vascular maturation and subsequent bleeding. This follows a drastic reduction of pericytes associated with the developing blood vessels, a key process in vascular maturation (Liu, Wada et al. 2000). Since LPA and S1P signaling have been reported to signal through

similar pathways and exert overlapping effects on various cell types, it is likely that LPA signaling is involved in the interaction between pericytes and endothelial cells.

1.6 Conclusions

LPA signaling has been implicated in various aspects of cortical development, such as cortical growth and folding, neurogenesis, neuronal migration and cell survival. Thus, LPA is well-positioned to influence cortical development. Similarly, various stages of vascular development in the brain involve LPA signaling. LPA influences the cells and signaling processes that mediate various aspects of vascular development. These effects include cellular processes such as endothelial cell survival, migration, proliferation and also physiological processes such as angiogenesis and vascular maturation. Collectively, the studies described here indicate that LPA signaling plays a significant role in the development of the cortex and vasculature, and requires further investigation in this context towards understanding how receptor-mediated LPA signaling affects this system.

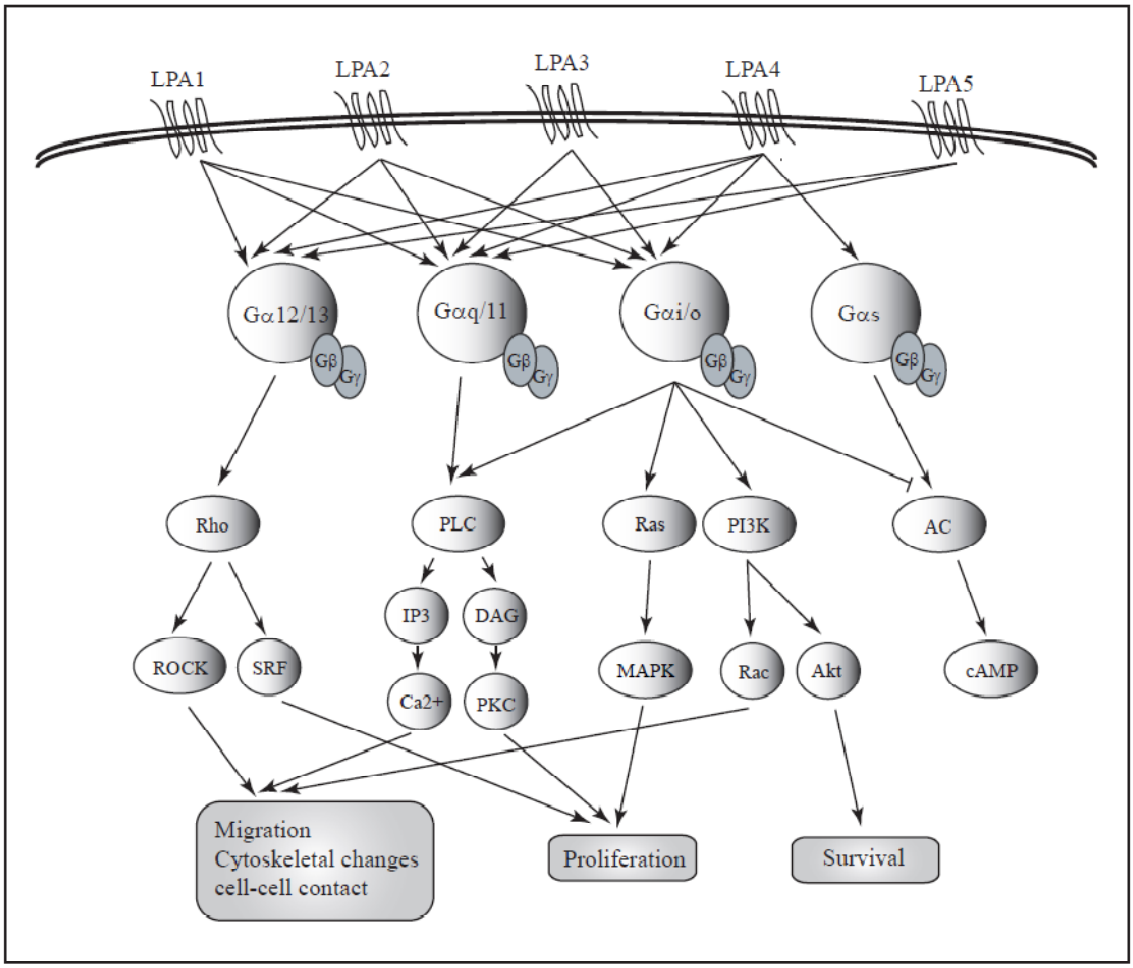

Figure 1.1 LPA signaling pathways mediated by LPA receptors

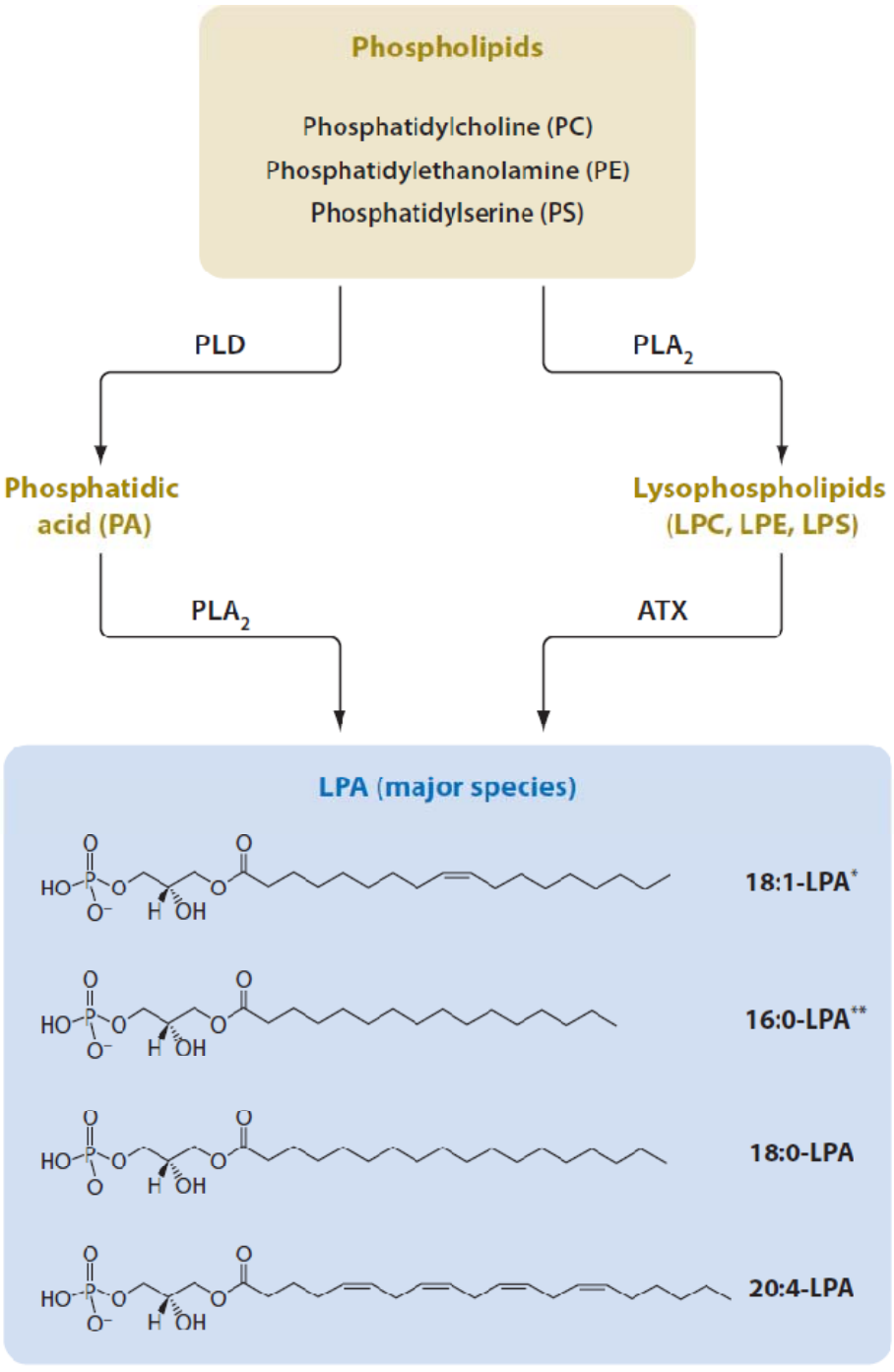

Figure 1.2 LPA metabolism. The production of LPA from membrane phospholipids through two major metabolic pathways is shown.

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II. STEREOTYPED FETAL BRAIN DISORGANIZATION IS INDUCED BY HYPOXIA AND REQUIRES LPA_1 SIGNALING

Siew T. Teo, Deron R. Herr, Chang-Wook Lee & Jerold Chun

Department of Molecular Biology, Dorris Neuroscience Center, The Scripps Research Institute, 10550 North Torrey Pines Road, DNC-118, La Jolla, CA 92037

2.1 Abstract

Fetal hypoxia is a common risk factor that has been associated with a range of CNS disorders including epilepsy, schizophrenia, and autism. Cellular and molecular mechanisms through which hypoxia may damage the developing brain are incompletely understood but likely to involve disruption of the laminar organization of the cerebral cortex. Lysophosphatidic acid (LPA) is a bioactive lipid capable of cortical influences via one or more of 6 cognate G protein-coupled receptors (GPCRs), LPA_{1-6}, several of which are enriched in fetal neural progenitor cells (NPCs). Here we report that fetal hypoxia induces cortical disruption via increased LPA_1 signaling involving stereotyped effects on NPCs: N-cadherin disruption, displacement of mitotic NPCs, and impaired neuronal migration. Importantly, genetic removal or pharmacological inhibition of LPA_1 prevented the occurrence of these hypoxia-induced phenomena. Hypoxia resulted in over-activation of LPA_1 through selective inhibition of GPCR kinase 2 (GRK2) expression and selective activation of downstream pathways including $G_{\alpha i}$ and Rac1. These data identify stereotyped and selective hypoxia-induced cerebral cortical disruption requiring LPA_1 signaling, inhibition of which can reduce or prevent disease-associated sequelae, towards therapeutically treating fetal hypoxia-induced CNS disorders, and possibly other forms of hypoxic injury.

2.2 Introduction

During fetal development, the embryonic brain is susceptible to hypoxic insults (Arbeille, Maulik et al. 1999) that can contribute to a range of neurological and psychiatric abnormalities including autism, schizophrenia, and epilepsy (Bergamasco, Benna et al. 1984; Cannon, van Erp et al. 2002; Kolevzon, Gross et al. 2007). These diseases or disorders are strongly associated with cerebral cortical abnormalities in neuronal migration (Gleeson and Walsh 2000; Geschwind and Levitt 2007; Fatemi and Folsom 2009), which are thought to occur during the neurogenic period to produce disruption (cortical dysplasia) in the laminar organization of the cortex. Despite the identification of fetal hypoxia as a significant risk factor for these and other afflictions of the brain, mechanistic information is lacking on how hypoxia might contribute to these pathologies.

LPA is a bioactive lysophospholipid that can influence a range of developmental processes within the embryonic cerebral cortex through the activation of one or more LPA receptors (Choi, Herr et al. 2010). As a family, these G protein-coupled lysophospholipid receptors couple to at least four distinct G protein families ($G_{\square q}$, $G_{\square i}$, $G_{\alpha s}$, and $G_{\alpha 12/13}$) that activate multiple downstream signaling pathways to influence myriad cellular physiologies (Choi, Herr et al. 2010). LPA_1 gene expression is enriched in the ventricular zone (VZ), a neurogenic region of the embryonic cerebral cortex (Hecht, Weiner et al. 1996). Over-activation of LPA signaling rapidly displaces the normal, apical location of mitotic NPCs to more superficial locations as they undergo interkinetic nuclear migration (INM) and these effects are prevented by genetic removal of $LPA_{1,2}$ (Kingsbury, Rehen et al. 2003). Receptor-mediated LPA signaling has been shown to

alter the neural expression of cell adhesion molecules like N-cadherin or beta-catenin (Weiner, Fukushima et al. 2001; Ishii, Fukushima et al. 2004). Importantly, cortical dysplasia has been reported in cell adhesion molecule knockout brains (N-cadherin, beta-catenin) after birth, which had shown a similar displacement of mitotic NPCs during fetal life (Machon, van den Bout et al. 2003; Kadowaki, Nakamura et al. 2007). In addition, LPA signaling has been shown to affect neuronal migration *in vitro* and *in vivo* (Fukushima, Weiner et al. 2002). In all, this suggests that LPA signaling might play an important role in neuronal migration during development.

A correlative thread amongst fetal hypoxia, LPA signaling, and cerebral cortical defects led to an examination of these elements in the early embryonic cortex. Using genetic approaches combined with LPA receptor-specific pharmacological tools, alterations to the embryonic cerebral cortex were assessed both *ex vivo* and *in vivo* under hypoxic conditions. Here we report the results of this analysis that identifies cellular and molecular links between fetal hypoxia and receptor-mediated LPA signaling in the embryonic cerebral cortex.

2.3 Materials and Methods

Cortical hemisphere cultures

Animal protocols were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute and conform to National Institutes of Health guidelines and public law. *Ex vivo* cortical cultures at E13.5 were performed as described previously (Kingsbury, Rehen et al. 2003; Rehen, Kingsbury et al. 2006). Embryos from *Lpar1*$^{+/-}$ females were genotyped by PCR. Brains of embryos were dissected in serum-free medium: Opti-MEM I (Gibco/BRL) containing 20 mM D-glucose, 55 μM β-mercaptoethanol and 1% penicillin-streptomycin. The cortical hemispheres of each brain were separated along the midline. One hemisphere was cultured under hypoxia (1.8% O_2 or 1.7 KPa), and the other in control medium in 21% O_2 in a humidified 5% CO_2 chamber as is used routinely for explant cultures (Dammerman, Noctor et al. 2000; Bultje, Castaneda-Castellanos et al. 2009). Hypoxia was achieved in a hypoxia chamber with an attached oxygen sensor (Biospherix), which was calibrated before each experiment. Hemispheres were cultured at 37 °C for 17h, shaking at 65 r.p.m. After 17h of culture, matched hemispheres were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (pH 7.4), cryoprotected and embedded in Tissue-Tek (Sakura) and rapidly frozen on dry ice. Tissue was cut coronally at 20 μm on a cryostat and mounted onto Superfrost Plus slides (Fisher Scientific).

Treatment of cortical hemisphere cultures

AM095 (Amira Pharmaceuticals, Inc.), an LPA_1 specific antagonist(Swaney, Chapman et al. 2010), was added to the cultures at a final concentration of 1 μM. Ki16425, an LPA_1/LPA_3 specific antagonist (Kirin Brewing Co./DebioPharm Group)

(Ohta, Sato et al. 2003), was added to the cultures at a final concentration of 10 μM 15 min prior to incubation. NSC23766, a Rac1 inhibitor, was used at a final concentration of 100 μM and added 2h prior to treatment. Pertussis toxin (List Biological Laboratories) was used at a final concentration of 100 ng/ml and added 6h prior to hypoxia treatment. Y-27632 (Sigma) was used at a final concentration of 30 μM and added 15 min prior to treatment A GRK2-specific inhibitor (Calbiochem) and heparin were used at a concentration of 1 mM and 1 μM, respectively.

Immunohistochemistry

The antibodies used were rabbit anti-phospho-H3 (Upstate Biotechnology), mouse Tuj1 (Covance) and mouse-BrdU (Roche). Primary antibodies were detected with AF568-conjugated donkey anti-rabbit antibody (Invitrogen) and AF488-conjugated anti–mouse antibody (BD Biosciences). Tissue was processed as described previously (Kingsbury, Rehen et al. 2003).

Quantification of mitotic displacement

Ventricular mitotic cells were defined as phospho-histone3 positive cells within 5 μm of the ventricular surface. The percentages of displaced and non-displaced cells were then quantified using ImageJ software. 2-tailed paired t-tests were used for all statistical calculations.

BrdU labeling

E13.5 timed pregnant BALB/c mice were injected intraperitoneally with 1 ml per 100g of body weight with BrdU reagent (Invitrogen), and sacrificed after 1h. The brains of embryos were then prepared for cortical *ex vivo* cultures.

Western blot

The cortices were washed in ice-cold 1X phosphate-buffered saline before the addition of ice-cold lysis buffer (1X RIPA buffer, complete protease inhibitor cocktail (Roche Diagnostics, Co.), sodium fluoride, sodium orthovanate for 15 minutes at 4 degrees on a rotator. The lysate was then centrifuged at 14,000 g for 15 minutes, and transferred to a new tube. 30 μg of total lysate protein was separated on a 4-12% SDS-PAGE gel, then transferred and blocked overnight. The blot was then incubated with rabbit anti-GRK2 (Santa Cruz Biotechnology, Inc.) diluted 1:200, secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1:10000, and subsequently visualized using the West Femto kit (Thermo Scientific, Inc.).

cAMP assay

HA- LPA_1 over-expressing cell lines were generated by transfecting B103 cells with linearized HA-tagged LPA_1-pcDNA3.1 (Invitrogen) using Effectene transfection reagent (Qiagen). Stable transfectants were selected using 1 mg/ml of geneticin (Invitrogen) and clonally expanded. Cells were seeded at 100,000 cells/well, and serum-starved overnight and treated with 5 μM forskolin, 0.5 μM 3-isobutyl-1-methylxanthine, and increasing concentrations of LPA. cAMP content was determined according to the protocol supplied by the cAMP ELISA kit (Cayman Chemical). Number of viable cells per well was determined by MTT assay of identically-treated replicate plates (see below) to allow calculation of cAMP/cell. The EC_{50} values were calculated using the Prism 4.0 program.

MTT assay

B103 over-expressing HA- LPA_1 were seeded at 100,000 cells/well, and serum-starved overnight after 4h. MTT reagent (1mg/ml) was added to each well and incubated

for 1.5h, which after the reagent was aspirated, and MTT solvent was added. After 15 min of agitation on a rotator, the absorbance was read on a plate reader at a wavelength of 590 nm. The number of viable cells was then calculated using a standard curve.

Neurite retraction assay

TSM-1 cells were seeded at 20,000 cells/well, and serum-starved overnight. They were then either exposed to normoxia or hypoxia for 6h before the addition of 100 nM LPA. After 30 min, the cells were fixed and stained with phalloidin and DAPI for cell morphology. The number of cells with retracted neurites and the number of total cells were counted in three separate field for each sample, and the percentage of cells with retracted neurites was calculated.

Maternal hypoxia

E13.5 embryos were exposed to hypoxia *in vivo* using a modification of a previously described protocol (Golan, Mane et al. 2009). Pregnant C57Bl/6J or Balb/cByJ female mice (E13.5) are put in a hypoxia chamber at 9% oxygen for 2 hours then returned to atmospheric air. After 17 h, the cortices of the embryos were fixed in 4% paraformaldehyde.

The PaO_2 in ovine embryos is known to drop from 3.4 KPa to ~2 KPa when the pregnant dam is subject to 9% O_2 (Richardson, Korkola et al. 1996). Thus, this maternal hypoxia model is effective in inflicting a hypoxic insult to the embryo. Since the amount of oxygen that is actually delivered to the embryo is not only dependent on the PaO_2 of blood but also dependent on additional physiological factors such as arterial flow rate and hemoglobin saturation (Meschia 1978), it is not possible to directly compare the level of

hypoxia obtained *in vivo* with that generated by our *ex vivo* model. However, the *in vivo* hypoxic insult is likely to be much milder in extent and duration compared to our *ex vivo* system, in which the cortices are subject to a lower oxygen pressure (1.7KPa) for 17h.

2.4 Results

Hypoxia induces displacement of mitotic cells in the embryonic brain via LPA_1.

To identify possible relationships between hypoxia and receptor-mediated LPA mechanisms affecting the embryonic brain, an *ex vivo* method of culturing intact embryonic cerebral cortices (Kingsbury, Rehen et al. 2003) was employed. This method maintains normal cortical architecture and neurogenic gradients up to 24 hours in culture and allows a direct comparison of hemispheres from the same animal. Control explants were grown under "normoxia" (21% atmospheric oxygen), which allows normal development to occur in culture (Kingsbury, Rehen et al. 2003; Bultje, Castaneda-Castellanos et al. 2009). Cortices cultured under normoxia showed mitotic cells at the apical boundary (ventricular surface) of the cerebral wall that formed an expected band of M-phase NPCs identified by phosphorylated histone-H3 immunolabeling, as observed *in vivo* (embryonic day 13.5 (E13.5); Figure 2.S1*A*). Matching contralateral hemispheres were grown under "hypoxia" (1.8% oxygen or a partial pressure of 1.7 kPa) approximating conditions associated with hypoxic neuronal damage in culture (Rashidian, Iyirhiaro et al. 2005). These hemispheres displayed superficially (basally) displaced mitotic NPCs (Figure 2.1*A*-*C*, Figure 2.S1*B*), with displacement commencing between 6-12 hours of hypoxic exposure and maximal at 1.8% oxygen (Figure 2.S2, 3). Hypoxia doubled the percentage of basally displaced NPCs (Figure 2.1*F*).

This hypoxic response was similar to that produced by over-activation of LPA signaling through LPA_1 in the embryonic cerebral cortex (Kingsbury, Rehen et al. 2003), suggesting a possible mechanistic relationship. This possibility was assessed by hypoxic challenge of cortices from LPA receptor-null mouse mutants. Prior gene expression

studies of the embryonic cerebral cortex identified LPA_1, LPA_2, and LPA_4 as the most highly expressed LPA receptors (Dubin, Herr et al. 2010). Therefore, constitutive receptor-null mutants were initially screened, which revealed a prominent effect in cortices from mice lacking LPA_1 (*Lpar1*$^{-/-}$). These cortices did not display the increased basal displacement of mitotic NPCs when subjected to hypoxia as compared to littermate controls (Figure 2.1*D-F*), thus linking LPA_1 signaling to hypoxic effects on NPC positioning.

It was possible that the constitutive nature of the $LPA_1^{-/-}$ mutant (Contos, Fukushima et al. 2000) could have produced artifactually altered cellular responses to hypoxia. To eliminate this possibility, wild-type cortices were exposed to two chemically distinct LPA_1 antagonists (Figure 2.1*G-K*) followed by hypoxic challenge. Ki16425 is an LPA_1/LPA_3 selective antagonist (Ohta, Sato et al. 2003), and its exposure to cortices significantly reduced the hypoxia-mediated displacement of mitotic NPCs. Nearly identical results were obtained using a different LPA_1-specific antagonist, AM095 (Swaney, Chapman et al. 2010). Thus, pharmacological LPA_1 inhibition phenocopied the results from genetic deletion studies, supporting LPA_1 signaling as an essential component of hypoxia-induced NPC displacement.

To confirm that these observations reflected processes that could also occur *in vivo*, E13.5 pregnant dams were subjected to 2 hours of maternal hypoxia (MH) (9% [O_2]) (Hallak, Hotra et al. 2000), which is known to produce a 40% reduction in the partial pressure of oxygen (PaO_2) of embryonic blood (Richardson, Korkola et al. 1996) (Methods). This level of hypoxia represented a less severe insult than that employed for *ex vivo* cultures, which was necessary to limit maternal distress (Hallak, Hotra et al.

2000), and a significant, albeit reduced level of NPC displacement was observed with MH. This mitotic displacement was again absent in the *Lpar1*$^{-/-}$ cortices from MH-exposed embryos (Figure 2.1*L*-*O*).

Hypoxia induces altered NPC positions and migration *via* LPA_1.

Additionally, stereotyped disorganization of the cerebral wall was also identified that affected NPCs and young neurons. First, newly post-mitotic neurons were neuroanatomically displaced from their normal positions in the cortical plate (future grey matter) as identified by two different markers for post-mitotic neurons (Tuj1 and DCX) (Francis, Koulakoff et al. 1999). Under hypoxia, these neurons were aberrantly positioned throughout the cerebral wall, consistent with migration defects, as manifested through the abnormal expression patterns revealed by Tuj1 or DCX immunolabeling (Figure 2.2*A*-*D*). Importantly, this disorganization was absent in the *Lpar1*$^{-/-}$ cortices, indicating a dependence on LPA_1 (Figure 2.2*G*-*J*).

Second, the distribution of the cell adhesion molecule, N-cadherin, was also disorganized following hypoxia. N-cadherin is expressed on apical NPCs (Kadowaki, Nakamura et al. 2007), and LPA signaling can alter N-cadherin in neural cells (Weiner, Fukushima et al. 2001). Notably, NPC displacement was also reported in N-cadherin, neural-specific null mutants (Kadowaki, Nakamura et al. 2007). In control wild-type cortices, N-cadherin immunolabeling appeared as a prominent apical band along the ventricular surface (Figure 2.2*E*, *M*). Under hypoxia, this N-cadherin band appeared diffuse, forming a honey-comb pattern indicating disruption of normal N-cadherin organization (Figure 2.2*F*, *N*). These changes were reduced or absent following hypoxic

challenge in LPA_1-null cortices (Figure 2.2*K-L*, *O-P*), again identifying LPA_1-dependent effects associated with hypoxia.

To further investigate hypoxic effects on cell migration, cortices were briefly pulsed with BrdU to label a subset of NPCs actively undergoing DNA synthesis prior to normoxia or hypoxia, and then assessed after 17 hours in culture. These analyses revealed fewer cells reaching their normal post-mitotic locations within the cortical plate following hypoxia (Figure 2.3*A-B*, *E*), which is consistent with defects in neuronal migration. This hypoxic effect was again absent in the *Lpar1*$^{-/-}$ cortices, consistent with a dependence on LPA_1 (Figure 2.3*C-D*, *F*).

Hypoxia selectively activates LPA_1 downstream signaling pathways by potentiating LPA_1 activity.

To evaluate the downstream signaling pathways of LPA_1-mediated hypoxic effects, pharmacological blockade of known LPA_1 downstream effectors was assessed. LPA_1 activates G-proteins known to influence cell migration including $G_{\alpha i}$, which can be blocked by pertussis toxin (PTX) (Hsia, Moss et al. 1984), which in turn can activate the small GTPase Rac1 that can be inhibited by NSC23766 (Gao, Dickerson et al. 2004). LPA_1 also activates $G_{\alpha 12/13}$ which, in addition to activating Rac1 (Kjoller and Hall 1999), can also activate RhoA (Kranenburg, Poland et al. 1999) that can be inhibited by the Rho kinase inhibitor Y-27632 (Uehata, Ishizaki et al. 1997). These inhibitors were applied to *ex vivo* cortices under hypoxic conditions; both PTX and NSC23766 prevented NPC displacement as well as disruption of N-cadherin organization, which is consistent with LPA_1 activation of $G_{\alpha i}$ and Rac1, respectively (Figure 2.4*A-F*, Figure 2.S4). In contrast, Y-27632 exacerbated NPC displacement during hypoxia (Figure 2.4*G-I*), which possibly

involves the known antagonistic relationship between RhoA and Rac1 (Norman, Price et al. 1996), and underscoring downstream signaling pathway selectivity of the LPA_1-hypoxia response. Overall, these data support preferential over-activation of LPA receptor pathways that include $G_{\alpha i}$ and Rac1 in mediating the effects of hypoxia.

The observed hypoxic effects on NPCs that required LPA_1 were consistent with receptor potentiation, resulting in an over-activation of LPA signaling. To examine this possibility, LPA_1 activity under hypoxic conditions was assessed by two independent, quantitative assays (Figure 2.4*J*-*M*). First, a well-defined LPA response – neurite retraction – was quantified via LPA dose-response under normoxia in a neuronal cell line that endogenously expresses LPA_1 (TSM-1) (Chun and Jaenisch 1996) (Figure 2.4*J*-*L*). This was compared to LPA exposure under hypoxia, which resulted in a 30% increase in neurite retraction that was inhibited by Ki16425, suggesting that hypoxia potentiates LPA_1 activity during LPA exposure. Second, heterologous expression of LPA_1 in LPA-unresponsive B103 neuroblastoma cells (Fukushima, Kimura et al. 1998) (Figure 2.S5) was used to assess $G_{\alpha i}$ activation through the inhibition of adenylate cyclase and subsequent cyclic AMP (cAMP) production. B103 cells (Fukushima, Kimura et al. 1998) stably expressing LPA_1 showed a dose-dependent inhibition of cAMP when exposed to LPA. Under hypoxia, a more potent inhibition of cAMP production at each relevant LPA concentration was observed, reaching a maximum of ~50% more inhibition relative to normoxia at 250 nM LPA (Figure 2.4*M*). These results support cell-autonomous potentiation of LPA_1 by hypoxia.

Hypoxia potentiates LPA_1 activity by inhibition of GRK2 expression.

The potentiation of LPA_1 signaling by hypoxia could occur through several mechanisms, including increased availability of ligand, increased receptor expression, or altered activity of existing receptors. Unexpectedly, there was neither change in gene expression for LPA_1 nor in the major LPA-producing enzyme, autotaxin, in hypoxic cortices (Figure 2.S6). These results suggested the operation of other receptor mechanisms through which hypoxia may modulate LPA_1 activity. One identified mechanism involves inhibition of LPA_1 by GRK2, implying that inhibition of GRK2 might increase LPA_1 signaling (Aziziyeh, Li et al. 2009). To examine this possibility, a GRK2-specific inhibitor (Methyl[(5-nitro-2-furyl)vinyl]-2-furoate) was used in *ex vivo* cortical cultures under normoxic conditions, which resulted in mitotic NPC displacement that had also been observed under hypoxic conditions (Figure 2.5*A*-*B*, *E*). Heparin, a non-specific but robust GRK2 inhibitor (Benovic, Stone et al. 1989), similarly induced mitotic displacement in the absence of hypoxia (Figure 2.S7), providing further support that GRK2 inhibition potentiates LPA_1 activity. Critically, no significant basal displacement of the mitotic NPCs was observed in LPA_1-null mice despite inhibitor exposure (Figure 2.5*C*-*E*, Figure 2.S7). GRK2 was also evaluated by qRT-PCR and Western blot. Hypoxia specifically reduced transcript levels of GRK2 but not GRK5, another major member of the GRK family, consistent with selective GRK2 reduction (Figure 2.5*F*). GRK2 protein levels were reduced significantly upon hypoxia as well (Figure 2.5*G*-*H*). These data support transcriptional and translational GRK2 inhibition by hypoxia as a mechanism for LPA_1 potentiation.

2.5 Discussion

This study identifies LPA signaling as an essential mediator of fetal brain hypoxia, providing functional linkage between lipid signaling and oxygen levels. Prior hypoxia studies in other systems identified transcriptional alterations mediated by HIF-1α (Majmundar, Wong et al. 2010). However, the protective effects of genetic removal or pharmacological inhibition of LPA_1, independent of any direct perturbation of HIF-1α, support HIF-1α-independent mechanisms mediated through LPA_1. The hypoxia-induced changes were prevented by both genetic removal of LPA_1 as well as pharmacological antagonism using two chemically distinct LPA_1 antagonists, supporting the direct involvement of LPA_1 in the observed cortical effects induced by hypoxia. These pharmacological results also eliminated developmental artifacts hypothetically produced by constitutive deletion of LPA_1. Notably, the LPA receptor-dependent hypoxic effects showed selectivity through both proximal pathways (GRK2 vs. GRK5) and defined downstream pathways ($G_{\alpha i}$ and Rac1). Combined with the observed stereotyped cellular responses and their dependence on LPA signaling, these results identified a finite and molecularly accessible set of interactions induced by fetal hypoxia, contrasting with an alternative scenario of non-specific hypoxic damage.

The stereotyped changes affecting NPCs reported here are similar to defects known to disrupt the normal laminar structure of the cerebral cortex (Machon, van den Bout et al. 2003; Kadowaki, Nakamura et al. 2007). The range of observed effects included cell adhesion disruption, altered positions of mitotic NPCs, and impaired cell migration as identified by the position and expression of the neuronal markers Tuj1 and

DCX. The relationships between independent reports on these cortical alterations, such as null-mutants for N-cadherin or beta-catenin, support the existence of other cellular and molecular changes initiated by fetal hypoxia that also depend on LPA signaling.

The dependence of stereotyped and selective hypoxic changes on LPA signaling, combined with previously reported associations between fetal hypoxia and various CNS disorders (Bergamasco, Benna et al. 1984; Cannon, van Erp et al. 2002; Kolevzon, Gross et al. 2007), underscores therapeutic implications of this study. Importantly, periventricular heterotopias observed in these diseases have been associated with a defect in migration of early postmitotic neurons, which we observed in our study (Sarkisian, Bartley et al. 2008). Targeting of LPA receptors by subtype selective agents and/or their selectively activated downstream pathways may provide new ways for interrupting or preventing deleterious sequelae of fetal hypoxia. The feasibility of targeting lysophospholipid receptors such as LPA_1 is further supported by the recent FDA approval of a brain-penetrant medicine (fingolimod) that targets related lysophospholipid receptor family members (Choi, Gardell et al. 2011). The relevance of fetal hypoxia-LPA signaling to other forms of hypoxic insult such as stroke may represent future disease processes that could be therapeutically addressed by modulation of lysophospholipid signaling.

2.6 Acknowledgements

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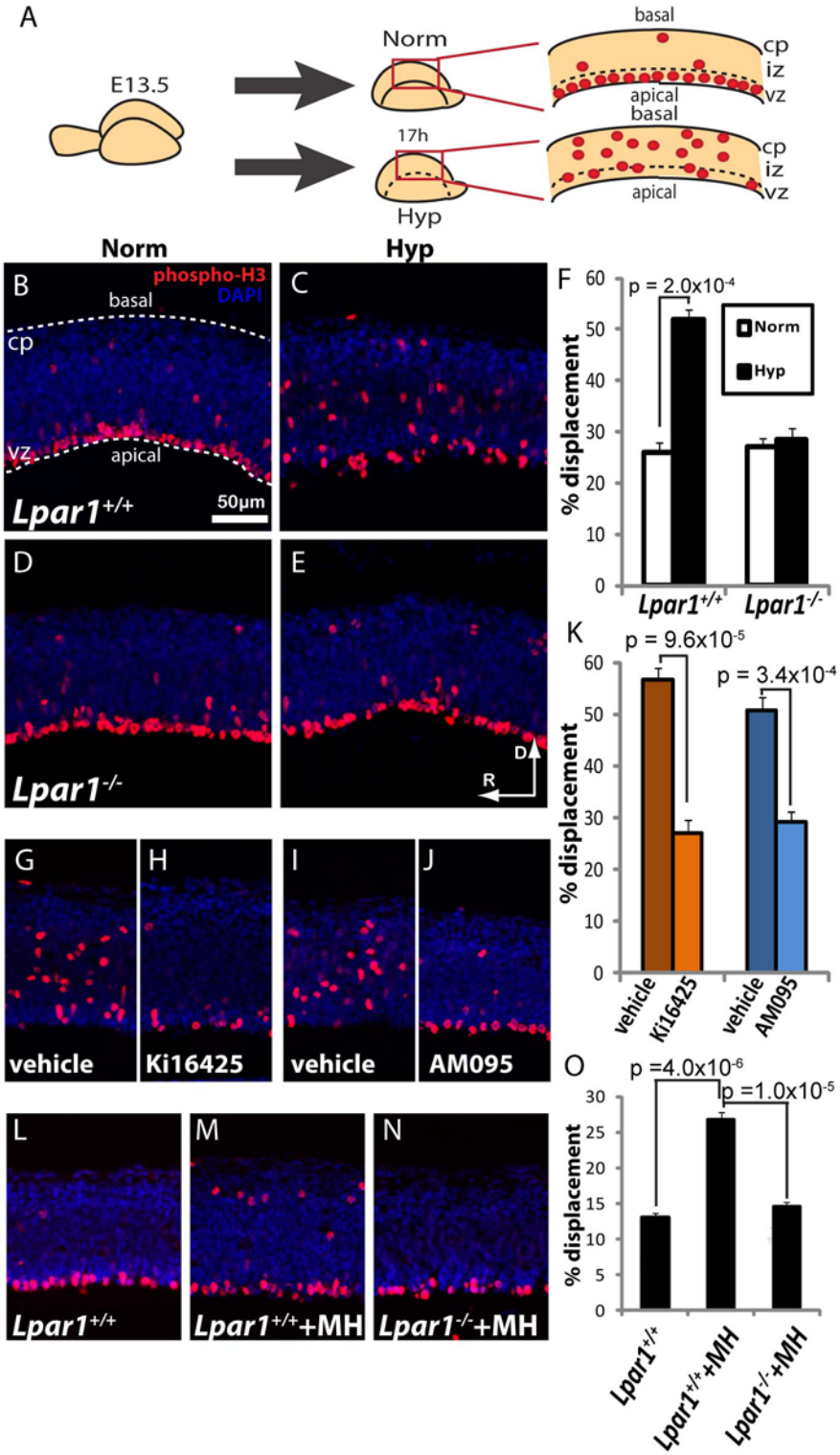
Figure 2.1 Hypoxia induces mitotic displacement in the embryonic brain *via* LPA_1.

(*A*) E13.5 cortices were cultured in normoxic (Norm) or hypoxic (Hyp) conditions. The red dots depict mitotic cells which were displaced basally upon hypoxic exposure. (*B-E*) Sagittal sections of cortices were immunolabeled with phospho-histone H3 (mitotic cell marker) and DAPI (nuclear stain). Wild-type (*Lpar1*$^{+/+}$) cortices were exposed to normoxia (*B*) or hypoxia (*C*) (n=8 matched pairs). *Lpar1*$^{-/-}$ cortices were exposed to normoxia (*D*) or hypoxia (*E*) (n=9 matched pairs). Orientation marker indicates rostral (R)-dorsal (D) direction for all panels. (*F*) Quantification of the displaced mitotic cells. (*G-J*) Hypoxic cortices were treated with vehicle or the LPA_1 antagonists: Ki16425 (n=7 matched pairs) (*G-H*) and AM095 (n=11 matched pairs) (*I-J*). (*K*) Quantification of (*G-J*). (*L-N*) Cortices were exposed to maternal hypoxia (MH) in utero. MH-exposed cortices (*M*) exhibited greater mitotic displacement compared to controls (*L*). This effect was absent in *Lpar1*$^{-/-}$ cortices (*N*). (*O*) Quantification of (*L-N*). All statistics were performed on matched cortices using the paired t-test (2-tailed). vz=ventricular zone. cp=cortical plate. Scale bars, 50 μm.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apical
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p = 2.0x10-4
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Lpar1+/+
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Lpar1-/- +MH
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Lpar1+/+
M
Lpar1+/+ +MH
N
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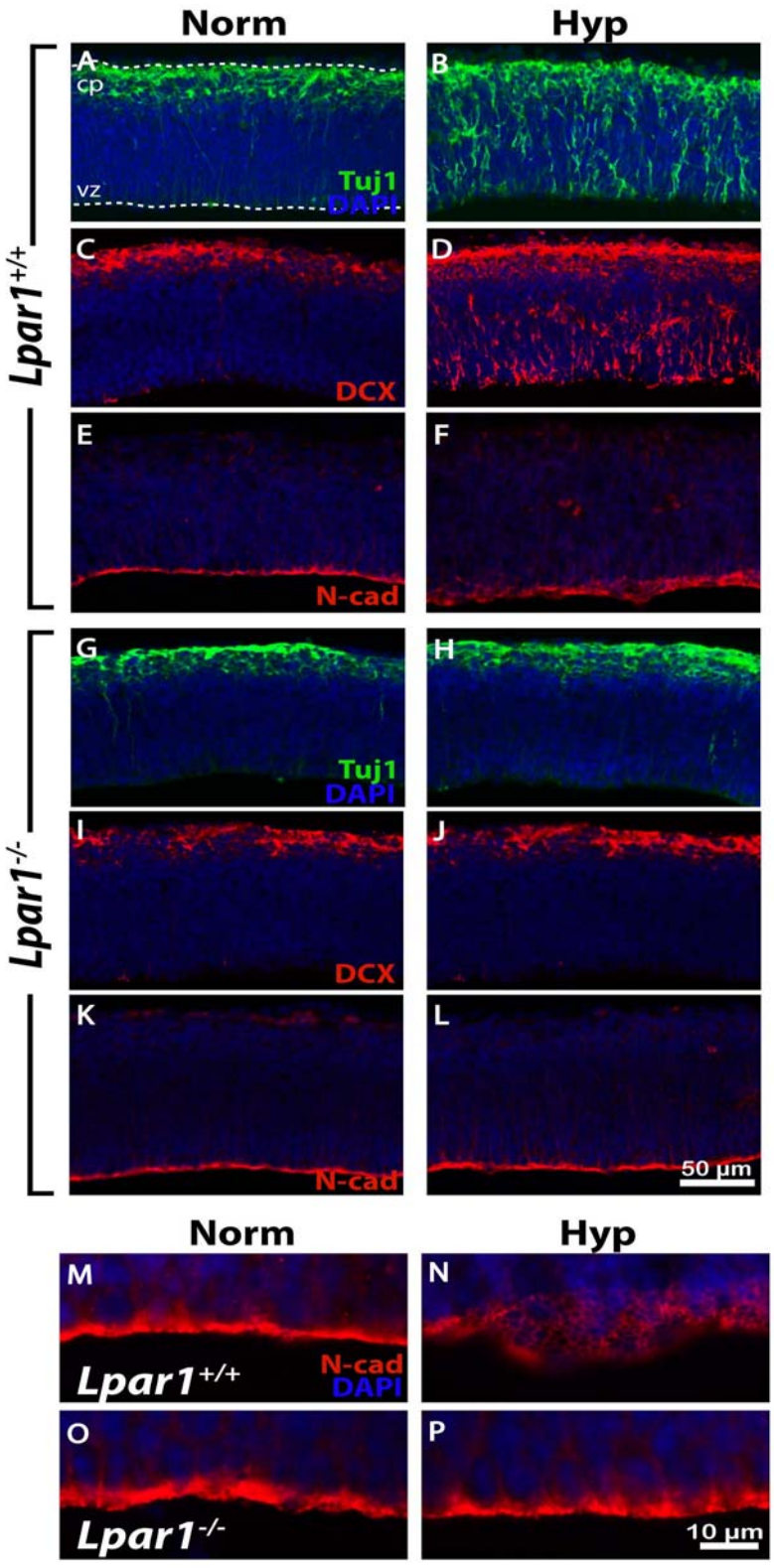
Figure 2.2 Hypoxia induces cortical disorganization *via* LPA_1.

(*A-L*) Sagittal sections of *Lpar1*$^{+/+}$ or *Lpar1*$^{-/-}$ *ex vivo* cortices subjected to normoxia or hypoxia were immunolabeled for Tuj1 (*A-B*, *G-H*; n=5 and 7 matched pairs, respectively), DCX (*C-D*, *I-J*; n=3 matched pairs in each group), and N-cadherin (*E-F*, *K-L*; n=15 and 5 matched pairs, respectively). (*M-P*) High magnification images of N-cadherin immunolabeling in *Lpar1*$^{+/+}$ (*M-N*) or *Lpar1*$^{-/-}$ (*O-P*) cortices cultured under normoxic or hypoxic conditions. Scale bars 50 μm (a-l), 10 μm (m-p).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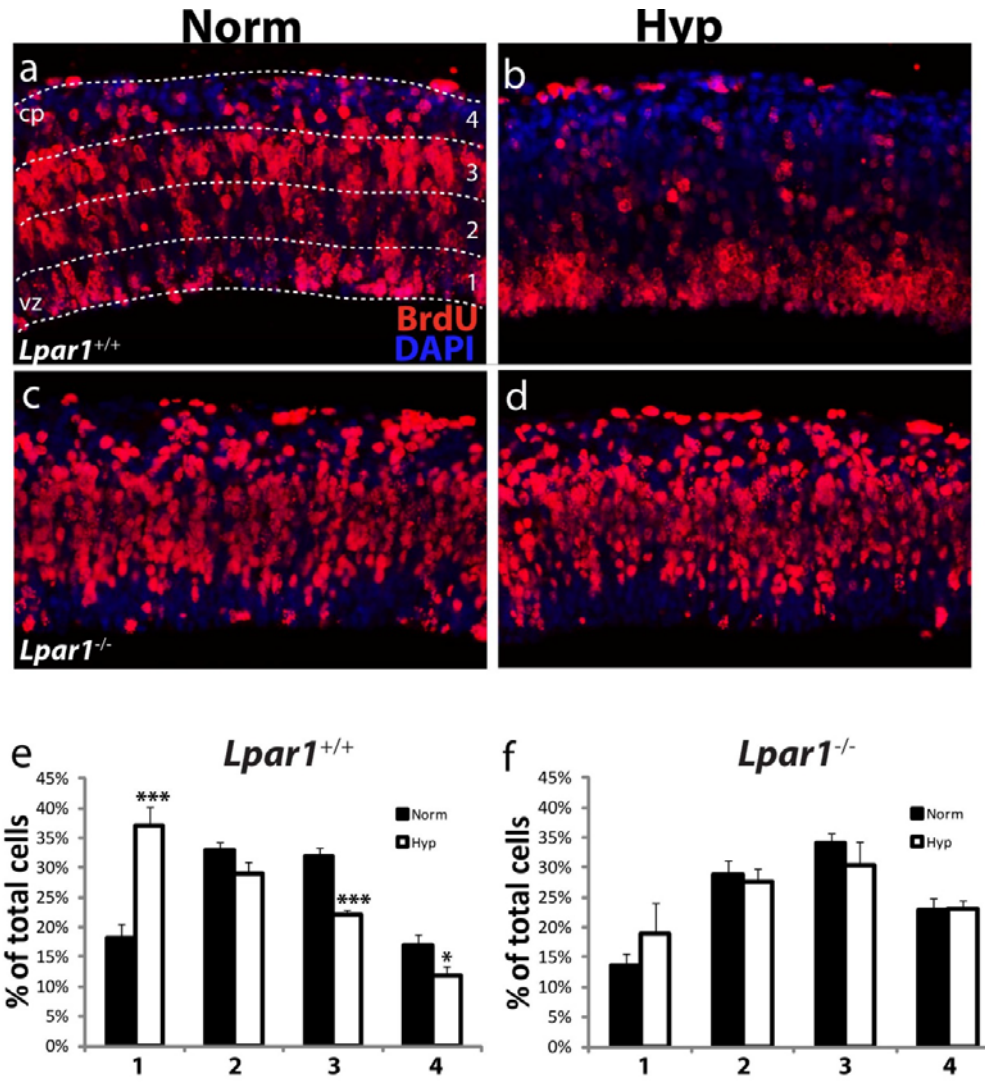
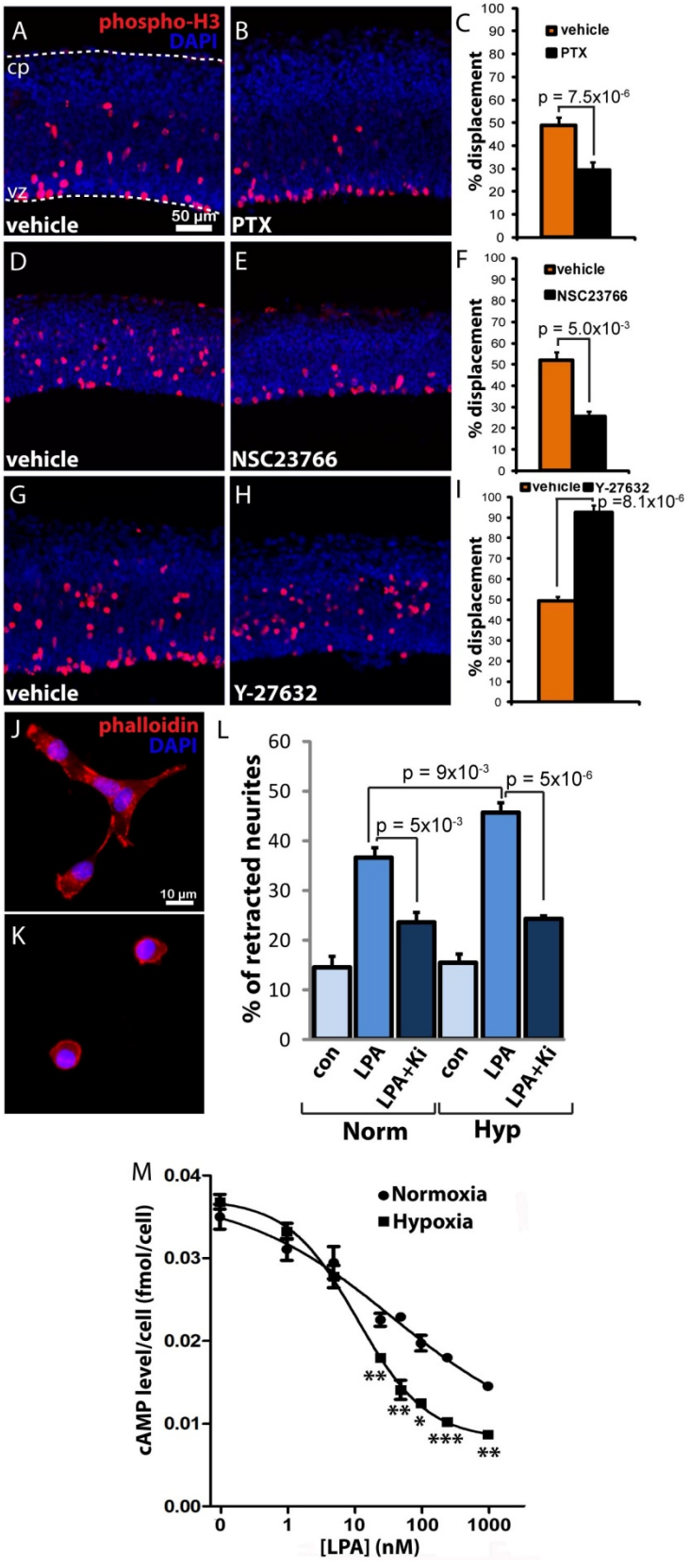


Figure 2.3 Hypoxia induces delayed migration of neural cells *via* LPA_1 signaling.

(*A-D*) Matched wild-type (*Lpar1*$^{+/+}$) or *Lpar1*$^{-/-}$ cortices were pulsed with BrdU 1h prior to normoxia or hypoxia for 17h, and subsequently immunolabeled for BrdU antibody and counterstained with DAPI. (*E-F*) Quantification of *A-D* (n=9, 6 matched pairs for wild-type or *Lpar1*-null cortices, respectively). Each cortical section was divided into 4 equal zones shown in (*A*), and the percentage of cells in each region was then calculated: 1 approximating the ventricular zone, 2 and 3 approximating the sub-ventricular zone, and 4 approximating the intermediate zone/cortical plate. *P <0.05, **P <0.01, ***P<0.001.

Figure 2.4 Hypoxia activates LPA_1 signaling pathways by potentiating LPA_1 activity.

PTX (*A-C*) and NSC23766 (*D-F*) inhibited hypoxia-induced mitotic displacement whereas Y-27632 treatment (*G-I*) increased displacement of mitotic cells (n=10, 6, and 7 matched pairs, respectively) compared to cortices treated with vehicle. (*J-K*) Representative images of LPA-induced neurite retraction. (*L*) LPA-induced retraction of TSM-1 neurites can be partially blocked by inhibiting LPA_1 with Ki16425. Other Ki16425-insensitive LPA receptors are expressed by TSM-1 cells. This retraction is potentitated by hypoxia and is Ki16425-sensitive (LPA_1-specific). (*M*) cAMP inhibition assay. B103 cells stably expressing LPA_1 were treated with increasing LPA concentrations in hypoxia, showing a maximal inhibition at 250nM (~50% over normoxia). cAMP levels normalized to cell number. *P <0.05, **P <0.01, ***P <0.001. Scale bars 50 μm (A-H), 10 μm (J-K).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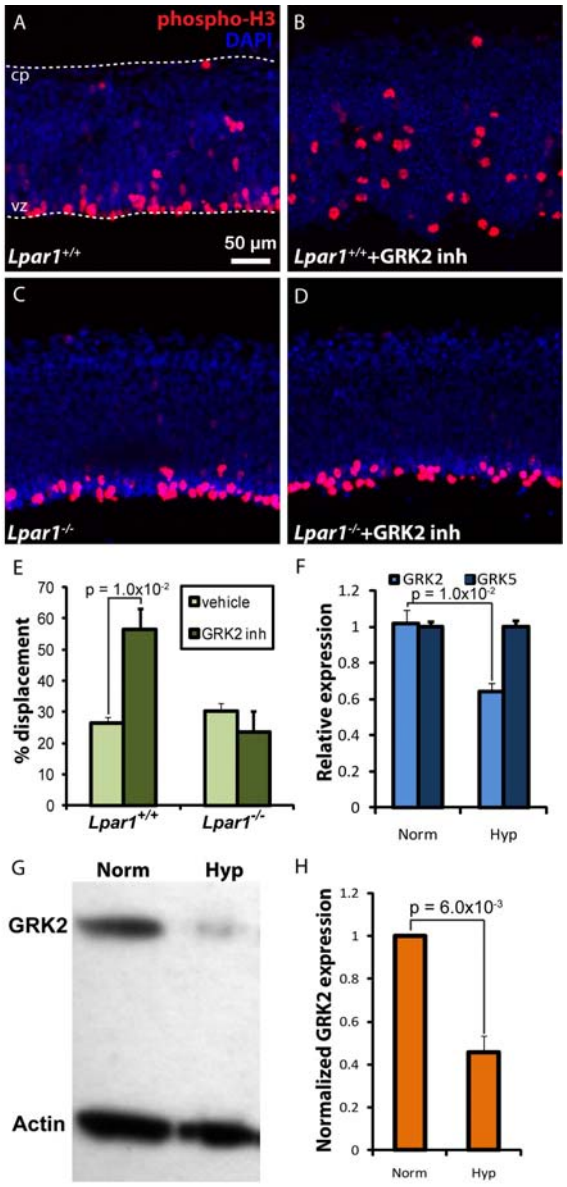

Figure 2.5 Hypoxia potentiates LPA_1 activity by inhibition of GRK2 expression.

(*A-D*) GRK2 inhibitor-treated cortices (*B*) show an increase in mitotic displacement compared to control hemispheres (*A*) (n=15 matched pairs). This effect is absent in *Lpar1*$^{-/-}$ cortices (*C-D*) (n=9 matched pairs). (*E*) Quantification of (*A-D*). (*F*) qRT-PCR quantification of GRK2 and GRK5 transcript levels in *ex vivo* cortices under normoxia or hypoxia (n=6). (*G*) Representative western blot of GRK2 protein in normoxic and hypoxic cortices and (*H*) corresponding quantitative analysis (n=4). Scale bars 50 µm

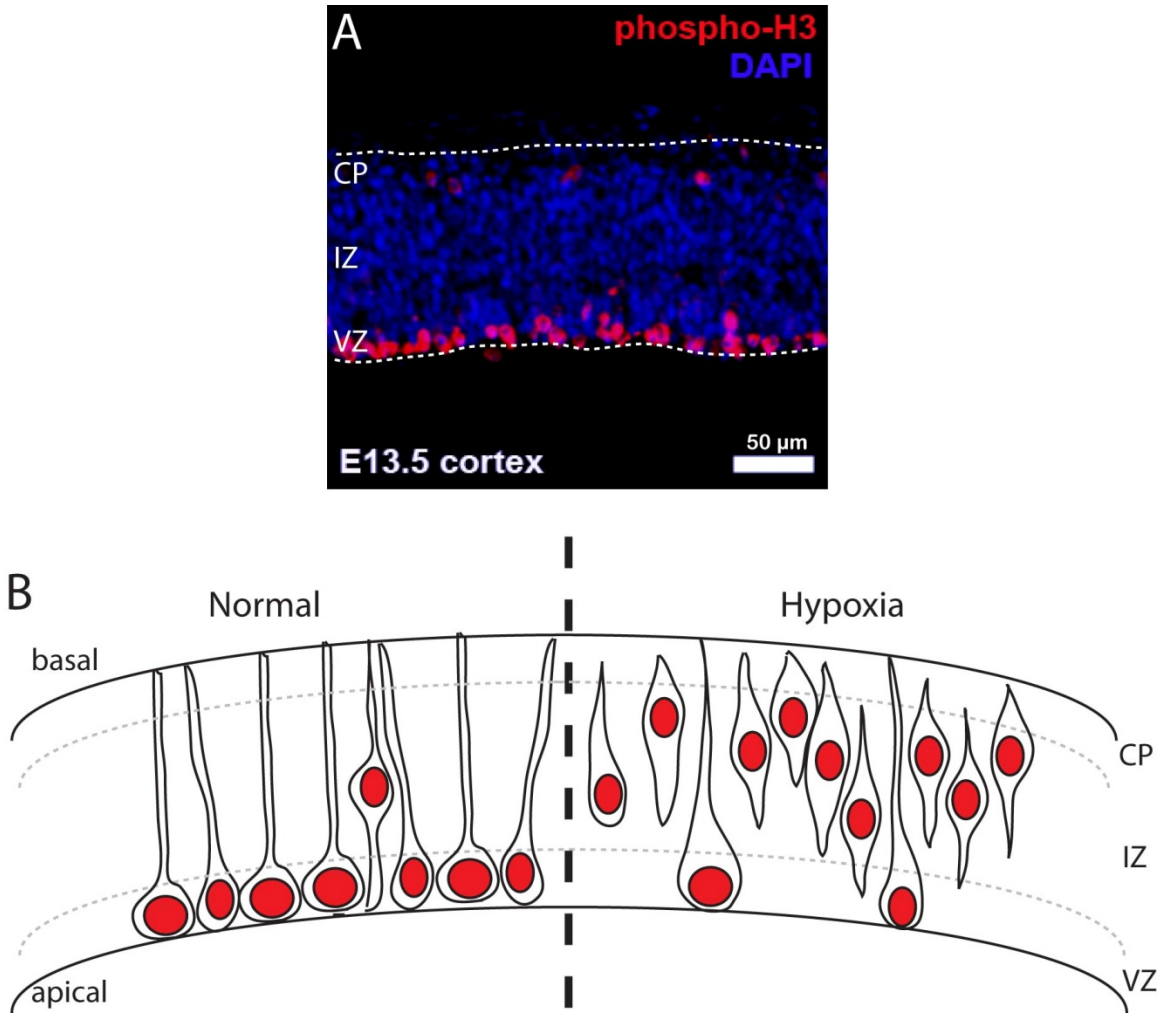

Figure 2.S1 Hypoxia induces displacement of mitotic cells.

(A) Representative image of an E13.5 cortex *in vivo*. Cells in M-phase (mitotic phase) are immunolabeled for phospho-histone 3 (phospho-H3) in red, and DAPI staining is shown in blue to show the morphology of the cerebral wall. *In vivo*, mitotic cells form an expected band in the ventricular zone (vz). (B) Schematic of interkinetic nuclear migration (INM) in the embryonic cortex. Cells in M-phase (mitotic phase) are shown in red. Upon hypoxia, displacement of the mitotic cells occur, as depicted. VZ = ventricular zone, IZ = intermediate zone, CP= cortical plate.

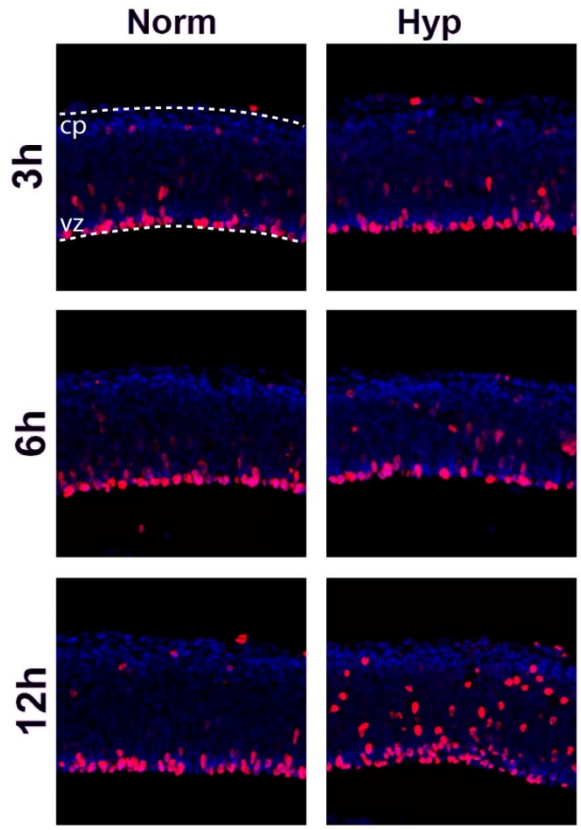

Figure 2.S2 Time course of *ex vivo* hypoxia treatment.

Mitotic displacement becomes prominent between 6h to 12h following hypoxic treatment.

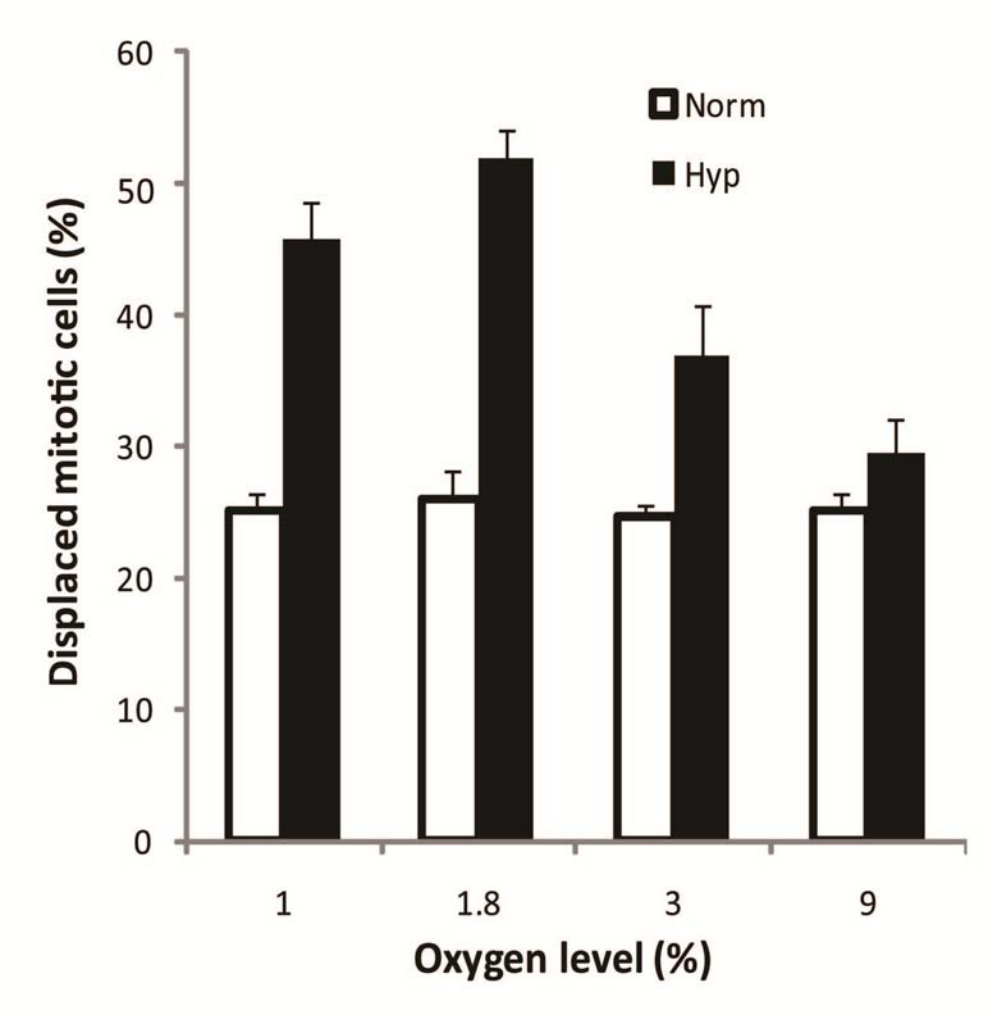

Figure 2.S3 Mitotic displacement was most prominent under 1.8% oxygen.

Ex vivo cortices were subject to varying oxygen levels, and the percentage of mitotic displacement is compared to matched control cortices. **P* <0.05. ***P* <0.01. ****P* <0.001.

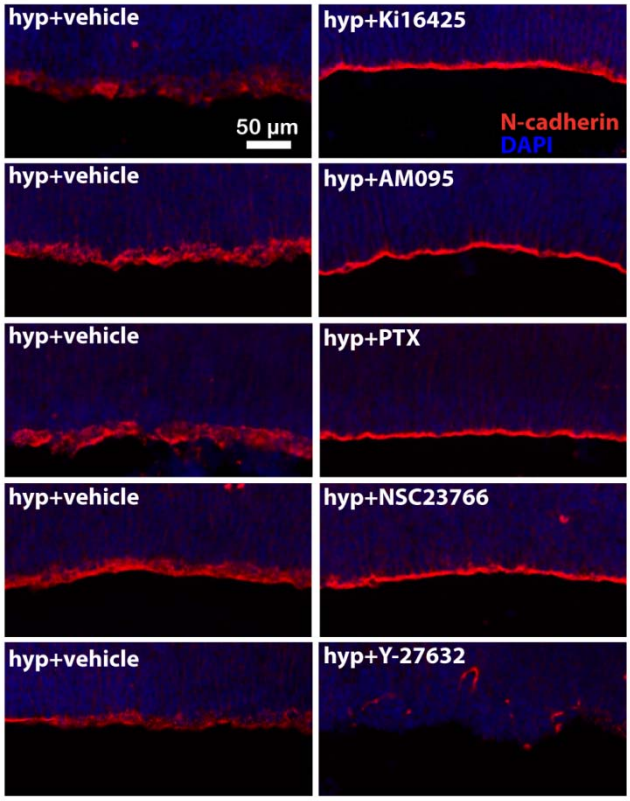

Figure 2.S4 Hypoxia induces disruption of N-cadherin distribution *via* LPA_1 signaling.

Sagittal cross-sections of cortices following *ex vivo* culture were immunolabeled for N-cadherin antibody and counterstained with DAPI. Treatment of cortices with Ki16425, AM095, PTX, or NSC23766 prevented disruption of N-cadherin distribution, whereas Y-27632-treated control cortices showed increased N-cadherin disruption (n=3 matched pairs for all). Scale bar, 50 µm.

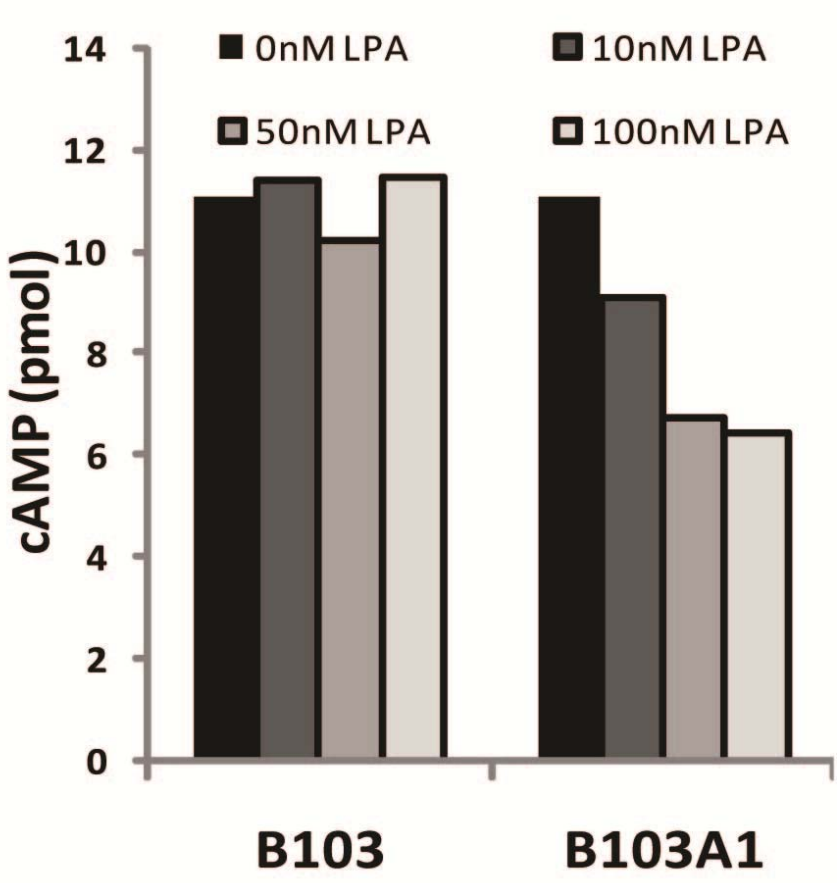

Figure 2.S5 cAMP inhibition following LPA exposure of B103 cells requires LPA_1.

Native B103 cells, without the heterologous expression of LPA_1, did not respond to LPA, whereas LPA_1-stably-expressing B103 cells show a robust response to LPA.

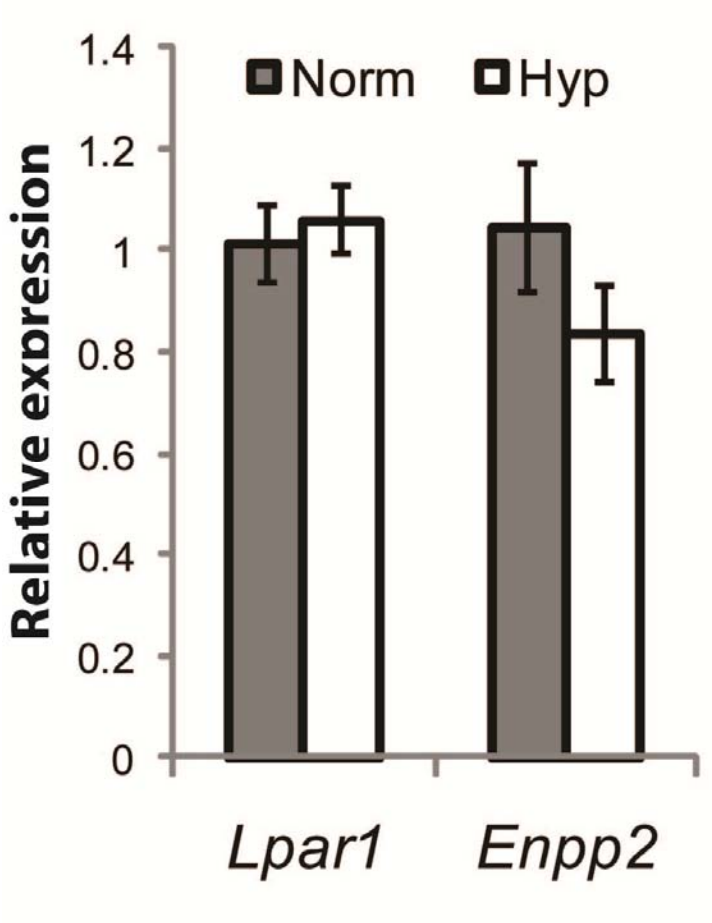

Figure 2.S6 Hypoxia does not significantly alter gene expression of *Lpar1* or ATX (*Enpp2*).

Real-time PCR of normoxic and hypoxic cortices was performed using primers for *Lpar1* and *Enpp2*, the gene encoding autotaxin (ATX). There was no significant difference in *Lpar1* and *Enpp2* transcript levels upon hypoxia. *P* values = 0.68 and 0.22, respectively.

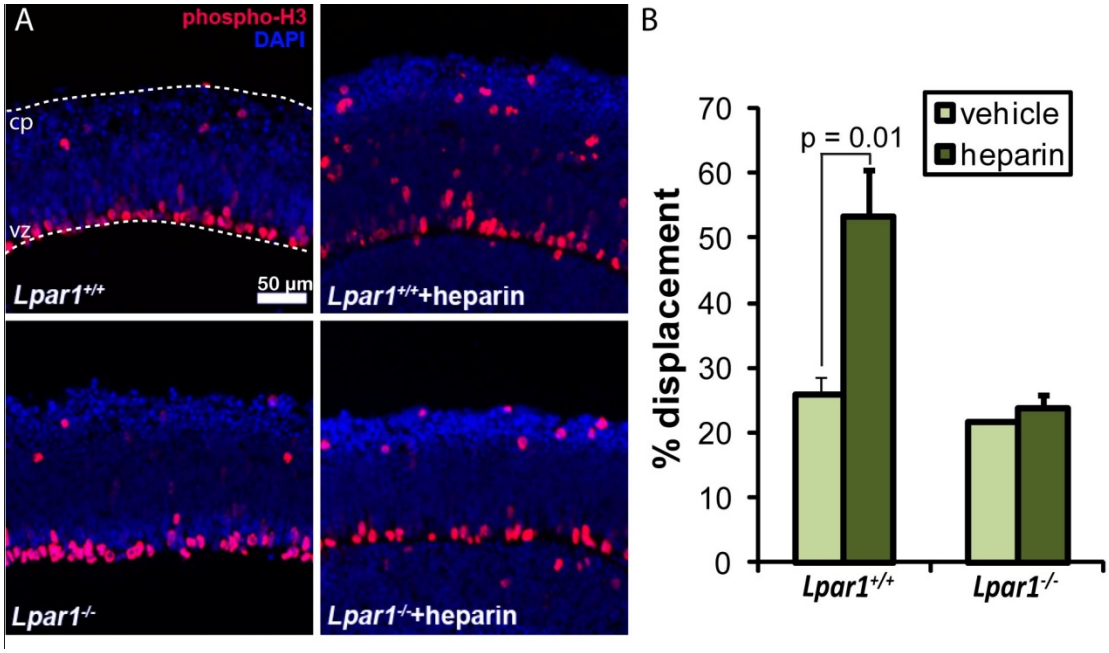

Figure 2.S7 Absence of mitotic displacement induced by heparin in *Lpar1*-null cortices.

Heparin, a non-specific but potent inhibitor of GRK2, was used as a secondary proof of the involvement of GRK2 in this signaling pathway. (A) Top panel: Wild-type (*Lpar1*$^{+/+}$) cortices treated with heparin showed an increase in displaced mitotic cells compared to controls (n=7 matched pairs). Bottom panel: *Lpar1*-null (*Lpar1*$^{-/-}$) cortices treated with heparin did not show any increase in mitotic displacement (n=3 matched pairs). (B) Quantification of the percentage of mitotic displacement in wild-type or *Lpar1*-null cortices treated with heparin. Scale bar, 50 µm.

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III. FURTHER DISSECTION OF THE MECHANISM OF HYPOXIA-INDUCED FETAL BRAIN DISORGANIZATION VIA LPA_1 SIGNALING

3.1 Introduction

We have previously shown that LPA_1 is an essential mediator of hypoxic-induced cortical disorganization during the fetal period (Chapter Two). In addition, we have presented the mechanism of this interaction, and several effectors of the pathway involved. We have shown that hypoxia potentiates LPA_1 activity through the inhibition of GRK2. Also, we have demonstrated that hypoxia activates LPA_1 and subsequently selectively the Gi pathway and downstream effectors.

Several experiments have since been performed to characterize this hypoxia-LPA signaling pathway further in depth. This chapter serves to provide the findings of more intricate experiments which have been designed to understand the signaling pathway and determine the involvement of other players in this system. Also, work has been done to extrapolate our findings in Chapter Two to other relevant systems, which will be presented in this chapter as well.

3.2 Assessment of the involvement of other LPA receptors in hypoxic response

LPA_1 expression is most highly expressed in the embryonic cortex during development, but other LPA receptors, namely LPA_2 and LPA_4, are also expressed at a significant level in the embryonic cortex. In order to determine their involvement in the hypoxia-LPA signaling pathway, genetic null mice cortices for these receptors were prepared for ex vivo culture and subject to hypoxic insult. These cortices were then immunolabeled for mitotic neural progenitor cells in order to determine the extent of mitotic displacement, which is one of the hypoxic effects described in Chapter Two.

As shown in Figure 3.1, neither *Lpar2* nor *Lpar4* null mice exhibit a significant difference in mitotic displacement compared to their respective littermate controls, indicating that the loss of LPA_2 or LPA_4 did not affect the hypoxic response. Thus, this suggests that other LPA receptors expressed in the embryonic cortex were not involved in the hypoxic response. This is in line with the previous data presented using *Lpar1* null cortices, where the loss of LPA_1 obliterated the various hypoxic effects on the embryonic cortex.

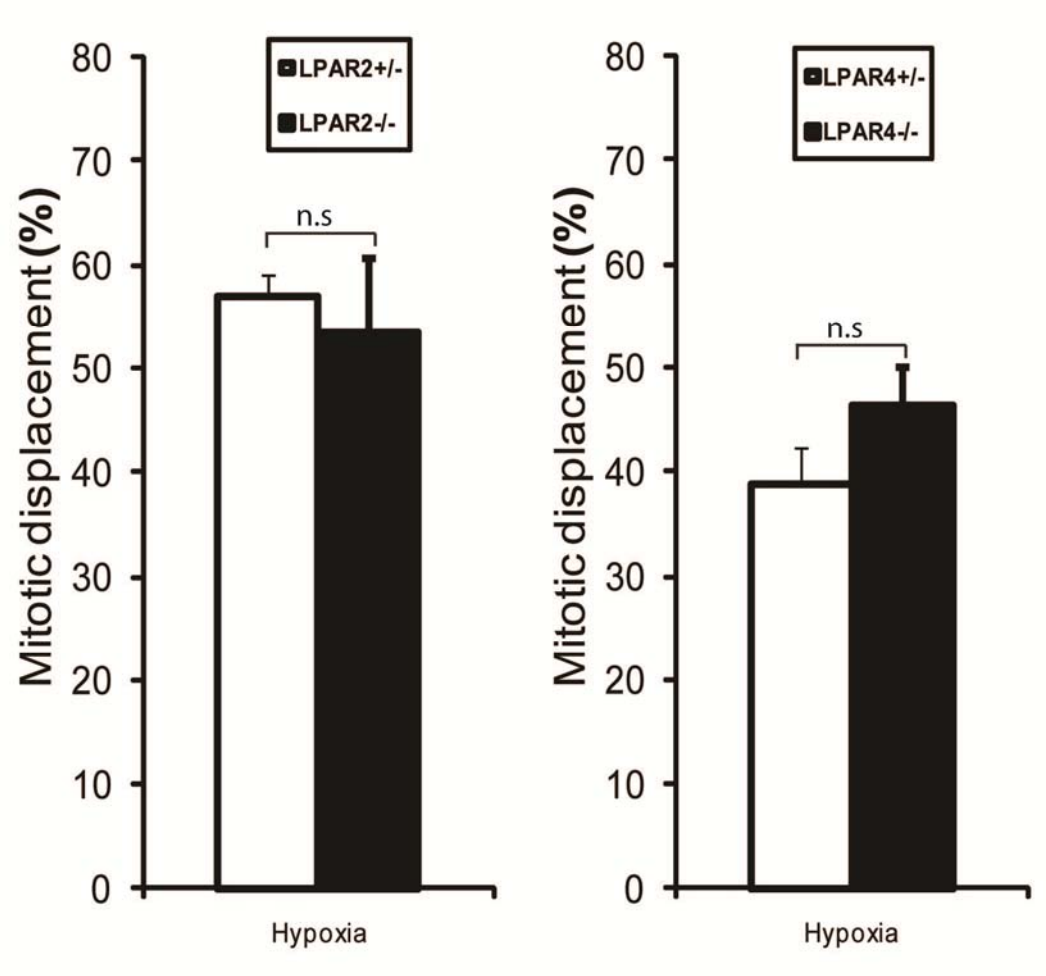


Figure 3.1 Neither LPA_2 nor LPA_4 are involved in the hypoxic-LPA signaling pathway.

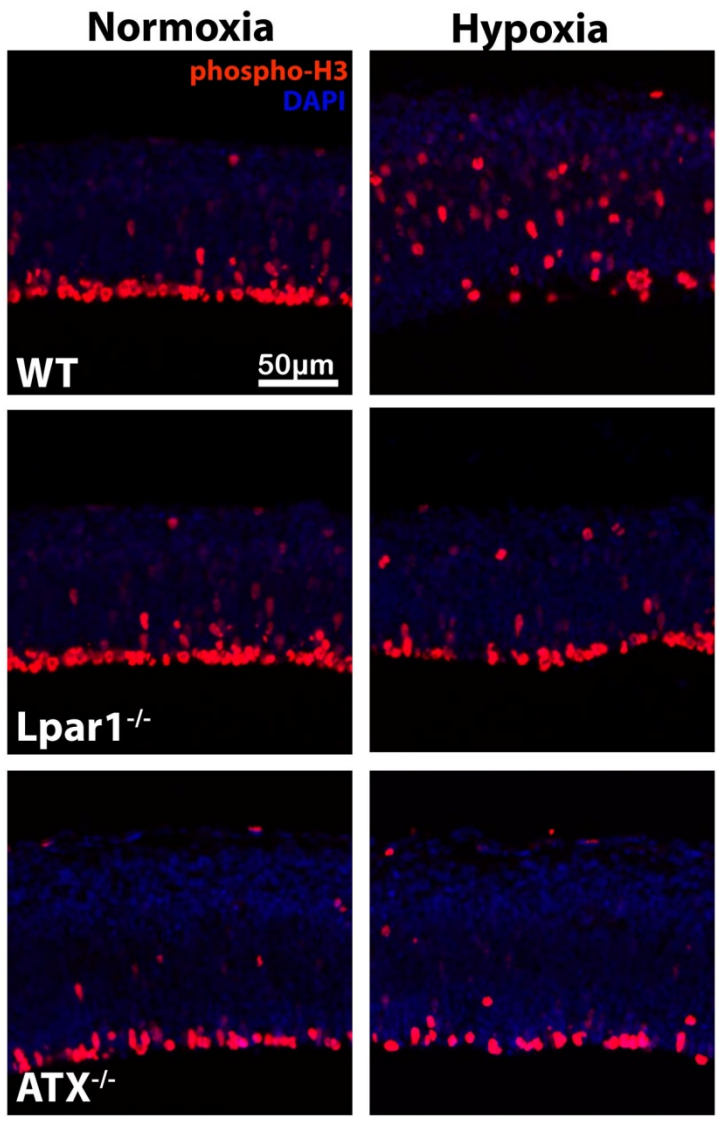
In order to investigate the possible roles of other LPA receptors expressed in the cortex, *Lpar2* (n=5 null cortices) and *Lpar4* (n=4 null cortices) were subject to hypoxia and assess for displacement of mitotic neural progenitor cells. As shown, the percentage of mitotic displacement under hypoxia was not significantly different in these null mice compared to littermate controls. Hence, LPA_2 and LPA_4 are not involved in this hypoxic response. Notably, the extent of mitotic displacement in the *Lpar4* null mice and littermate controls was lesser due to a different background strain of these mice compared to the rest of the mice used in this study.

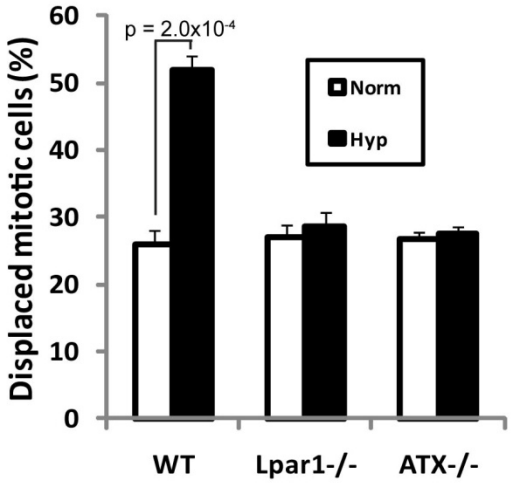
3.3 The hypoxic effect via LPA_1 is dependent on ligand (LPA) availability

We have presented previously in Chapter Two that hypoxia potentiates LPA_1 activity by the down-regulation of GRK2 which normally prevents LPA_1 from over-activation. Since it is commonly agreed upon that GRKs targets G-protein-coupled receptors for internalization and desensitization only after the receptors have been activated, GRK2 down-regulation will only cause an over-activation of LPA_1 if LPA_1 is activated to a significant level by its ligand, LPA in our system. In order to prove that this is the case, we looked at autotaxin nestin-cre conditional mutant mice. As mentioned previously, autotaxin is the main LPA-producing enzyme *in vivo*. Hence, the loss of autotaxin in the neural compartment would result in a significant loss of LPA in our system. When these cortices were subject to hypoxia, there was an absence of mitotic displacement, similar to that observed in the Lpar1 null cortices (Figure 3.2). This is in accord with our theory that the hypoxic response is dependent on the presence of sufficient LPA.

Figure 3.2 The hypoxic effect is dependent on the expression of autotaxin in the neural compartment.

Conditional nestin-cre autotaxin cortices subject to hypoxia and compared to matched control cortices (n=10 matched cortices). The bottom graph shows the percentage of mitotic displacement. As shown, autotaxin conditional null cortices displayed an absence of mitotic displacement, similar to that observed in *Lpar1* null cortices. Hence, this shows that the hypoxic response is dependent on the presence of the ligand LPA, since autotaxin is the main LPA-producing enzyme *in vivo*.


Normoxia
Hypoxia
phospho-H3
DAPI
WT
50µm
Lpar1-/-
ATX-/-


p = 2.0x10-4
Norm
Hyp
Displaced mitotic cells (%)
60
50
40
30
20
10
0
WT
Lpar1-/-
ATX-/-

3.4 HIF-1-alpha is involved in the hypoxia-LPA signaling pathway.

One of the most established downstream effectors of hypoxia is hypoxia-inducible-factor 1 alpha (HIF-1a). HIF-1a is a transcription factor which activates a variety of downstream genes to induce many hypoxic effects in various systems. Under normoxia conditions, the protein is rapidly degraded, hence limiting its activity. Upon hypoxia, HIF-1a is prevented from degradation, hence activating downstream effectors. Since we are effecting pathological hypoxia in our system, we wanted to determine if HIF-1a is involved in the hypoxic response as well.

In order to assess that, we used two non-specific but potent inhibitors of HIF-1a, which has been shown to inhibit HIF-1a activity effectively (Laughner, Taghavi et al. 2001; Zhang, Qian et al. 2008). When hypoxic cortices were treated with each of these inhibitors (rapamycin, ouabain), the extent of mitotic displacement was significantly reduced when compared to matched hypoxic cortices (Figure 3.3). Since the inhibition of HIF-1a reduced mitotic displacement significantly, this shows that HIF-1a is involved in this hypoxic response.

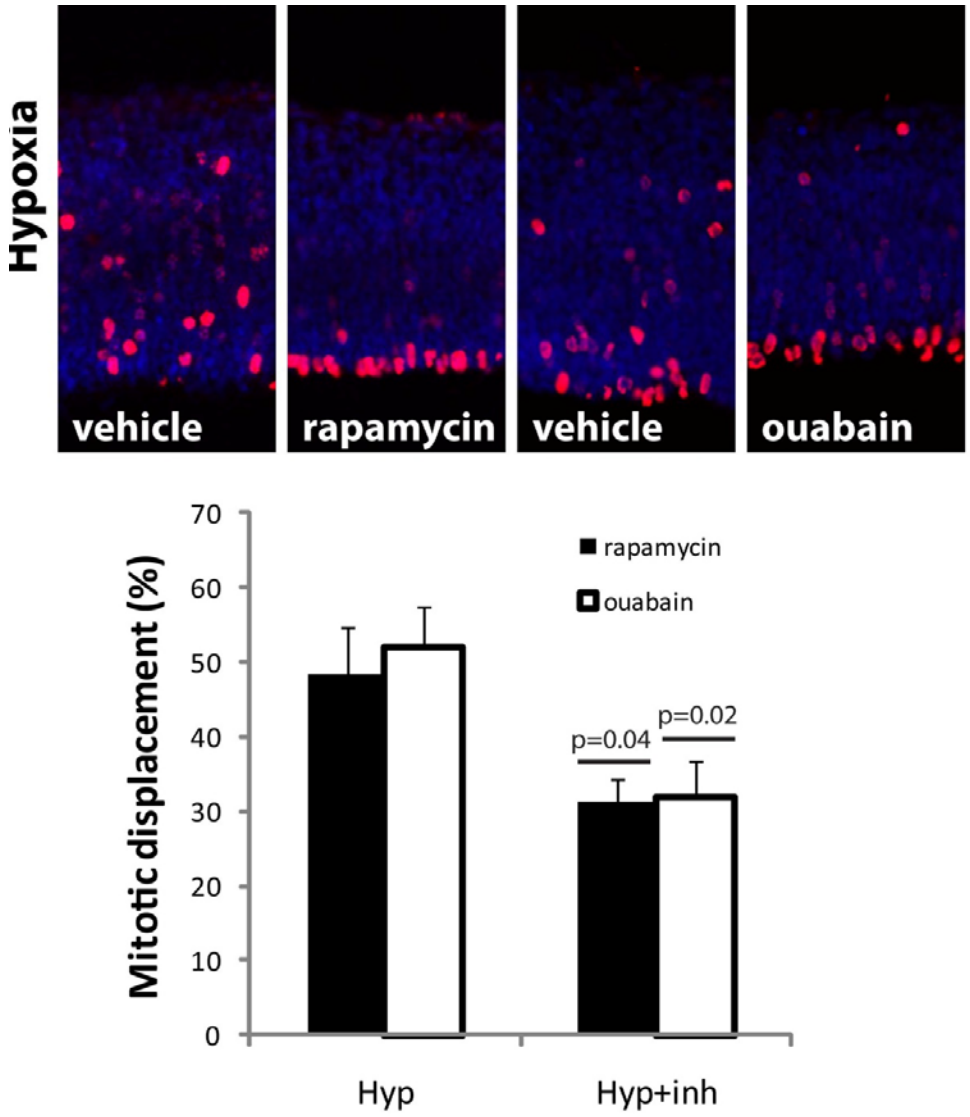


Figure 3.3 HIF-1a is involved in the hypoxia-LPA signaling pathway.

Hypoxic cortices were treated with two different non-specific but potent HIF-1a inhibitors- rapamycin (n=7 matched cortices) and ouabain (n=6 matched cortices), of which the percentage of mitotic displacement was subsequently quantified in each case. The inhibition of HIF-1a was found to cause a significant reduction in mitotic displacement in both cases.

3.5 HIF-1a activation is downstream of GRK2 inhibition by hypoxia.

Since we have established that HIF-1a is involved in the hypoxic response in our system, it will be of importance to understand if HIF-1a is upstream or downstream of LPA_1 signaling in the system. In order to elucidate this, hypoxic cortices exposed to the two HIF-1a inhibitors were assessed for any change in GRK2. GRK5 levels was also assayed as a control gene, since it shares similar biological roles to GRK2 (Harris, Cohn et al. 2008). Surprisingly, GRK2 levels remained unchanged even upon HIF-1a inhibition, arguing for HIF-1a as a player downstream of GRK2 inhibition. Since GRK2 inhibition directly affects LPA_1 activity, it is highly plausible that HIF-1a is downstream of LPA_1 signaling. In order to ascertain that, we have plans to look at HIF-1a levels in hypoxic *Lpar1* null cortices in the future. If the loss of LPA1 brings about no change in HIF-1a levels compared to littermate cortices, it would imply that HIF-1a is downstream of LPA_1.

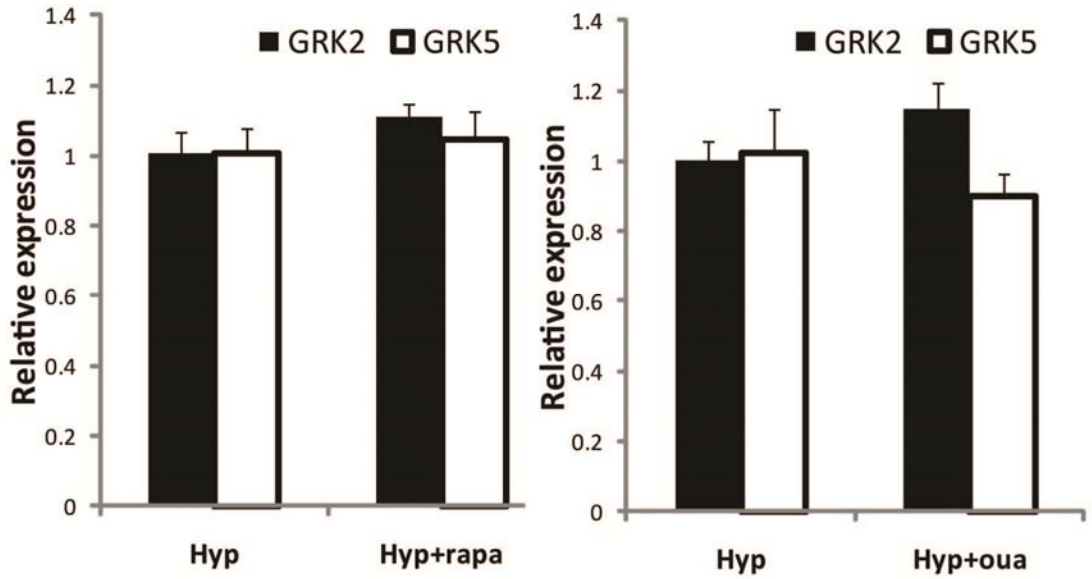


Figure 3.4 GRK2 levels remained unchanged by HIF-1a inhibition

Hypoxic cortices were treated with two different non-specific but potent HIF-1a inhibitors- rapamycin (n=4 matched cortices) and ouabain (n=4 matched cortices), of which the gene expression level of GRK2 and GRK5 (control gene) was assessed through quantitative PCR in each case. The inhibition of HIF-1a did not induce any significant change in GRK2 in both cases.

3.6 Hypoxia potentiates LPA_1 activity in breast cancer cells expressing endogenous LPA_1.

Previously, we have shown that hypoxia increased LPA1-induced neurite retraction in a cell-autonomous assay (Chapter 2). LPA signaling has been associated in passing with hypoxia in a handful of cancer studies previously, where hypoxia was shown to increase LPA-induced responses (Kim, Sengupta et al. 2006; Park, Jeong et al. 2007). As a first step to relate our findings to the cancer field, we measured cell rounding of breast cancer cells which have been shown to express endogenous levels of LPA_1 and respond to LPA by rounding (Figure 3.5). It was observed that the results obtained were similar to that in our neurite retraction assay: Hypoxia increases LPA-induced cell rounding by 10% compared to normoxia conditions. Importantly, Ki16425 treatment was able to inhibit this cell rounding response to the same extent for both normoxia and hypoxia, indicating that hypoxia induces the increase in cell rounding through LPA_1. Thus, this suggests that there might be similarities in the effect of hypoxia on cortical or cancer development, and that the mechanism that we proposed for the modulation of LPA signaling by hypoxia might be applicable to fields other than cortical development.

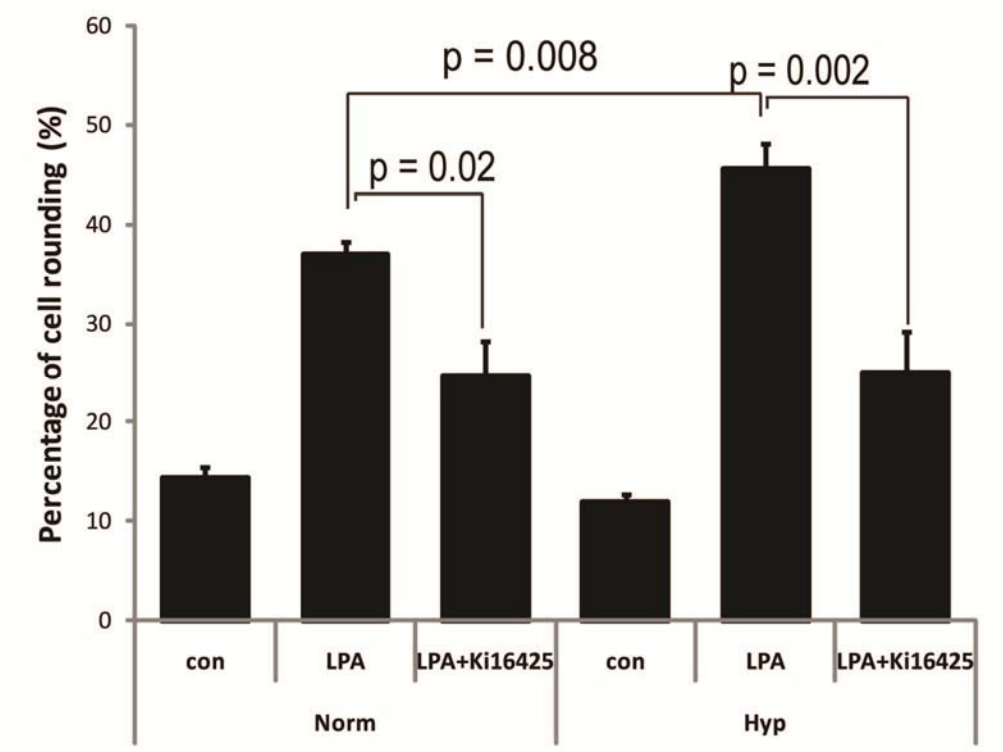


Figure 3.5 LPA_1-mediated cell rounding is potentiated by hypoxia in MDA-MB231 breast cancer cells.

MDA-MB231 cells are known to express significant levels of LPA1 and respond to LPA by cell rounding. In this assay, 100nM LPA was added under both normoxia and hypoxia, in the presence or absence of the LPA1 antagonist Ki16425, which after the percentage of cell rounding was calculated. These results were similar to that of the neurite retraction assay in Chapter 2.

3.7 Hypoxia does not increase LPA production

Previously, we have shown that hypoxia effects were dependent on the presence of the ligand LPA (Chapter 3.3). We wanted to know if hypoxia influences LPA production as well on top of GRK2 inhibition. In order to assess that, we added a fixed amount of LPA (25 nM LPA) to ATX-nestin-cre conditional cortices, and subject one hemisphere to hypoxia. In this system, since the LPA-producing enzyme (ATX) is absent and a fixed amount of LPA was added, any decrease in the potentiation of LPA signaling upon hypoxia would be due to a lack of increased LPA production. As shown in Figure 3.6, hypoxia was found to elicit a similar level of mitotic displacement as in wild-type cortices (Figure 2.1). Hence, this shows that hypoxia does not regulate LPA production to increase LPA signaling, and its sole mechanism is the inhibition of GRK2 to increase LPA_1 activity.

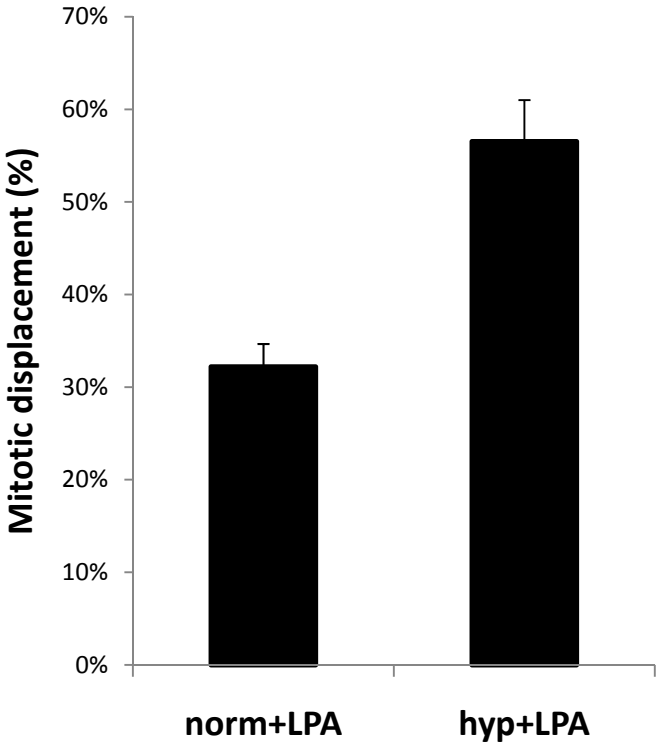


Figure 3.6 Hypoxia does not increase LPA production

Autotaxin-nestin-cre conditional knockout cortices were subject to 25nM LPA and subject to either normoxia (control) or hypoxia. The extent of displacement of mitotic NPCs was then quantified and represented in the graph. Hypoxia was found to elicit a similar amount of mitotic displacement to wild-type cortices, in the absence of autotaxin, the main LPA-producing enzyme. Hence, hypoxia does not increase LPA signaling through the regulation of LPA production.

3.8 Hypoxia causes LPA_1-dependent ectopic positioning of early neurons *in vivo*.

Previously, we have shown that maternal hypoxia induces a significant displacement of mitotic NPCs, similar to that observed *ex vivo* (Chapter 3). In order to investigate if other hypoxic effects observed *ex vivo* was also physiologically relevant, we immunolabeled cortices exposed to maternal hypoxia with the early post-mitotic neuronal marker Tuj1. We observed an ectopic positioning of early neurons in cortices exposed to maternal hypoxia, similar to that observed in *ex vivo* cultures. Importantly, this ectopic positioning was absent in LPA_1 knockout cortices, underlining the importance of LPA_1 in this process (Figure 3.7). Hence, this shows that hypoxia causes LPA_1-dependent ectopic positioning of early neurons *in vivo*, and that ex vivo results are relevant physiologically.

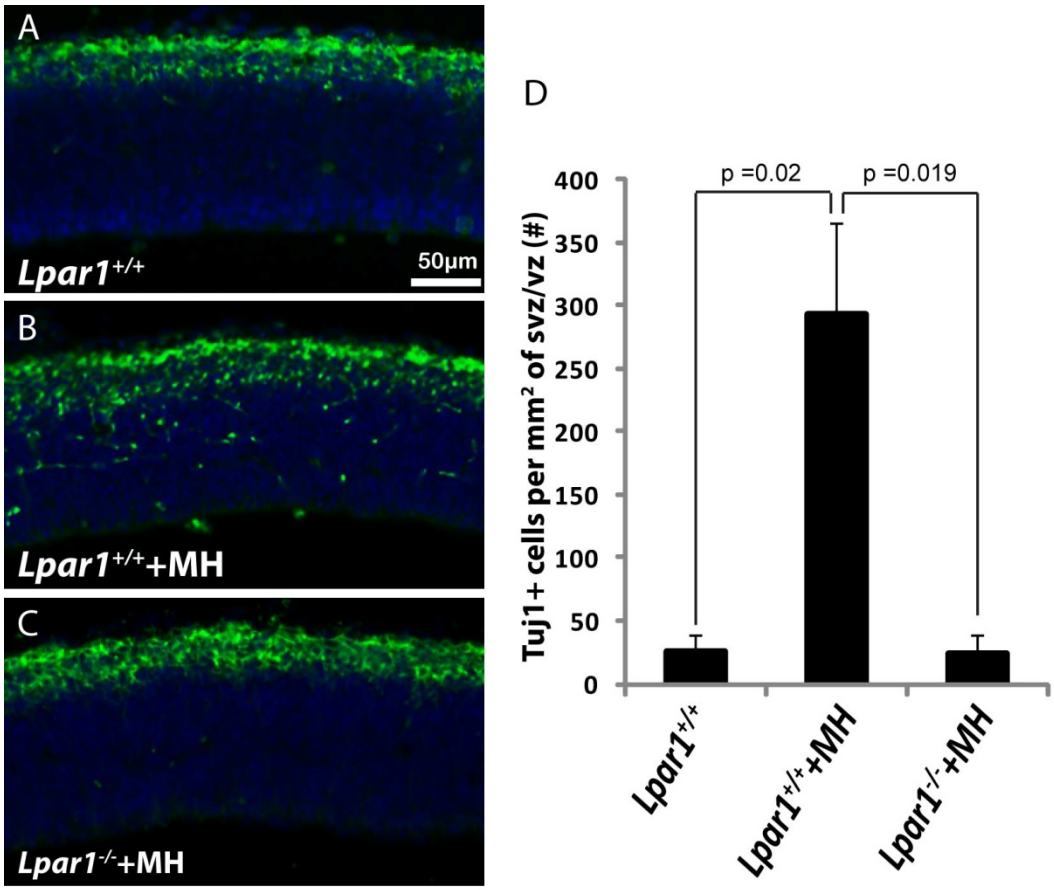


Figure 3.7 Hypoxia causes LPA_1-dependent ectopic positioning of early neurons *in vivo*.

Wild-type and LPA_1 knockout pregnant dams were subject to maternal hypoxia at E13.5. The embryos were then dissected out at E14.5, of which sagittal sections were immunolabeled with early postmitotic neuronal marker Tuj1. On the left panel, wild-type cortices exhibited an ectopic positioning of early neurons, similar to that observed *ex vivo*. This is absent in the LPA_1 knockout. The right panel shows the quantification of ectopic early neurons in the sub-ventricular zone and ventricular zone (svz/vz). Maternal hypoxia causes a significant ectopic displacement of early neurons which is dependent on LPA_1.

3.9 Conclusions

In this chapter, the hypoxia-LPA signaling pathway is further characterized. In addition, we also extrapolated our findings to the cancer field. A brief summary of the findings is presented below (Table 3.1). From these data, one can conclude that LPA_1 is the primary LPA receptor involved in mediating the effects of hypoxia. The fact that the ligand, LPA, is required for the hypoxic effects underscores the importance of the state of activation of the LPA_1 receptor. In addition, we demonstrated that HIF-1a is involved in this process, and shed new light on its role in the signaling pathway. Also, we show that hypoxia does not influence LPA production. We also show additional analysis of cortices exposed to hypoxia in vivo, strengthening our point that our ex vivo results are relevant physiologically. Importantly, we were able to demonstrate that a similar potentiation of LPA_1 activity occurs in breast cancer cells, which could aid the understanding of previously reported synergy between hypoxia and LPA in cancer and provide a rationale for LPA_1 antagonists as a therapeutic for cancer.

Summary of findings
• **Other LPA receptors expressed significantly in the embryonic cortex are not involved in the hypoxia-LPA signaling pathway**
• **The potentiation of LPA_1 by hypoxia is dependent on the presence of LPA**
• **HIF-1a is involved in the hypoxia-LPA signaling pathway**
• **HIF-1a activation is downstream of GRK2 inhibition**
• **Hypoxia potentiates LPA_1 activity in breast cancer cells as well**
• **Hypoxia does not increase LPA production**
• **Hypoxia causes LPA_1-dependent ectopic positioning of early neurons *in vivo***

Table 3.1 Summary of findings

3.8 References

Harris, D. M., H. I. Cohn, et al. (2008). "GPCR signalling in hypertension: role of GRKs." Clin Sci (Lond) **115**(3): 79-89.

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Zhang, H., D. Z. Qian, et al. (2008). "Digoxin and other cardiac glycosides inhibit HIF-1alpha synthesis and block tumor growth." Proc Natl Acad Sci U S A **105**(50): 19579-19586.

IV. LPA_1 AND LPA_2 ARE REQUIRED FOR PROPER BLOOD VESSEL FORMATION IN THE CORTEX DURING DEVELOPMENT

Siew T. Teo, Yun C. Yung, Deron R. Herr & Jerold Chun

Department of Molecular Biology, Dorris Neuroscience Center, The Scripps Research Institute, 10550 North Torrey Pines Road, DNC-118, La Jolla, CA 92037

4.1 Abstract

LPA signaling has been implicated in various processes of endothelial cells, including endothelial migration, proliferation and survival. Due to the enriched expression of LPA receptors LPA_1 and LPA_2 in the embryonic cortex, we investigate the role of these receptors in the formation of the vasculature in the developing cortex. We found that the loss of LPA_1 and LPA_2 results in leaky blood vessels, specifically in the cortex. Importantly, the leakiness of the blood vessels occurs only during the critical period of just prior to birth and extending to early postnatal ages, and is observed specifically in the cortex, suggesting a role for these receptors in vascular formation during development. Also, the ablation of only LPA_1 or LPA_2 did not induce any leakage of the cortical blood vessels, indicating that both LPA_1 and LPA_2 are required for the proper formation of cortical blood vessels.

In addition, *Lpar1/Lpar2* null cortices exhibited a greater branching of blood vessels in the meningeal layer and a significant increase in vascular density within the cortex as well. At the electron-microscopic level, a significant population of endothelial cells in *Lpar1/Lpar2* null cortices was abnormally electron-light, indicating "unhealthy" metabolically compromised endothelial cells. In all, these data suggest a role of LPA_1 and LPA_2 in cortical vascular development.

4.2 Introduction

Lysophosphatidic acid is a bioactive lipid which acts as an extracellular signaling molecule by coupling to at least 6 known cognate G-protein-coupled-receptors, LPA_{1-6}. Out of these receptors, LPA_1 and LPA_2 are known to be enriched in expression in the embryonic cortex during development. As such, these receptors have been implicated in various aspects of cortical development, such as neurogenesis, neuronal migration, survival and cortical growth. However, little is known about the role of LPA signaling in vascular development in the cortex, despite various studies which have hinted at the importance of LPA signaling in vascular processes.

Importantly, the first definitive study that linked LPA to vascular development was that of the autotaxin (ATX/ Enpp-2)-deficient phenotype. ATX is the major LPA-producing enzyme *in vivo* (Tanaka, Okudaira et al. 2006), and functions as a secreted lysophospholipase D that converts lysophosphatidylcholine (LPC) into LPA (Tokumura, Majima et al. 2002). ATX-deficient mice die at E9.5 with profound vascular defects in the yolk sac and embryo, which coincides with the age of increased ATX and LPA receptor expression in normal embryos (van Meeteren, Ruurs et al. 2006). Notably, severe vascular defects, such as absence of yolk sac vascularization and the presence of enlarged embryonic blood vessels, were apparent by E9.5. This strongly suggests that LPA signaling is critical for vasculature development.

Furthermore, individual LPA receptor (LPA_{1-3}) knockouts showed moderate vascular defects with the presence of frontal hematomas in 2.5% of LPA_1 single knockout and 26% of $LPA_{1,2}$ double knockout embryos (Contos, Fukushima et al. 2000; Contos,

Ishii et al. 2002). The lack of more severe vascular defects might be due to redundancy of the LPA receptors, as well as the continued presence of S1P lysophospholipid receptors that have proven roles in vascular development and integrity (Ozaki, Hla et al. 2003; Hla 2004; Hla, Venkataraman et al. 2008). It is possible that more severe disruptions of vascular development will only occur with the simultaneous deletion of additional LPA receptors and/or in conjunction with S1P receptors.

In addition, LPA signaling has been involved in various aspects of angiogenesis, which is the predominant mechanism of vascular development in the cortex. Angiogenesis is a process which involves the proliferation and migration of endothelial cells to form new sprouting blood vessels from existing vasculature. LPA has been implicated in both the proliferation and migration of endothelial cells. In a study using adult bovine aortic and human umbilical vein endothelial cells, LPA was observed to stimulate both endothelial cell migration and proliferation in a wound healing in vitro assay (Lee, Goetzl et al. 2000). Also, LPA was shown to induce the migration of several endothelial cell types, including fetal bovine heart endothelial cells and bovine aortic endothelial cells (Panetti, Nowlen et al. 2000). In addition, LPA was found to evoke the formation of new blood vessels in a chicken chorio-allantoic membrane (CAM) assay, confirming it as an angiogenic compound (Rivera-Lopez, Tucker et al. 2008).

In view of these studies, it is highly likely that LPA signaling is involved in the development of the vasculature in the cortex. Through the use of LPA receptor-null mutant mice, we will investigate the role of LPA signaling in vascular formation in the developing cortex. Here we report the results of this analysis that identifies LPA_1 and

LPA_2 as essential mediators in the proper formation of the vasculature in the embryonic cerebral cortex.

4.3 Material and Methods

Vascular leakage assays.

1 ml of 10mg/ml Hoechst 33242 (Invitrogen) or DiI () was perfused into the E18.5 mouse embryo through the left ventricle, after the right atrium has been punctured to eliminate any unnecessary pressure of perfusion on the vasculature. The brain and other reference organs are then dissected out, fixed in 4% paraformaldehyde and subsequently sectioned using the vibratome (DiI) or cryostat (Hoechst 33242). For Evan's blue quantification, 200, 600 and 1200ul of 1% EB were perfused into the mouse embryo at E18.5, P7 and P14 respectively. Cortical or other reference tissues were then flash-frozen on dry ice. 1 ml of formamide was added to each sample, and the samples were incubated at 65 degrees for 72 h to extract the EB. The samples were then spun down at 13000 rpm for 10 min, and the absorbance was read at 620 nm. The concentration of EB was then calculated using a standard curve, and normalized to weight of the samples.

Whole mount immunolabeling.

Whole cortices were fixed in 4% paraformaldehyde and washed with 0.1% TritonX-100 in phosphate-buffered saline (PBS). They were then blocked with 3% BSA in 0.1% TritonX-100 in PBS. A rat-CD31 antibody (BD Biosciences) was used at a dilution of 1:1000 overnight on a rotator. The cortices were then washed extensively and a goat anti-rat biotin was used at a dilution of 1:400 for 2 h. The cortices were then

washed and subsequently developed using an ABC kit (Vector Labs) according to the manufacturer's instructions.

Immunohistochemistry.

The antibody used was rat-CD31 (BDBiosciences), and detected with a secondary AF488-conjugated donkey anti-rat antibody (Invitrogen) Tissue was processed as described previously (Kingsbury, Rehen et al. 2003).

Analysis.

The number of branch points per area for the whole mount cortices and the number of blood vessels per area of the cortical sections were quantified using the program ImageJ.

4.4 Results

The vasculature is known to permeate the brain as early as E10.5 onwards, and the blood-brain-barrier (BBB) is thought to be fully formed by E18.5. In order to evaluate blood vessel integrity in the developing brain, the vasculature was visualized by perfusing the embryo at E18.5 with a variety of dyes. DiI is a fluorescent dye which has been used previously to visualize retinal vasculature. When perfused with DiI, *Lpar1, 2* null cortices showed DiI widely dispersed around the blood vessels in the surrounding ependymal cells, whereas DiI was contained within the vessels in littermate controls (Figure 4.1a-d). A similar result was observed using Hoechst 33242, a nuclear stain which has been proven effective in visualizing intact blood vessels with an existing BBB (Figure 4.1e-h). This result was observed only in the brain, and not in other reference organs such as the liver or kidney (Figure 4.S1a-h). In order to obtain a quantitative assessment of this leakage, embryos were perfused with Evan's blue (EB), and the amount of dye was quantified in the brain. The amount of EB per gram of cortex tissue was significantly greater in the *Lpar1, 2* null mice compared to littermate controls. There was no difference in the midbrain, suggesting that the leakage was specific to the cortex (Figure 4.1i). Again, there was no difference in the reference organs (Figure 4.S1i). These data strongly indicate that *Lpar1, 2* double null mice exhibit compromised blood vessel integrity in the developing cortex.

In order to determine if this phenomenon persists at later developmental ages, mice at ages P7 and P14 were perfused with EB and subsequently measured for EB content in the cortices. Interestingly, there was still significantly greater EB content in the

double null cortices at P7 compared to littermate controls, suggesting that this leakage persists after birth (Figure 4.2a). This difference was again absent in the liver, indicating specificity to the brain (Figure 4.2b). At P14, there was no difference in the EB content of the double null cortices. Hence, this leakage is present till the developmental age of P14 (Figure 4.2c-d).

We also observed a greater vascular density on the meningeal layer of the cortex in the *Lpar1, 2* null E18.5 mice (Figure 4.3a-b). The number of branch points of vessels per surface area was assessed and shown to be significantly greater in the double null mice (Figure 4.3c). In addition, cortical sections were immunolabeled with a blood vessel marker (CD31), and the number of blood vessels per area of cortical section was quantified. This was again found to be significantly greater in the double null cortex (Figure 4.3d). The blood vessel density was similar in the midbrain, implying that this increased vessel density is specific to the cortex (Figure 4.3e).

To evaluate the blood vessel integrity further, endothelial cells in the double null cortex was observed at the EM level. Interestingly, we observe the presence of abnormal "electron-light" endothelial cells only in the double null cortex, and not in the littermate cortex (Figure 4.3f-g). This was reported previously, and thought to be representative of "unhealthy" metabolically compromised endothelial cells (Sato, Tozawa et al. 1995). Additionally, these abnormal endothelial cells were absent in other reference tissues (hindbrain, kidney), implying that the abnormality of the blood vessels is restricted to the cortex (Figure 4.S2).

This compromised blood vessel integrity in the developing cortex could be due to the loss of *Lpar1*, *Lpar2*, or both receptors. In order to determine the receptor(s) responsible for this phenomenon, single null mice were assessed for leakage using EB. Surprisingly, both *Lpar1* null and *Lpar2* null frontal brains did not exhibit any leakage, as shown by the comparable EB content to littermate controls (Figure 4.4a-b). These data indicate strongly that blood vessel integrity in the developing frontal brain is dependent on both LPA_1 and LPA_2.

4.5 Discussion

In all, these data suggest that the presence of both LPA_1 and LPA_2 is essential for the proper development of the vasculature in the developing embryonic brain during a critical period, specifically the period just prior to birth extending to early postnatal ages. In the absence of these receptors, the blood vessels in the cortex were found to exhibit a lack in integrity, as demonstrated by three independent leakage assays in our study. Interestingly, the leakiness of the blood vessels was not observed in any other reference tissues, such as the midbrain and liver. Hence, this phenomenon is cortex-specific.

Importantly, single *Lpar1* or *Lpar2* null cortices did not display any significant leakage, indicating that the loss of both LPA1 and LPA2 was required for the abnormal leakiness and suggesting receptor redundancy. In addition, *Lpar1* /*Lpar2* null cortices were found to exhibit a greater extent of branching on the meningeal layers, and greater vascular density within cortical tissue. We also demonstrated that a significant population of endothelial cells in the double null cortices were electron-light, which has been associated with unhealthy endothelial cells previously. The presence of such cells was again specific to the cortex, and not found in any reference tissue. In all, we have identified several aberrations in vascular development brought about by the loss of LPA_1 and LPA_2.

Tie-1 is a vascular endothelial cell-specific receptor tyrosine kinase which belongs to the same family as Tie-2, another RTK which is important for angiogenesis. Notably, a similar increased vascular density and leakage in the form of edemas and hemorrhages were observed in the Tie1 null mice (Sato, Tozawa et al. 1995).

Furthermore, the presence of electron-light endothelial cells was also observed in the Tie1 mutant mice. These effects were attributed to a lack of structural integrity of the blood vessels. In light of the findings of our study, there might be interplay between LPA signaling and Tie1/2 signaling which contributes to the phenotype of the *Lpar1 /Lpar2* null mice, which remains to be assessed.

4.6 Acknowledgements

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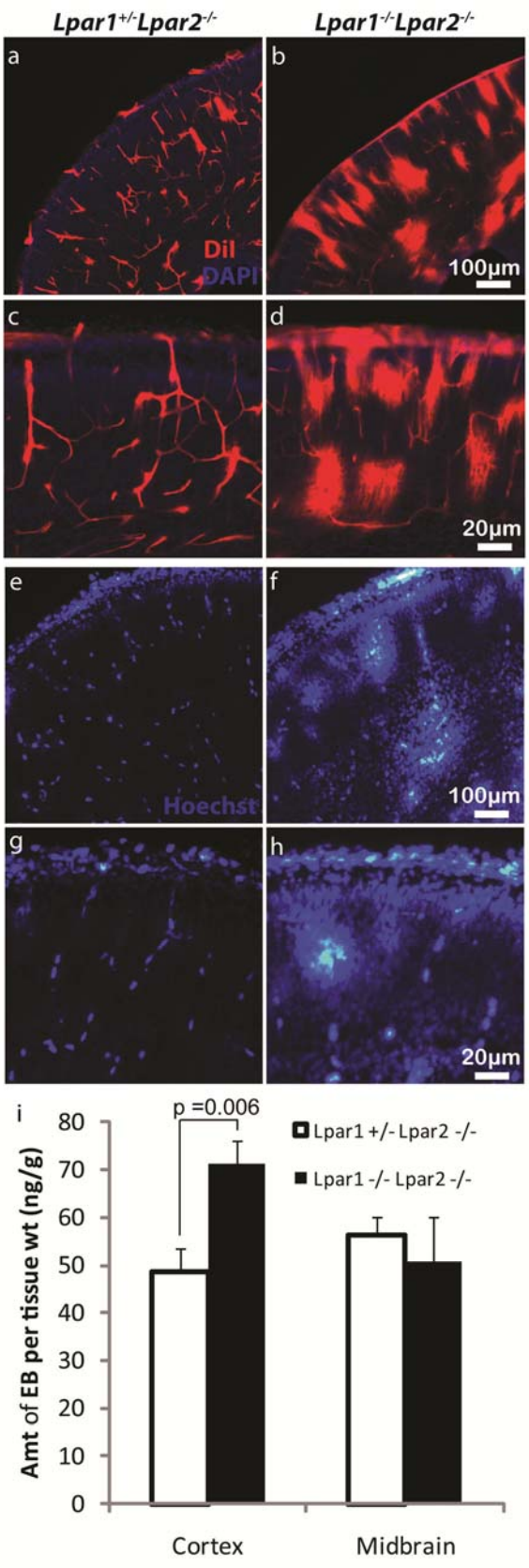
Figure 4.1 *Lpar1, 2* double null mice display a compromised blood vessel integrity in the developing brain.

(a-d) Representative images of *Lpar1, 2* null and littermate control cortical sections of E18.5 embryos perfused with DiI at low magnification (a-b), and at high magnification (c-d). 6 out of 8 controls did not display any leakage outside the vessels and 9 out of 9 double null displayed widespread leakage. (e-h) Representative images of *Lpar1, 2* null and littermate control cortical sections of E18.5 embryos perfused with Hoechst 33242 at low magnification (e-f), and at high magnification (g-h). 6 out of 7 controls did not display any leakage outside the vessels and 11 out of 11 double null displayed widespread leakage. (i) E18.5 embryos were perfused with Evan's blue (EB) and subsequently assessed for EB content in the cortex (n=7, 8 for double null and control respectively) and midbrain (n=5, 6 for double null and control respectively). There is increased leakage of EB in the double null cortex but not the midbrain.


Lpar1+/-Lpar2-/-
Lpar1-/-Lpar2-/-
a
b
DiI
DAPI
100μm
c
d
20μm
e
f
Hoechst
100μm
g
h
20μm
i
p =0.006
Lpar1 +/- Lpar2 -/-
Lpar1 -/- Lpar2 -/-
Amt of EB per tissue wt (ng/g)
80
70
60
50
40
30
20
10
0
Cortex
Midbrain

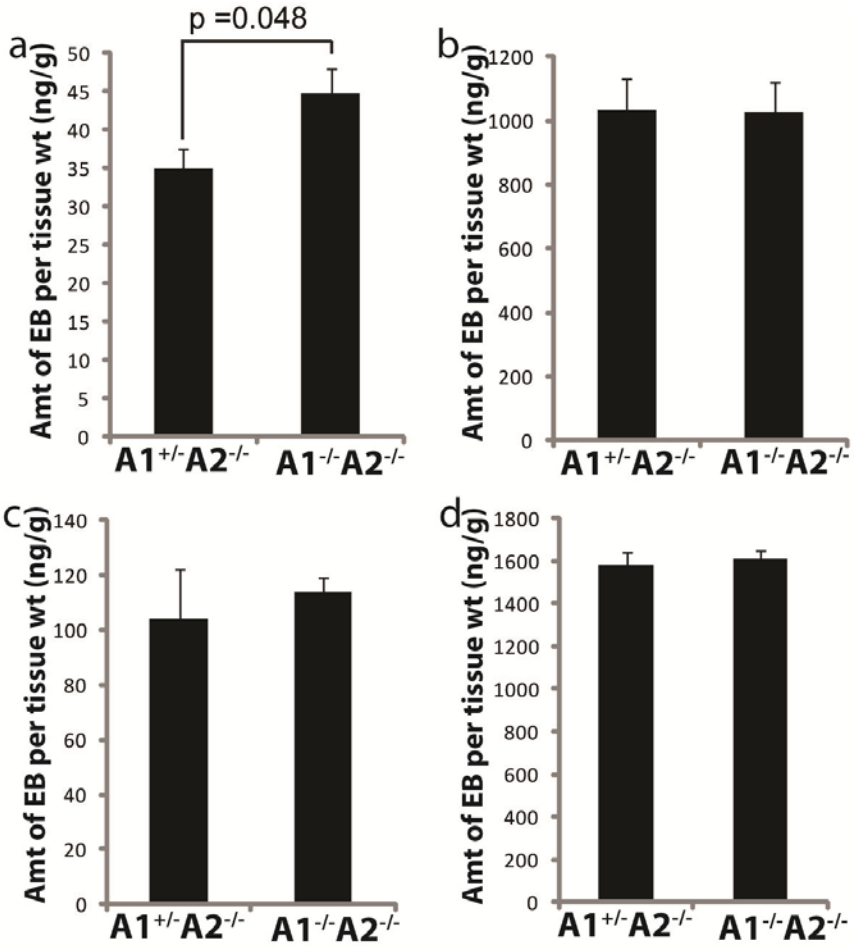
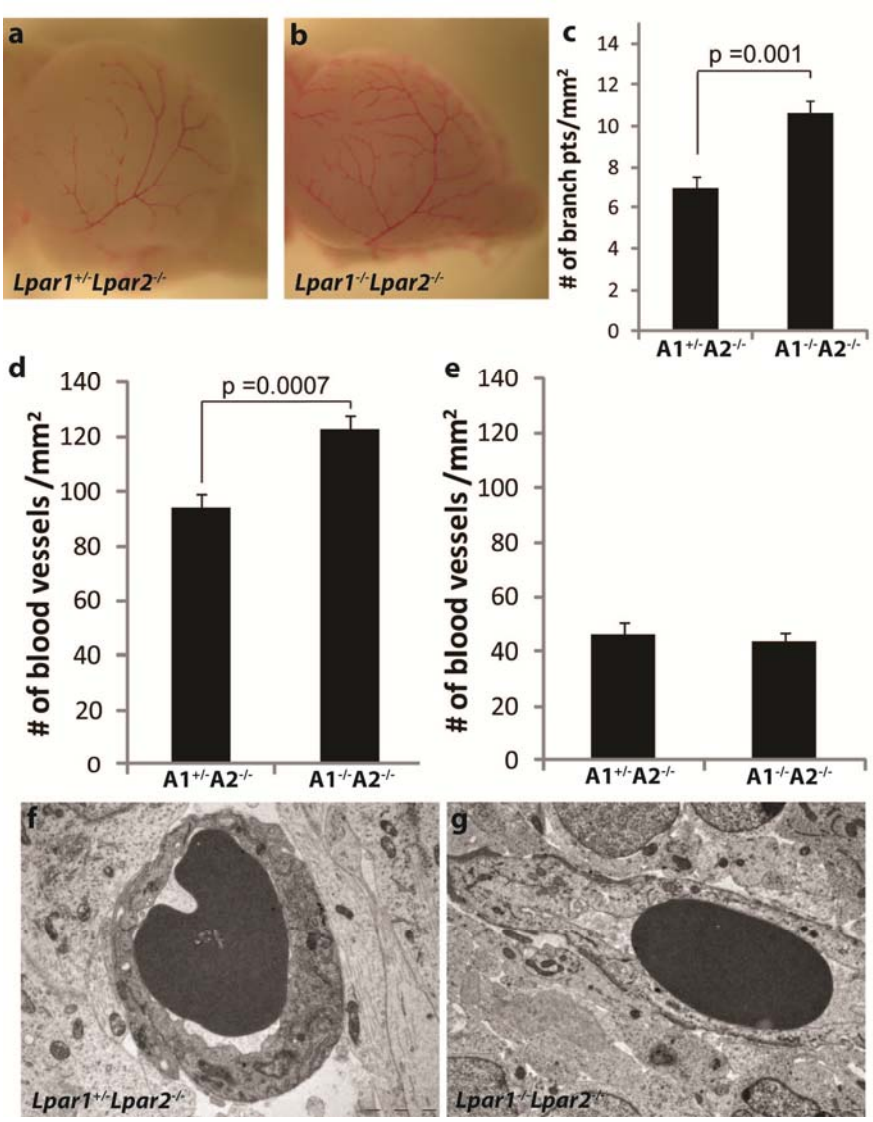


Figure 4.2 The compromised blood vessel integrity persists till after birth.

(a-b) P7 mice were perfused with EB and subsequently assessed for EB content in the cortex (a; n=6, 8 for double null or control mice respectively) or a reference organ, liver (b; n=7, 8 for double null or control mice respectively). (c-d) P14 mice were perfused with EB and subsequently assessed for EB content in the cortex (c; n=6, 7 for double null or control mice respectively) or a reference organ, liver (d; n=6, 7 for double null or control mice respectively).

Figure 4.3 *Lpar1, 2* double null frontal cortices possess a greater blood vessel density and abnormal electron-light endothelial cells.

(a-b) Representative images of the meningeal surface of cortices perfused with DiI. *Lpar1, 2* double null cortices (a) display a greater vascular density compared to littermate controls (b). (c) Quantification of branch points per surface area of meningeal layer (n=6, 11 respectively). (d-e) E18.5 brain sections were immunolabeled with a endothelial cell marker (CD31) and the number of blood vessels in the cortex (d) (n=11, 13 for double null, control respectively) or midbrain (e) (n=6, 11 for double null, control respectively) was quantified and shown here. (f-g) EM of endothelial cells in control (f) (n=15) or *Lpar1, 2* double null (g) (n=15) cortices. Almost half of all *Lpar1,2* double null endothelial cells (7 out of 15) are electron-light.


a
Lpar1+/-Lpar2-/-
b
Lpar1-/-Lpar2-/-
c
# of branch pts/mm²
p =0.001
A1+/-A2-/-
A1-/-A2-/-
d
# of blood vessels /mm²
p =0.0007
A1+/-A2-/-
A1-/-A2-/-
e
# of blood vessels /mm²
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Lpar1-/-Lpar2-/-

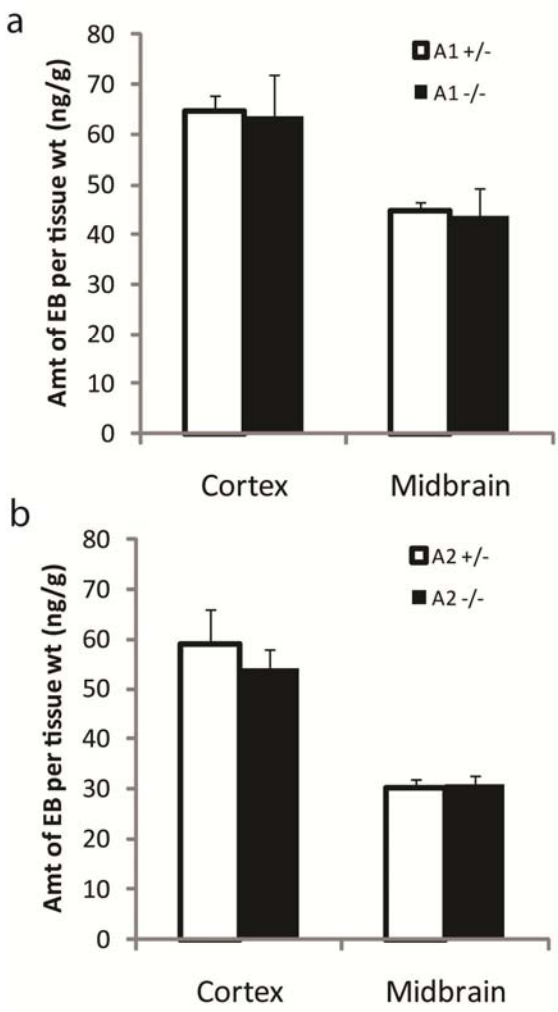
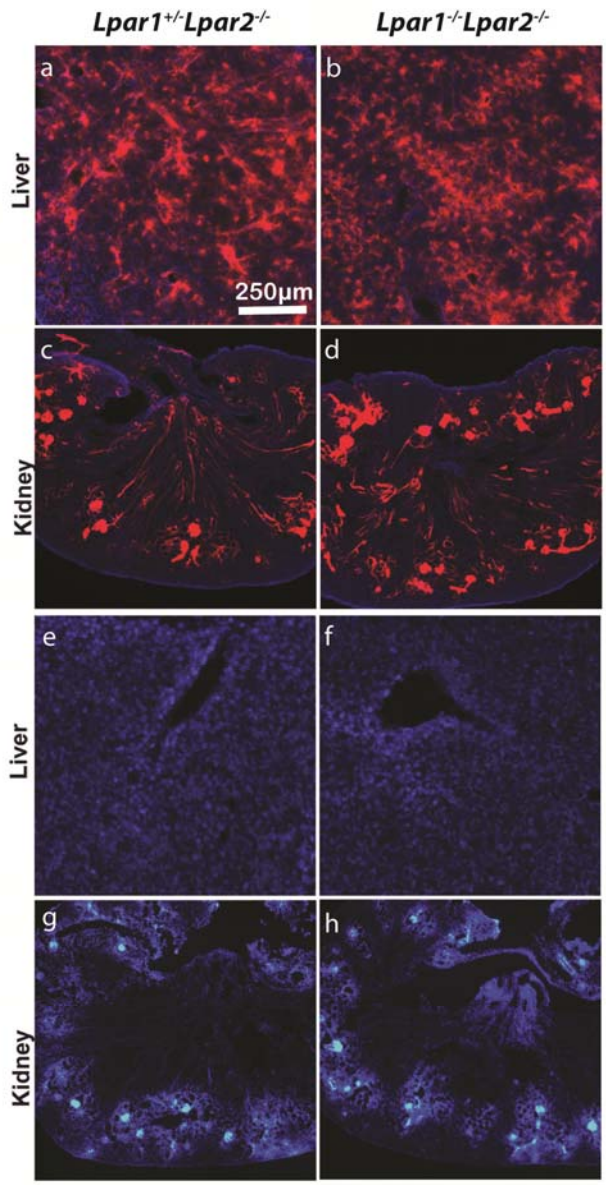


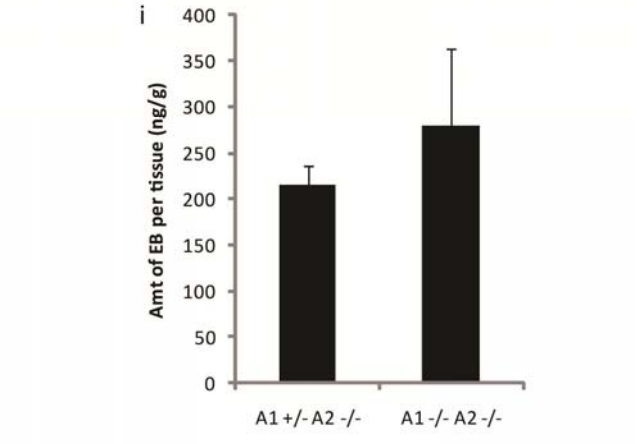
Figure 4.4 Both LPA_1 and LPA_2 are required for the maintenance of blood vessel integrity in the developing brain.

(a-b) E18.5 *Lpar1* null (a; n=14, 7 for double null or control mice respectively) or *Lpar2* null (b; n=3, 12 for double null or control mice respectively) mice were perfused with EB and subsequently assessed for EB content in the cortex or the midbrain. No difference in EB content was observed in either of the single null mice.

Figure 4.S1 No difference in blood vessel integrity was observed in tissue outside of the brain.

(a-h) Representative images of *Lpar1, 2* null and littermate control liver (a-b, e-f) or kidney tissue (c-d, g-h) of E18.5 embryos perfused with either DiI or Hoechst 33242 respectively. (i) E18.5 mice were perfused with EB and subsequently assessed for EB content in liver tissue (n=7,8 for double null or control mice respectively).


Lpar1+/-Lpar2-/-
Lpar1-/-Lpar2-/-
a
b
Liver
250μm
c
d
Kidney
e
f
Liver
g
h
Kidney


i
400
350
300
250
200
150
100
50
0
Amt of EB per tissue (ng/g)
A1 +/- A2 -/-
A1 -/- A2 -/-

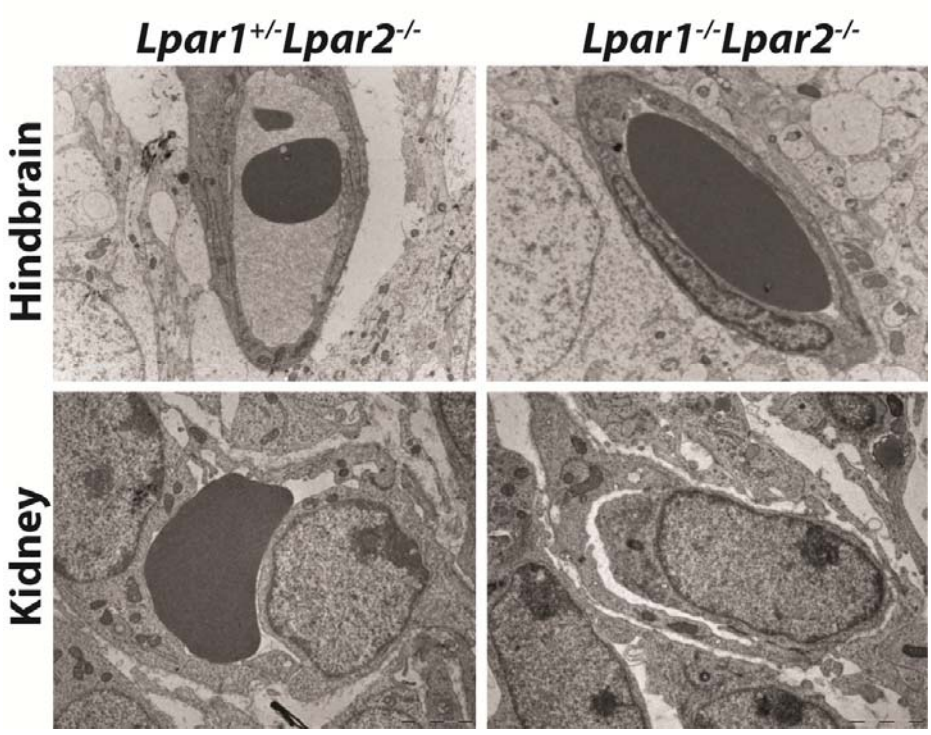


Figure 4.S2 Endothelial cells do not appear to be abnormally electron-light in tissue outside of the cortex in *Lpar1, 2* double null mice.

Endothelial cells were observed under EM in hindbrain (a-b; n=7, 8 for double null or control mice respectively) and kidney (c-d; n=5, 3 for double null or control mice respectively) sections.

4.7 References

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V. CONCLUDING REMARKS

The purpose of this dissertation was to elucidate the role of LPA signaling in two aspects of cortical development. First, we strived to investigate the role of LPA signaling as a mediator of pathological hypoxia on fetal cortical development (Chapter Two). We started out by characterizing the effects of pathological hypoxia on the embryonic cortex during development. Using an *ex vivo* cortical explant culture method, which has been shown to replicate the *in vivo* state, we were able to dissect out various effects of hypoxia on cortical development. Hypoxia was found to induce specific, non-generalized changes to the cortical cerebral wall, resulting in a stereotyped disorganization of the cortex. Importantly, one of the first phenotypes observed was a basal displacement of mitotic neural progenitor cells, which are essential for giving rise to new neurons through the process of interkinetic nuclear migration in the cortex. This ectopic positioning of the mitotic cells suggested two implications: 1) adhesion of these mitotic cells to the ventricular zone was disrupted. 2) new neurons arising from these ectopic mitotic cells might be abnormal. Indeed, N-cadherin, a major adhesion molecule for these neuroepithelial cells, was found to be disrupted under hypoxia. Also, early postmitotic neurons were found to be aberrantly positioned throughout the width of the cerebral wall, instead of migrating and forming a thick band at the cortical plate, suggesting a defect in migration. This was confirmed by the tracking of cortical cells marked with a Brdu pulse, where cortical cells accumulate in the ventricular zone instead of their normal path towards the cortical plate under hypoxia.

In all, these data depict a stereotyped disorganization of the embryonic cortex brought about by hypoxia. Interestingly, these effects were strikingly similar to the

effects involving LPA signaling in several studies by our lab. Thus, we employed both a genetic and a pharmacological approach to understanding the possible role of LPA signaling in this pathological process. Using mutant mice null for several LPA receptors which are expressed strongly in the embryonic cortex, we found that LPA_1 null mice did not display any of these hypoxic effects, implicating LPA_1 as an essential mediator of hypoxia. Similar results were obtained through the use of two chemically distinct LPA_1 antagonists, ascertaining the role of LPA1 in this hypoxic pathway.

Since LPA receptors are known to activate one or more G-proteins to activate a variety of downstream cascades, we next sought to dissect out the predominant downstream effectors of hypoxia through LPA_1. Using various chemical inhibitors for many downstream effectors, it was identified that hypoxia signals through predominantly the $G\alpha_i$ pathway, subsequently activating Rac1, which is antagonistic to ROCK. Since the nature of the effect of hypoxia on LPA_1 was unclear, we went on to assess the effect of hypoxia on LPA_1 through two different cell-autonomous assays. Hypoxia was found to potentiate LPA_1 activity, which could be achieved by an increase in receptor expression or ligand availability. Surprisingly, gene expression of LPA_1 and autotaxin (ATX), the main LPA-producing enzyme in vivo, was not altered significantly by hypoxia. An alternative mechanism could involve the G-protein-coupled-receptor kinases (GRKs), which phosphorylates and targets activated G-protein-coupled-receptors for internalization and desensitization. Interestingly, hypoxia was found to down-regulate GRK2, the GRK associated with LPA_1, at both the gene and protein level, hence leading to an overactivation of LPA_1. Through the use of a GRK2-specific inhibitor, GRK

inhibition was proven to induce similar effects to the embryonic cortex as hypoxia, validating it as a mechanism for the modulation of LPA_1 by hypoxia in this system.

Since one of the key downstream targets of hypoxia is hypoxia-inducible-factor 1 alpha (HIF-1a), we would like to assess if HIF-1a is involved in this hypoxic response in the developing cortex. Using two distinct potent HIF-1a inhibitors, we found that the inhibition of HIF-1a inhibits the displacement of mitotic neural progenitor cells. Thus, this confirms that HIF-1a is involved in this system. When GRK2 levels were assessed in the presence of these inhibitors, GRK2 was found to be unchanged, suggesting that HIF-1a is activated downstream of GRK2 and LPA_1, which remains to be assessed in the future.

LPA signaling has been associated only in passing with hypoxia in a handful of cancer studies previously, where hypoxia conditions were found to increase LPA-induced responses in ovarian cancer cells. No mechanism has been identified for this interaction in these studies. Hence, our study identifies the first link between hypoxia and LPA signaling in cortical development. Also, we present a novel mechanism for this interaction. Importantly, several fetal-hypoxia-linked CNS disorders, such as schizophrenia, autism and epilepsy possess pathologies of abnormal neuronal migration after birth, which could be attributed to early hypoxic events during fetal cortical development. Thus, our study provides a rationale for LPA_1 antagonists to be used as a therapeutic or as prevention of these fetal hypoxia-associated CNS disorders.

Second, the role of LPA signaling in vascular formation during cortical development was investigated (Chapter Four). LPA signaling has been implicated in

many aspects of vascular development previously, such as endothelial cell migration, proliferation and survival. Importantly, the loss of LPA in the autotaxin-null mice led to immense vascular defects in the embryo. In addition, a significant percentage of *Lpar1* and *Lpar1/Lpar2* null mice exhibit hematomas in the brain, implying a role for LPA signaling in vascular development. In light of these previous studies, and the enriched expression of LPA_1 and LPA_2 in the embryonic cortex, we assessed blood vessel integrity in the *Lpar1/Lpar2* double mutant mice during development.

Through the use of three established, independent leakage assays, we observed that *Lpar1/Lpar2* null embryos, at embryonic day 18.5, exhibited a significant increase in leakage of the blood vessels in the cortex when compared to littermate controls. Interestingly, this increase in permeability of the vessels were restricted only to the cortex, and not detected in other reference tissue, such as the midbrain or liver. This phenomenon was found to persist after birth, up to the age of postnatal day 14. Hence, this is a temporal and tissue-specific phenomenon, which might correlate with the expression levels of these receptors with development, or that of their interacting partners.

In addition, increased branching of the vessels in the meningeal layer of these double null cortices was observed, together with increased vascular density within the cortex. These data suggests increased angiogenesis, perhaps due to a lack of spatial cues which normally dictate this process, brought about by the loss of the LPA receptors. Furthermore, a significant population of endothelial cells in these double null cortices was found to be abnormally electron-light under electron microscopy, which has been associated with metabolically unhealthy cells previously. In conclusion, we describe an

aberrant development of the vasculature in the cortex, marked by unhealthy endothelial cells, a lack of vessel integrity and increased angiogenesis, all of which are brought about by the loss of both LPA_1 and LPA_2. Single null mutants for either of these receptors did not exhibit any abnormalities, confirming that the loss of both receptors is required for these effects. Further work needs to be performed in order to elucidate the mechanism behind these effects, particularly dissecting out the cell population (neural or endothelial) responsible for this phenomenon. In addition, possible cross-talk between LPA signaling and known angiogenic pathways should be investigated as well.

In the past five years, I have learned and grown so much, be it in a personal or professional capacity. Albeit frustrations and hurdles on this path of self-realization, my body of work has brought me much joy and pride. It is my hope that these findings will provide the basis for further elucidation of LPA signaling in these aspects of cortical development in the future.