

**SPHINGOSINE-1-PHOSPHATE RECEPTOR MODULATION IN
NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY**

An Undergraduate Research Scholars Thesis

By

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ABSTRACT

Sphingosine-1-Phosphate Receptor Modulation in Neonatal Hypoxic-Ischemic Brain Injury.
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Brain damage by hypoxia-ischemia is a devastating injury to neonatal infants, and can lead to long-term neurological defects, e.g. cerebral palsy. Recent revelations concerning the nature of sphingosine-1-phosphate (S1P) analogs led us to investigate their role in this serious health issue. Discovering the potential effectiveness of FDA approved Fingolimod in ameliorating hypoxia-ischemia brain injury in a neonatal mouse model was the end-goal, building off of reports of its efficacy in inhibiting demyelination induced by cuprizone; as well as the fact that central nervous system cells express S1P-receptors - its target receptor family. Temporal and spatial S1P receptor expression was characterized in developing mouse brains, and hypoxia-ischemia was induced in neonatal mice by cauterization to the right common carotid artery, followed by hypoxia exposure. To mimic neonatal infection and inflammation, mice in this condition additionally underwent Lipopolysaccharide peritoneal injection. Fingolimod was similarly administered, and animals were sacrificed four days post procedure and analyzed for the extent of brain injury. Combined hypoxia-ischemia with Lipopolysaccharide was found to cause

hypomyelination in the corpus callosum, striatum, and thalamus, as well as hippocampus neuron damage, more so than hypoxia-ischemia alone. The importance of this and future investigation lies in the need for a consistently effective treatment of newborn injury by ischemia, which, unlike in adults, is yet to be found. We hope these findings will further the pursuit of minimizing the large and deleterious impacts of this type of injury to infants.

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NOMENCLATURE

S1P	Sphingosine-1-Phosphate
S1Pr(x)	Sphingosine-1-Phosphate receptor (1,3,5)
FTY720	Fingolimod
P(x)	Postnatal day (x)
GFAP	Glial Fibrillary Acidic Protein
MBP	Myelin Basic Protein
H/I	Hypoxia and Ischemia

CHAPTER I

INTRODUCTION

Neonates are at high risk for brain injury given their immature vasculature regulation systems. Hypoxic-ischemic injury can be induced by various pre-natal and post-natal conditions such as infection, blood clotting disorders, and cardiac disease; resulting in lifelong debilitating disease, such as cerebral palsy (Nelson & Lynch, 2004; Vital, 2010). Concerning infection, varicella, meningitis, and tonsillitis are leading producers of arterial and venous ischemic stroke, with combinations of risk factors providing the greatest risk (Ganesan, Prengler, McShane, Wade, & Kirkham, 2003). Current treatment of neonates focuses on anti-coagulative drugs such as Heparin and Warfarin, stemming from common treatment of adult stroke (Sebire et al., 2005). Additionally, occasional surgical procedures to the end of reducing intracranial pressure are performed (Sandberg, Lamberti-Pasculli, Drake, Humphreys, & Rutka, 2001). Neurological dysfunction in cerebral palsy is characterized by dramatic impairment to motor systems associated with pathological changes in the white matter of the corpus callosum, the motor cortex, and the cerebellum – the latter receives blood from the basilar artery. Importantly, these structures, in addition to the hippocampus, are at an elevated risk for injury following ischemia due to relatively low blood flow to the areas (Miron, Schubart, & Antel, 2008; NIH, 2009).

Sphingosine-1-Phosphate (S1P) is an essential phospholipid involved in cellular signaling, and has been found to regulate angiogenesis, vascular stability, as well as B and T cell migration. Sphingosine is catabolized from ceramide by the enzyme ceramidase, resulting in an amine from the amide bond in ceramide; and is subsequently phosphorylated by Sphingosine Kinases to

produce Sphingosine-1-Phosphate (Christie, 2012).

FTY720, known as Fingolimod or its FDA approved drug name Gilenya, is a drug currently used in the treatment of relapsing and remitting multiple sclerosis, and is a structural analog of S1P. The protective effects of FTY720 are thought to arise from its inhibition of the migration of naïve and central memory lymphocytes from the lymph nodes; hence blocking trafficking into the brain where they would attack oligodendrocytes and increase inflammation with secretion of cytokines and other pro-inflammatory chemicals (V. Brinkmann et al., 2002). As concerns the drug's movement in vivo, it has been confirmed by Brinkmann, as well as in-lab in vivo experiments, that FTY720 crosses the blood brain barrier following intraperitoneal injection, exemplifying potential for direct action on CNS cells (Billich et al., 2003; Volker Brinkmann, 2009; Kim et al., 2011).

The receptors that bind S1P and FTY720 – which is phosphorylated to its active form FTY720P – are S1P receptors. There are five different S1P receptors, which are differentially expressed on various cell types of the CNS. For example, S1P receptor 1 is more abundantly expressed in astrocytes than neurons, and the opposite is true for S1P receptor 3 (Volker Brinkmann, 2009). These receptors are part of a G protein coupled receptor system that leads to the intracellular activity changes that bring about the cellular behaviors observed, i.e. proliferation and migration, produced by activation and subsequent calcium mobilization (Cuvillier et al., 1996; Olivera & Spiegel, 1993).

Especially significant is the relationship between the function of S1P receptors and the cell types.

As stated previously, S1P receptors are implicated in cell proliferation, and gliosis of astrocytes and microglia are signature events of encephalopathy (Sorenson et al., 2003). This connection makes the receptors a viable prospect for regulation of gliosis and consequent damage. It has been observed that astrocytes are intimately involved in modulating microglia-propagated inflammatory responses, and based on in vitro experiments with S1P analogs like FTY720 and CYM5442 – a molecule that binds S1P receptor 1 – astrocyte proliferation is significantly up regulated (Sorenson et al., 2003). Based on this observation, FTY720 administration would seem counter-intuitive in the treatment of brain injury; but in fact it has been reported that FTY720 treatment reduces gliosis of astrocytes and microglia and reduces injury to white matter in a cuprizone-induced demyelination model (Volker Brinkmann, 2009; Choi et al., 2008). The mechanism of this phenomenon is unknown, and could possibly involve desensitization and down-regulation of the activated S1P receptors.

While still shrouded in mystery, the idea that S1P analogs like FTY may have neuroprotective effects in other injury models was sought and explored. Efforts were specifically focused on a neonatal hypoxia-ischemia model in mouse pups, with and without systemic infection, due to the fact that greatly effective treatments for resulting injuries in premature human infants do not yet exist. At present, little is known about S1P receptor expression and function in the developing brain. In order to understand the potential therapeutic effect of S1P receptor modulators, the expression of S1P receptors would need to be characterized in the developing brain. From there, the neonatal brain injury model could be tested and the results of drug treatment on damage to the vulnerable neural areas discovered.

CHAPTER II

METHODS

S1P receptor developmental expression analysis

In addition to two adult C57BL/6 mice, a C57BL/6 mating pair was used to gather P3, P7, and P14 mice – two for each time point -, which were then intracardially perfused with 4% paraformaldehyde. The dissected brains were post-fixed overnight in 4% paraformaldehyde, followed by an overnight 30% sucrose incubation before being prepared for tissue sectioning in cryogel. For the adult conditions, both brains were used for sagittal sectioning and then stained for S1P receptor 1 with a 1:100 dilution of the Santa Cruz Rabbit EDG-1 primary antibody, and for S1P receptor 3 with a 1:100 dilution of the Cayman Rabbit primary antibody. In all tissue staining, tissue sections were permeabilized with 0.3% Triton X in PBS, blocked with 0.1% Triton X and 5% goat serum in PBS, and stained with primary antibody in 0.1% Triton X and 5% goat serum in PBS. Primary antibodies were tagged with a Invitrogen goat anti-rabbit secondary antibody with fluorescence at 594 nm in 0.1% Triton X and 5% goat serum in PBS .

To determine the cell types localized to S1Pr expression, a 1:100 dilution of the Millipore mouse NeuN primary antibody, in addition to a 1:200 dilution of the Millipore chicken anti-GFAP, were applied to the sections with Invitrogen goat anti-mouse and chicken secondary antibodies, respectively. P3, P7, and P14 brains were sectioned coronally at 10 μ m. Within each set, the sections were stained for S1P receptors 1 and 3, in addition to S1P receptor 5 (1:100 dilution of the Santa Cruz rabbit primary S1P receptor 5 antibody). The sections were also stained for neurons (NeuN), astrocytes (GFAP), and oligodendrocytes (1:200 dilution of the CalBiochem mouse anti-CC1 primary antibody). All stained tissues were also stained with

bisbenzamide (Hoechst 33258) with a 1:10,000 dilution for the nucleus and cell recognition. All images were captured with a fluorescence microscope equipped with an Olympus DP70 digital camera (model IX71; Olympus, Tokyo, Japan).

Protein quantification and western blot

Brains from developing C57BL/6 mice - one P0 and two P3,5, and 7 - were lysed and homogenized using 1.5 mL of lysis buffer (0.02 M Tris-HCl, 0.15 M NaCl, 0.001M EDTA, 0.001 M EGTA, 1% Triton X, 0.01 M Na₂P₂O₇, 0.01M NaF, 1X Protein Inhibitor Cocktail, 0.001 M Na₃VO₄, 0.001 M PMSF) and a Kantes Glass Co. homogenizer. The homogenized brains were then left on ice for 10 minutes before sonication, after which they were centrifuged at 17,000 xg and 4° C. For protein quantification, 1 mL of DC Assay Reagent A to 20 µL Reagent S were added to each well of a 96 well plate with which sample and standard would be added. Then 1 µg/uL BSA standard was loaded in two rows (duplicates) from 0 µg to 6 µg. Two rows of sample protein solutions (duplicates) were also added with 2 uL of each of the seven brain protein samples. 200 µL of DC Reagent B were then added and allowed 15 minutes at room temperature to incubate. The 96 well plate was analyzed using the Optima system. Using these values, 1 mg of protein was added to appropriate volumes of lysis buffer along with 55 µL of 5X SDS loading dye to produce 4.5 µg/uL concentrations of each protein sample. To separate the protein by size, the gels for SDS PAGE were prepared using 10% Separating (5 mL Acrylamide, 3.75 mL Separating buffer, 6.088 dH₂O, .15 mL APS, 12 µL TEMED) and 6% Stacking (1.3 mL Acrylamide, 2.5 mL Stacking buffer, 5.8 mL dH₂O, .4 mL APS, 8 µL TEMED) gels - since S1Pr protein weighs approximately 43 KDa. The gels were loaded with 5 µL of Precision Plus Protein™ Dual Color Standards (#161-0374, Bio-rad) in the first lane, and

50 µg of protein per subsequent lane - 11.1 µL of each 4.5 µg/µL protein-dye solution. The gels were run at 80 V for 45 minutes, then 110 V for 75 minutes. Transfer buffer was then prepared from a 10X transfer solution (100 mL 10X Transfer, 200 mL methanol, 700 mL dH₂O). The gels were removed and prepared to run at 4° C in the transfer buffer for 60 minutes at 300 milliamps. The membranes were stored in TBS-tween (100 mL 10X TBS, 895 mL dH₂O, 5 mL 20% Tween, pH 7.4) at 4 degree Celsius. The two membranes were then blocked in 5% skim milk in TBS-tween solution for 45 minutes before addition of primary antibodies. One membrane was incubated with a 1:500 dilution of Santa Cruz Rabbit anti S1Pr1 antibody (EDG-1) in milk TBS solution (8 µL antibody in 4 mL solution), while the other was incubated with a 1:500 dilution of Santa Cruz Rabbit anti S1Pr3 antibody (EDG-5) in blocking solution (8 µL antibody in 4 mL solution). Both membranes were shaken overnight at 4° C. After overnight incubation, the membranes were washed in TBS-tween for 15 minutes, then in new buffer for 5 minutes, then new buffer once again for 5 minutes in individual wash boxes. The Jackson-Immuno anti-Rabbit HRP secondary antibody was added to each membrane box at a 1:10,000 dilution in blocking solution and shaken for 45 minutes at room temperature. The membranes were subsequently washed again as noted previously, and then prepared for exposure and visualization. A 2 mL solution of 1:1 SuperSignal West Pico Luminol solution and Stable Peroxide solution were incubated with each membrane for 90 seconds before exposure and imaging. In order to lastly stain the membranes for actin, the membranes were stripped in 55° C (25 mM glycine-HCL, pH 2, 1% SDS) for 15 minutes, followed by three sets of 5 minute washing in TBS-tween. The membranes were then blocked with milk TBS solution for 30 minutes, and incubated in a 1:10,000 dilution of mouse anti-actin primary antibody milk TBS solution overnight while shaken at 4 degrees Celsius. After overnight incubation, the membranes, like before, were

washed in TBS-tween for 15 minutes followed by 3 five-minute washes. The secondary antibody was then added in a 1:10,000 dilution of the anti-mouse HRP antibody in milk-TBS solution for 45 minutes, then washed in the previously mentioned fashion. The membranes were then incubated with the previously detailed Pico solution and exposed. Western blot images were acquired using Molecular Imager ChemiDoc XRS+ System (BioRad, Hercules, CA) and specific S1P receptor bands were quantified using the QuantityOne software and normalized against β -actin, and graphed with t-tests.

Hypoxia-ischemia

P6 C57BL/6 and Ai14 mating pairs were anesthetized on ice for 10 minutes each before unilateral carotid ligation to the right common carotid artery. In this procedure, a midline incision below the neck was made; followed by the finding of the right common carotid artery by moving of the connective tissue and musculature that lie above the artery. Using a hook, the artery was isolated and subsequently cauterized using a Fine Science Tools cauterizing tip, after which the artery was checked to ensure that blood flow had been properly obstructed. The incision was mended using 3M Vetbond tissue adhesive, and the mouse was either intraperitoneally injected with 0.1 mg/kg FTY, or directly put on a heating pad for 30 – 60 minutes during recovery. Then two at a time, the mice were placed on a heating pad in a hypoxia chamber set at 8% Oxygen for 40 minutes; after which they would recover once again on a heating pad, receive a 0.3 mg/kg intraperitoneal injection of LPS, before being re-scented and returned to the mother. The hypoxia chamber was fed by a nitrogen gas cylinder, and calibrated by the BioSpherix ProOx 110 apparatus. Mouse pups were also used for the following controls: hypoxia alone, ischemia alone, Hypoxia and LPS, and LPS. At P10, the mice were

perfused using PBS solution by injecting a perfusion needle into the left ventricle of the heart and cutting the auricular vein. The brains were fixed in 4% paraformaldehyde overnight, and then incubated in 30% sucrose overnight. Coronal sections were then gathered and stained for S1P receptors 1, 3, and 5, as described above, and used for various histological and immunohistochemical stains: Cresyl Violet for motor neurons, rabbit anti-CDC68/Iba for microglia, rat anti- MBP for myelin, mouse anti- CC1 for oligodendrocytes, chicken anti-GFAP for astrocytes, and mouse anti-NeuN for neurons. Data for Cresyl violet, MBP, and CC1 staining are shown in Figures 7 and 8.

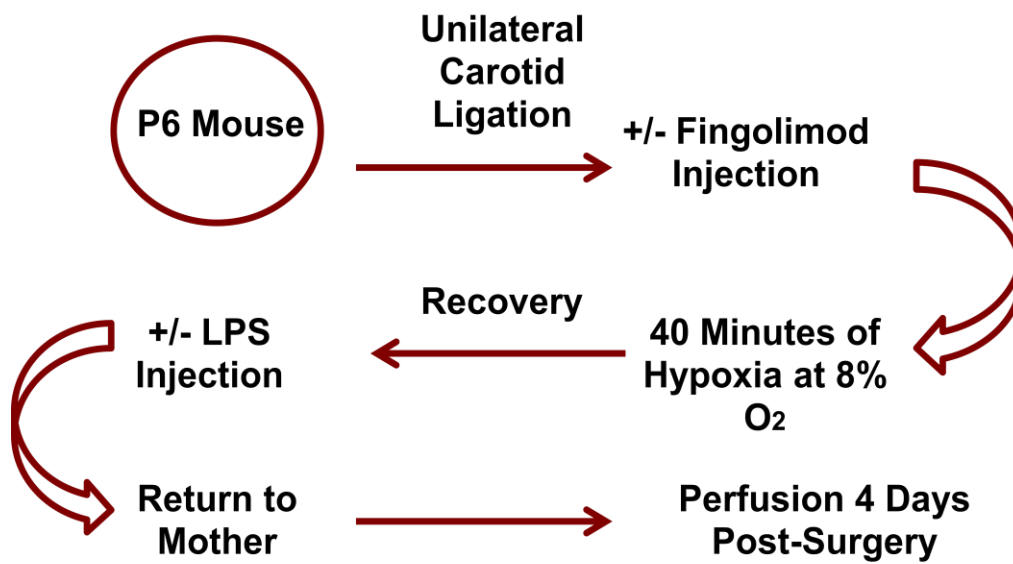


Fig. 1: Hypoxia-Ischemia Model

CHAPTER III

RESULTS

S1Pr expression in adult mouse brain

In the adult C57BL/6 mouse brain analysis, several brain areas were found to have relatively high expression of S1P receptors; these being the hippocampus, cerebellum, and the lining of the lateral ventricle (not shown). S1P receptor 1 showed high expression in gray matter throughout the brain, but not on neurons as seen in Figure 1 f-i, o-r. The S1P receptor 1 staining instead surrounds the cell bodies of the neurons. S1P receptor one also seems to colocalize with astrocytes in the hippocampus, but not completely. In the population of astrocytes in Figure 1 c,l, there are a number of astrocytes that do not exhibit S1P receptor 1 immunoreactivity. Little colocalization is seen in the white matter tract where astrocyte populations are high (Figure 1 k,l). Hippocampus and cerebellum S1P receptor 3 staining clearly colocalizes with the neurons present, as shown in Figure 2.

S1Pr expression in the developing mouse brain

Expression of S1P receptors 1,3, and 5 in the developing mouse brain at postnatal ages 3-14 days was examined. S1P receptors 1, 3, and 5 - exhibited various temporal and spatial changes during brain development. S1P receptor 1 did not show much change in expression, but colocalization with astrocytes and oligodendrocytes appeared to decrease substantially from P3 to P7; and from P7 to P14 an increase in gray matter expression was evident with a slight regain of astrocyte colocalization. CC1 colocalization with S1P receptor 1 appeared consistent throughout these developmental points with some decrease at P14. S1Pr3 showed a decrease in expression as well

as colocalization with neurons from P3 to P7 before increased expression and colocalization by P14. S1Pr5 showed a very slight decrease in expression while retaining colocalization levels with neurons from P3 to P7. Expression levels appeared to increase by P14, with neuron colocalization proportionally increasing. Low level S1Pr5 colocalization with oligodendrocytes was evident by CC1 staining in the corpus callosum. These observations were most obvious in the hippocampus, as seen in Figure 2. The western blots of the entire developing brain showed insignificant change in S1Pr1 expression with development to p7, while significant decrease in expression of S1Pr3 was observed, as shown in Figure 4. These findings however, illustrate cumulative S1Pr expression, and thus, specific brain structures could be incongruous with the western data.

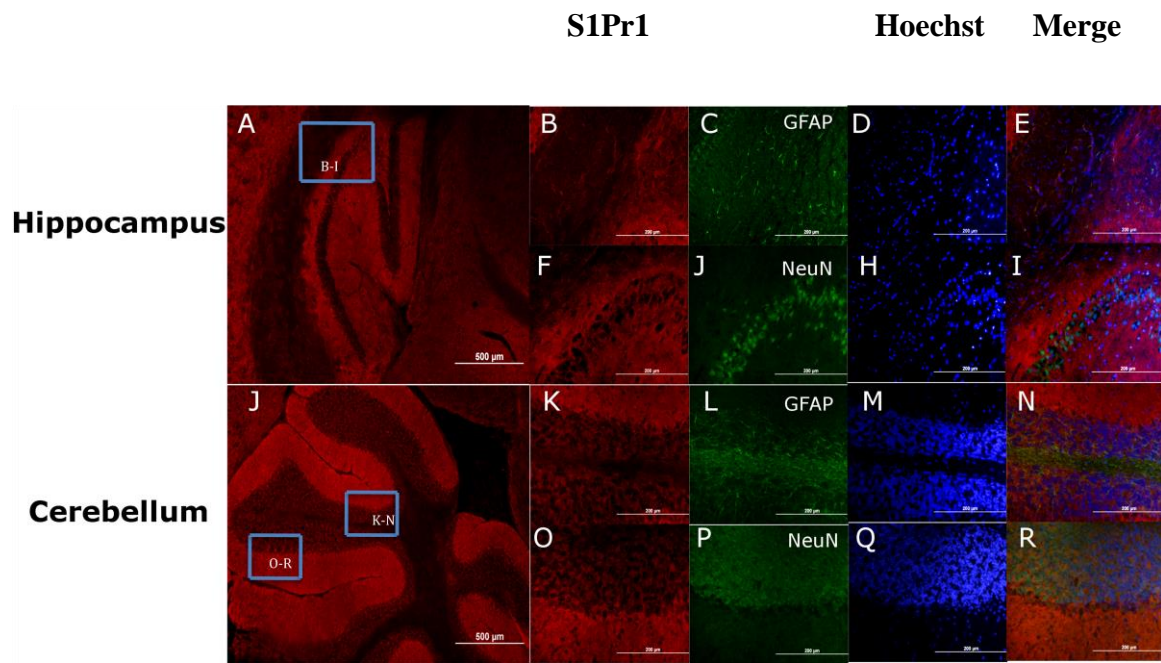


Fig. 1: S1P Receptor 1 Expression Patterns in Adult Mouse Brain

Adult C57BL/6 sagittal sections. A,J: 4X S1Pr1 staining. B,F,K,O: 20X S1Pr1 staining. C,L: 20X GFAP staining. L,P: 20X NeuN staining. D,H,M,Q: 20X Hoechst staining. E,N: S1Pr1+GFAP+Hoechst merge. I,R: 20X S1Pr1+NeuN+Hoechst merge.

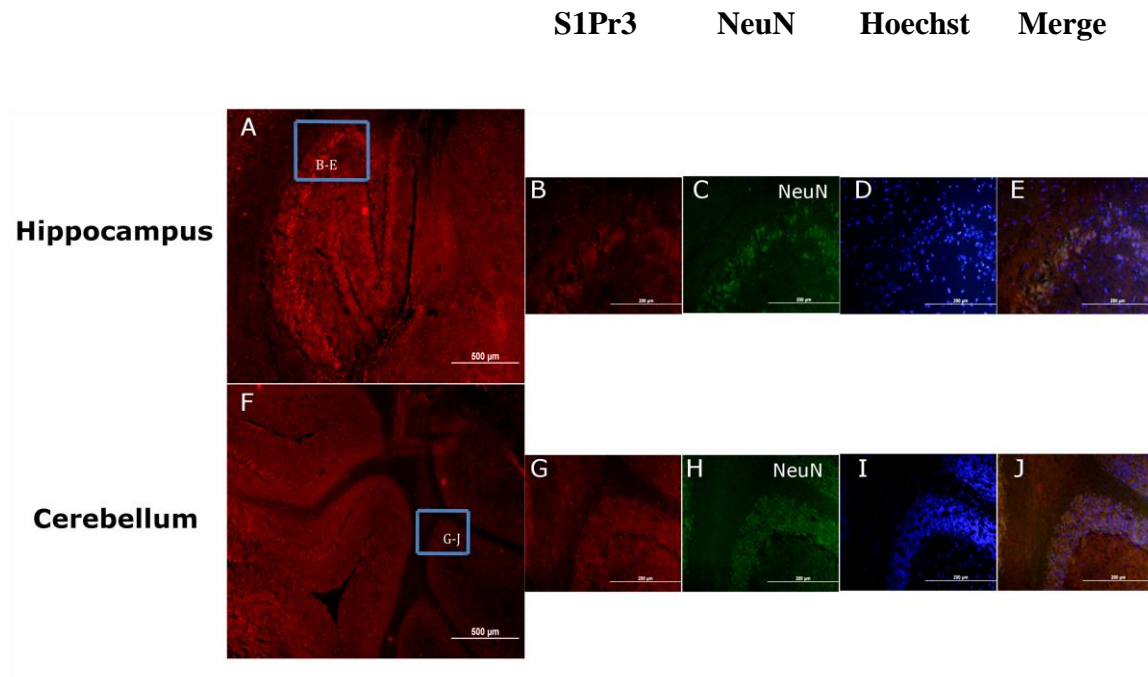


Fig. 2: S1P Receptor 3 Expression Patterns in Adult Mouse Brain

Adult C57BL/6 sagittal sections. A,F: 4X S1Pr1 staining. B,G: 20X S1Pr1 staining. C,H: 20X NeuN staining. D,I: 20X Hoechst staining. E,J: 20X S1Pr1+NeuN+Hoechst merge.

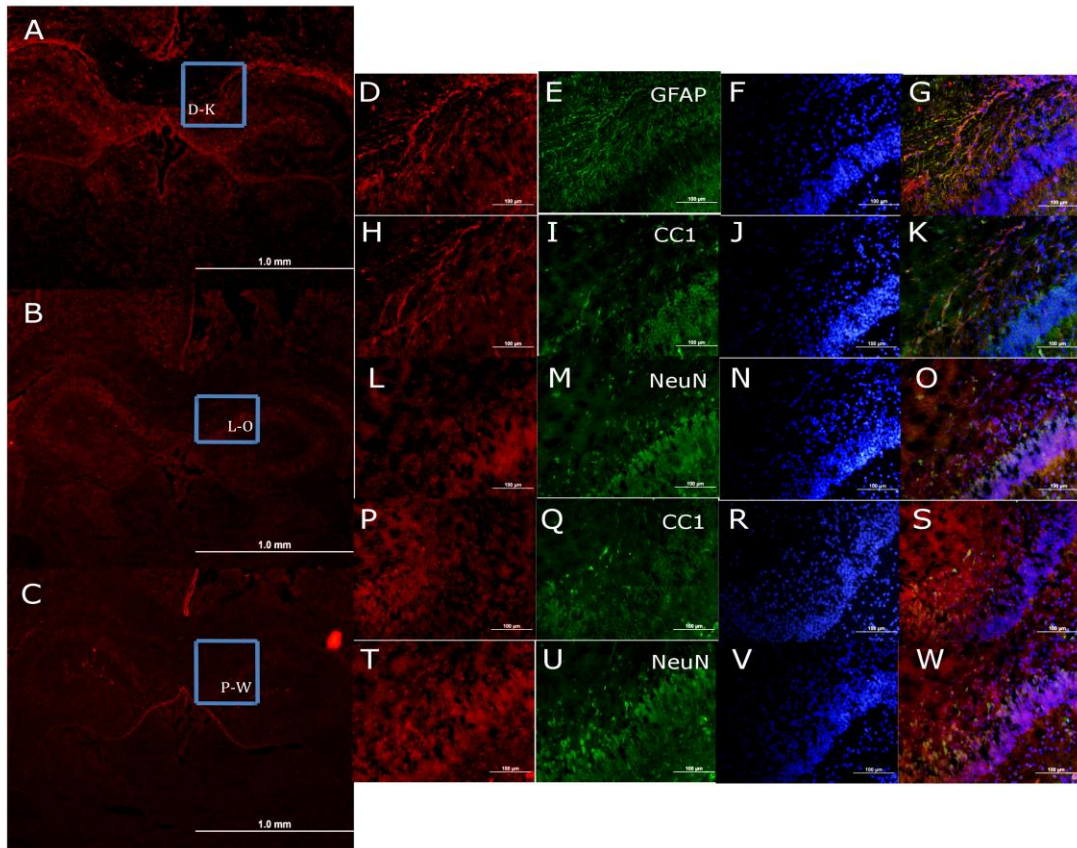


Fig. 3: P3 S1P Receptor Hippocampus Expression

P3 C57BL/6 coronal sections. A, F, B: 4X S1Pr1, r3, r5 staining respectively. D,H: 20X S1Pr1 staining. E: 20X GFAP staining. F-V: 20X Hoechst staining. G: Merge of D-F. I,Q: 20X CC1 staining. K: Merge of H-J. L: 20X S1Pr3 staining. M, U: 20X NeuN staining. O: Merge of L-N. P,T: 20X S1Pr5 staining. S: Merge of P-R. W: Merge of T-V.

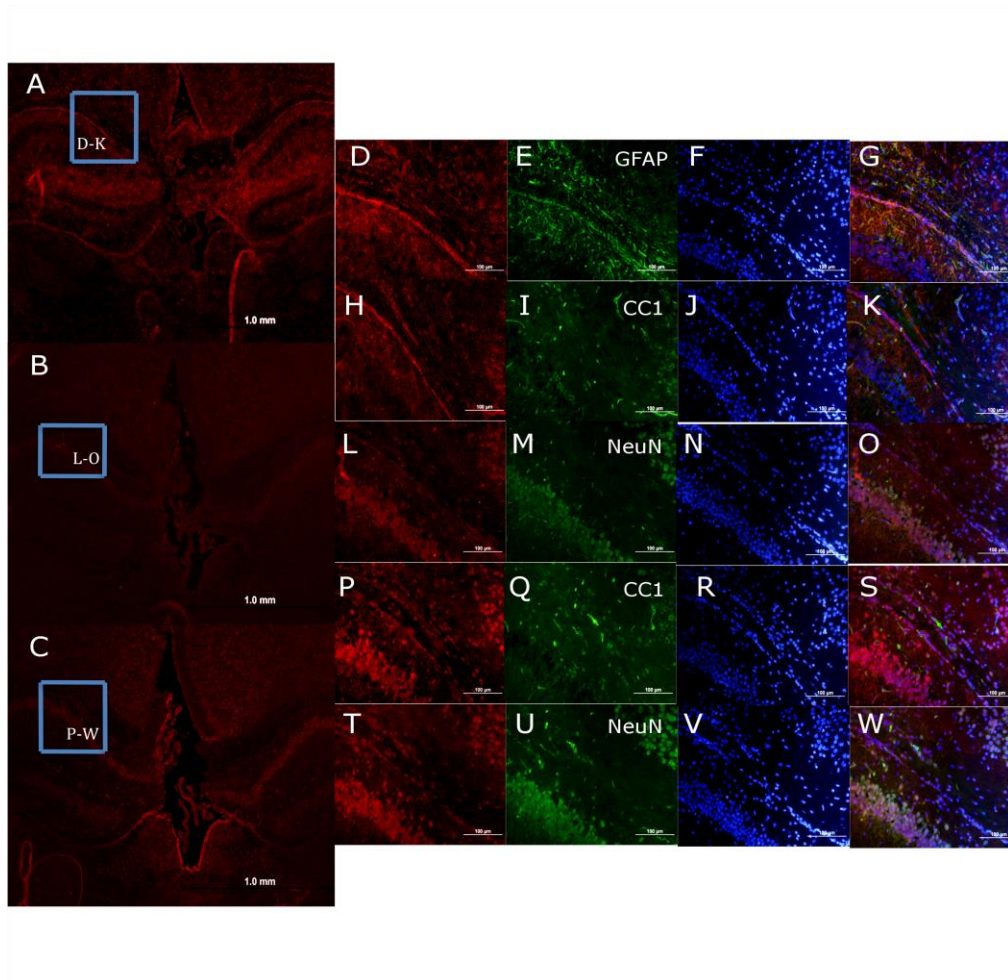


Fig. 4: P7 S1P Receptor Hippocampus Expression

P7 C57BL/6 coronal sections. A, F, B: 4X S1Pr1, r3, r5 staining respectively. D,H: 20X S1Pr1 staining. E: 20X GFAP staining. F-V: 20X Hoechst staining. G: Merge of D-F. I,Q: 20X CC1 staining. K: Merge of H-J. L: 20X S1Pr3 staining. M, U: 20X NeuN staining. O: Merge of L-N. P,T: 20X S1Pr5 staining. S: Merge of P-R. W: Merge of T-V.

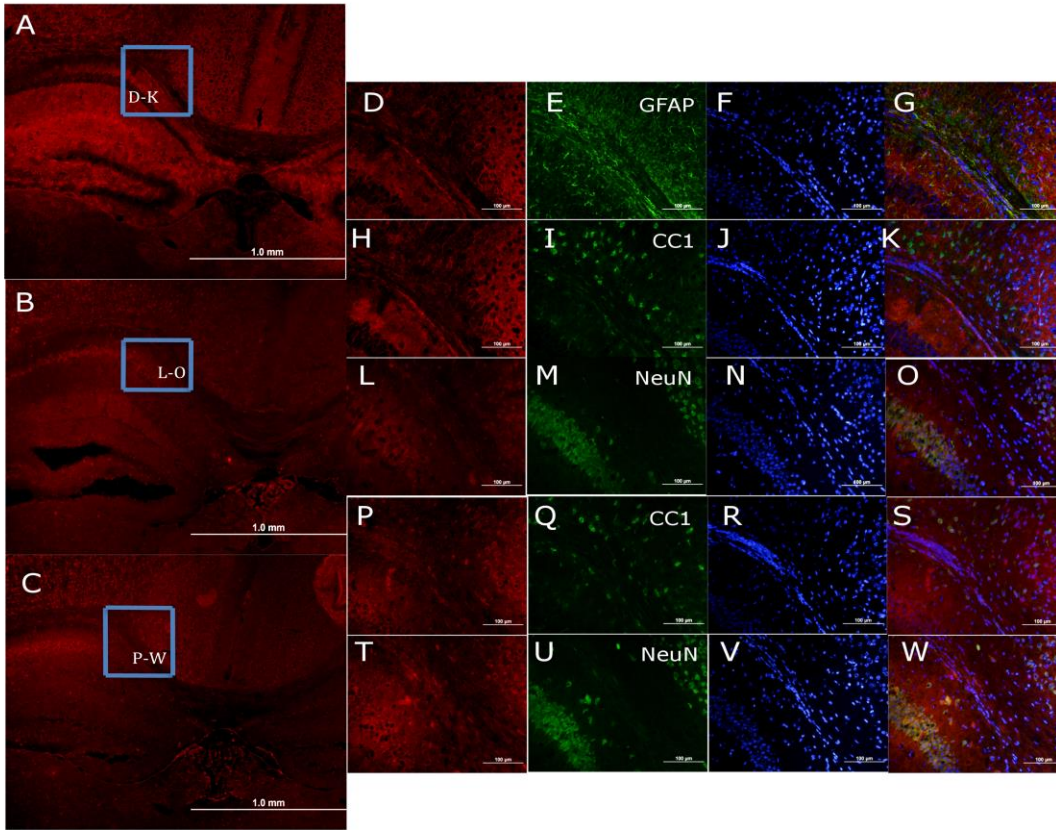
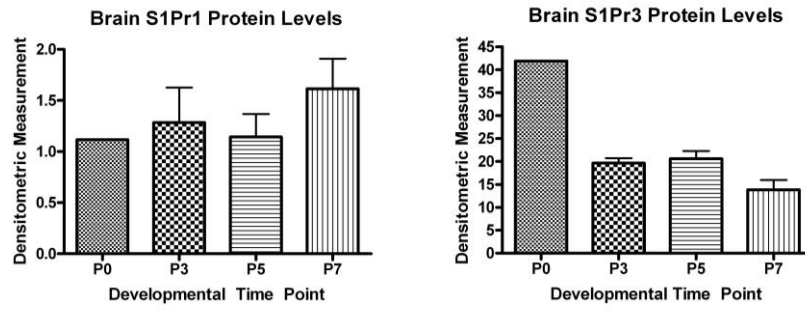


Fig. 5: P14 S1P Receptor Hippocampus Expression

P14 C57BL/6 coronal sections. A, F, B: 4X S1Pr1, r3, r5 staining respectively. D,H: 20X S1Pr1 staining. E: 20X GFAP staining. F-V: 20X Hoechst staining. G: Merge of D-F. I,Q: 20X CC1 staining. K: Merge of H-J. L: 20X S1Pr3 staining. M, U: 20X NeuN staining. O: Merge of L-N. P,T: 20X S1Pr5 staining. S: Merge of P-R. W: Merge of T-V.

A



B

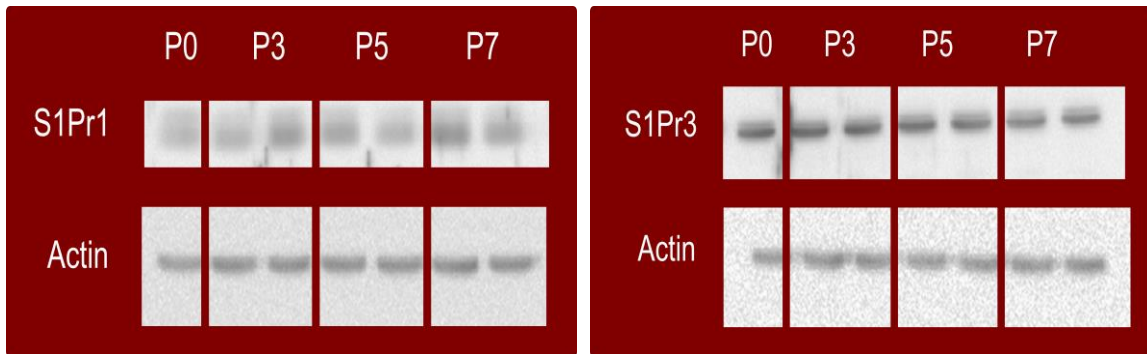


Fig. 6: Western Blot S1P receptor Expression Densitometry Analysis

Protein from C57BL/6 P0, P3, P5, and P7 mouse pups. A: Chemidoc densitometry of protein bands. B: Protein bands from western blot staining for S1Pr1,3 and actin.

Hypoxia-ischemia

Injury by hypoxia alone, ischemia alone, and hypoxia + LPS was minor and unremarkable compared to that of hypoxia-ischemia – especially with LPS which mimics systemic infection. In these first three conditions, the tissue of the ipsilateral – conditional hemisphere - and contralateral – unaffected hemisphere - hippocampus and vicinal areas appear indistinguishable. The added LPS condition produced visible and distinguishable damage to the ipsilateral CA1, 2, and 3 neurons of the hippocampus, in addition to oligodendrocyte damage in the caudate putamen as well as the cingulate gyrus and external capsule of the corpus callosum. The damage to neuron bodies, in addition to lesion formation can be seen in Figure 7 via Cresyl Violet staining for motor neurons. Hypomyelination and decreased oligodendrocyte populations are determined by MBP and CC1 immunostaining that label mature and post-mitotic oligodendrocytes, respectively, shown in Figure 8 a,b.

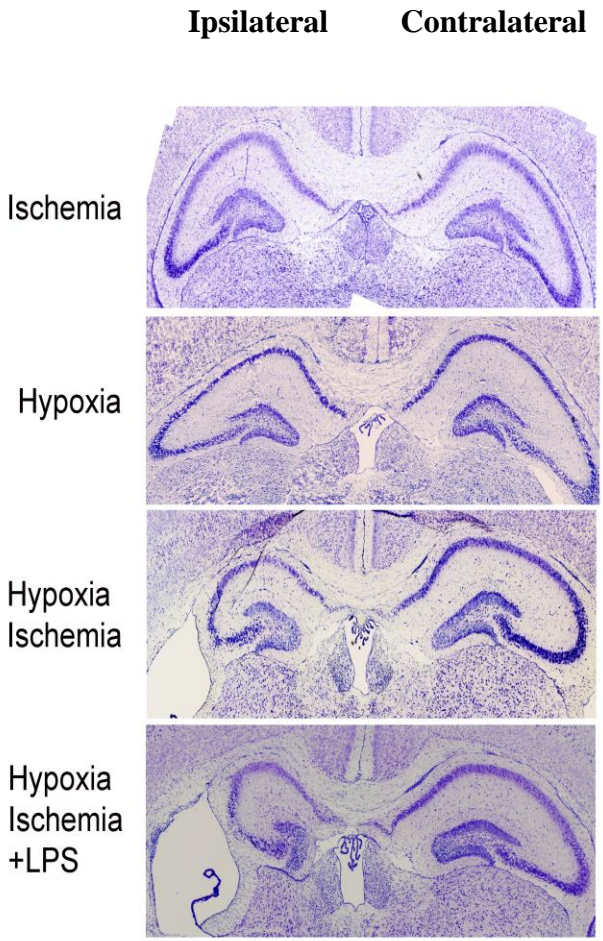


Fig. 7: Hypoxia-Ischemia Induced Hippocampus Damage

Cresyl Violet stain of hippocampus from P10 C57BL/6 mouse pups of listed conditions.

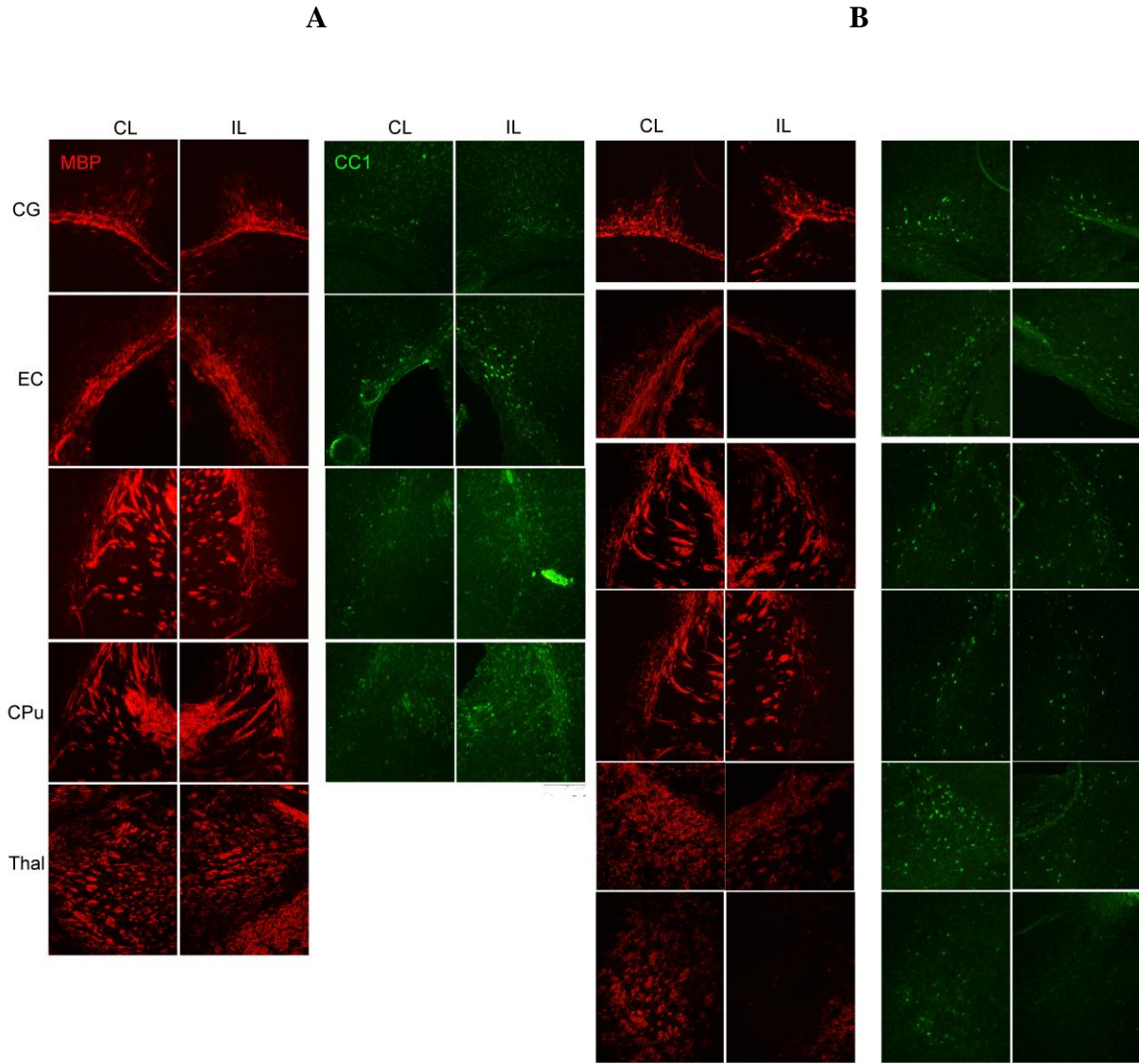


Fig. 8: Hypoxia-Ischemia & LPS Induced Hypomyelination and Oligodendrocyte Damage

A. MBP and CC1 staining of Hypoxia-Ischemia brain tissue. B. MBP and CC1 staining of Hypoxia-Ischemia brain tissue with LPS injection.

CG=Cingulate Gyrus; EC=External Capsule; CPu=Caudate Putamen; Thal=Thalamus.

CHAPTER IV

CONCLUSIONS

Given the clear need for powerful and effective treatment, brain damage by hypoxic and ischemic injury to neonates is a field in need of investigation and treatment research. In this current study, the question of whether modulating S1P receptor activity could lead to the minimization of inflammation and injury was surfaced for testing. To this end, the temporal and spatial expression of S1P receptors was examined. The expression of receptors 1, 3 and 5 in astrocytes, neurons, and oligodendrocytes of at-risk hippocampus and corpus callosum brain structures at specific developmental ages provide a rationale for discovering the potential role of these receptors in neonatal brain injury. While future quantification will be necessary in order to make confident conclusions of significance, several trends in receptor expression were found in this study. For example, S1P receptor 1 colocalization with post-mitotic oligodendrocytes, and especially astrocytes, appeared to decrease during early postnatal development while expression in gray matter but not neuronal cell bodies increased. Further exploration is necessary before concluding that that S1P receptor modulation of astrocytes peaks before P14, and that by the time this time point is reached, the potential impact of modulation has lowered. But with the hypothesis of the model, this in turn could mean that down regulation of inflammation by astrocytes has an ideal range before P14 before the natural lowering of S1Pr1 expression. For S1Pr3, overall expression seemed to decrease over development up to P14, but staining showed consistent colocalization with neurons in the hippocampus. S1Pr5, which showed a slight decrease in expression by P14, also exhibited high colocalization with neurons in the hippocampus, as well as in oligodendrocytes. Fingolimod binds to all three of the stained

receptors, but it remains unclear whether S1Pr3 or 5 would have a more profound impact on the inflammation response. While western blot data might not correspond to the expression in individual structures, western blot and densitometry will be furthered with later time points.

Importantly, these receptors have a strong presence in the areas seen to show damage in the hypoxic-ischemic injury model. The neuron colocalization in the CA1, 2, and 3 regions of the hippocampus as well as the astrocyte colocalization in the cingulate gyrus and the rest of the corpus callosum is substantial enough to surmise that modulation of the S1P receptors would impact the course of the injury model. This currently involves both decreases in hippocampus neuron and corpus callosum oligodendrocyte populations in addition to hypomyelination. As with the developmental S1P receptor expression pattern, quantification will be necessary in order to claim significance, but the injury seen in Figures 7 and 8 provides a basis to investigate the effect of FTY720 in future studies. LPS injection in conjunction with the hypoxia-ischemia provides ideal damage in the model. This is an appropriate setup given the fact that infection at birth places infants at an elevated risk for the injury of investigation (Ganesan et al., 2003).

These results have paved the way for specific investigation into the role that S1P receptors and Fingolimod can play in the hypoxia-ischemia brain injury animal model. Given the difficult nature of the procedure for inducing ischemia, this will take many trials to ensure confidence in the data; but the product will elucidate the potential of the drug in this field. Ultimately, the goal of this research is to improve the wellbeing of human infants who fall victim to ischemia and are left physically and mentally handicapped due to the limited options and impacts of current treatments. With promising results in a mouse model, clinical trials for human infants could

propel Fingolimod's entrance into this sphere of medicine.

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