How the Lysophospholipid Got its Receptor

The discovery of a new family of lipid receptors provides potential targets for diseases such as multiple sclerosis and autoimmunity.

By Jerold Chun

Illustrations by Thom Graves

In the early 1990s our lab was young and small; as developmental neurobiologists we were searching for novel molecules that might influence brain development. Lipids were not part of our formal training beyond being a source of membrane resistance or something to avoid in a California diet. However, in trying to find a ligand for a novel receptor we cloned – which we thought might trigger neural cell division – we had the good fortune to contribute answers to questions that had some excellent lipidologists stumped for years.

The lipid ligand in question was a "lysophospholipid," a simple phospholipid that influences a surprisingly wide range of biological processes. A key step in learning more about how this molecule effected physiological change was the cloning and identification of specific lysophospholipid receptors.

Lysophospholipids were likely recognized around the turn of the 20th century, if not earlier, as forms of the more common membrane phospholipids, the "bricks and mortar" of the cell membrane.[1] By the late 1960s, researchers had become aware that this somewhat obscure class of lipids had the remarkable ability to act like an extracellular signaling molecule.[2] Two prominent forms typify but do not limit lysophospholipids: lysophosphatidic acid (LPA), which is derived from glycerophospholipids such as phosphatidyl serine or phosphatidyl choline (known commonly as lecithin, as in egg yolk); and sphingosine 1-phosphate (S1P), which is derived ▶

from sphingolipids such as sphingomyelin, ceramide, and sphingosine.

Although rare compared to most membrane lipids, they could be found in relatively high concentrations (micromolar) within blood plasma and/or serum. How these lysophospholipids worked was unclear and much debated in the decade preceding the mid-1990s, with explanations invoking lysophospholipids as membrane disruptors (lysophospholipids look a bit like detergents), second messengers, ionophores, calcium chelators, as well as ligands for intracellular and extracellular receptors.

My lab, then at the University of California, San Diego, was trying to find genes that were turned on during embryonic cerebral cortical development, and we were focusing on G protein-coupled receptor (GPCR) genes, whose roles were unknown at the time. Two students in my lab, Jonathan Hecht, an MD-PhD student (now a pediatric neurologist at UCSF) and Joshua Weiner, a PhD student (now a faculty member at the University of Iowa) were drivers of the project. Using a strategy emulating that of Linda Buck and Richard Axel, degenerate PCR for GPCR genes was used to screen neuron-like cell lines that we had derived from the embryonic brain. Candidate genes were then screened for in situ expression. One gene lit up the neurogenic area of the brain and was named "Ventricular Zone Gene-1" or *VZG-1*, after the neurogenic area in the brain where it was expressed.[3] We had found a gene and the GPCR it encoded, but we had little idea of what it did and no idea of the ligand that triggered it.

We engineered neuron-like cell lines to overexpress the receptor, which was an unusual approach at the time. Most labs expressed orphan receptors (those without a known ligand) in fibroblast, HELA, or HEK cells, for a quick screen, but we never would have found the ligand with these cells because none are derived from neurons. For example, it is now known that LPA induces fibroblast cell bodies to flatten, a response which would not have been instructive compared to cell rounding that occurs in the actual neurogenic environment of the developing brain.

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These overexpressing neuron-like cells were thus grown in culture medium containing supplemented serum (a standard recipe) in which they did something peculiar: As soon as we added culture medium to them, their neural processes pulled back into the cell, and the cells rounded up. This behavior intrigued us, as it was reminiscent of what occurs in vivo. Neurons behave differently than most cell types during mitotic division. Within neurogenic regions the nucleus performs a little dance, in which it migrates from the bottom of the cell to the top, drops back down – the so-called interkinetic nuclear migration – after which a cell's process retracts, and only then does it divide. When we saw the processes retract into the cell, we thought that we might have found a receptor involved in triggering neural cell division.

We started to look for our VZG-1 receptor's trigger, which seemed to be something in the culture medium. We were biased to look for protein ligands, coming from a protein-centric field. We ruled out the cell-rounding effects that the protein thrombin was known to produce, and after exhaustive tests, we started to doubt that the ligand was a protein at all. We narrowed the possible ligand to something present in serum, and when we boiled the serum to denature all of the proteins, our cells still rounded up, ruling out proteins altogether.

With proteins out of the picture, we had to get a little creative in our search. Lipids were not initially on our radar screen. Few in the neuroscience community were thinking about lipids, at least not as signaling molecules. There were two exceptions: Ricardo Miledi's lab at UC, Irvine, as well as Wouter Moolenaar at the Netherlands Cancer Institute had identified neurite retraction and cell rounding as features of LPA exposure on neural cell lines. We gathered all the available lipids that we could find, including lysophosphatidylcholine, phosphatidic acid, arachadonic acid, and most importantly, LPA, and tested them one by one. Overall, this process and the subsequent receptor proof took several years after VZG-1 was cloned, but finally, we had found our culprit, and it was LPA.

By showing that LPA triggered our VZG-1 receptor, we had inadvertently pinpointed the elusive mechanism of action for this class of lipids and identified the receptor that lipidologists had been looking for. Our findings, however, were not met with enthusiasm in the field. Quite the contrary was true, in fact.

Lysophospholipids are lipid metabolites, which, with the right set of enzymes, can be created from basically any cell membrane, anywhere in the body. To put them in perspective, remove one of the two fatty acid chains from a membrane phospholipid, which is the main component of membranes, and the resulting structure is a lysophospholipid.

In the early 1800s, Nicolas Louis Vauquelin had recognized phospholipids in general,[4] and in the 1880s, Johann Ludwig Wilhelm Thudichum furthered phospholipids significantly with the identification of sphingolipids.[1] The lysophospholipids were surely part of the early phospholipid preparations in the 1800s, and were recognized by the early 1900s in studies of snake venom that acted on lecithin to produce lysolecithin, the "lyso-" reflecting

the hemolytic effects on red blood cells. By the late 1960s, scattered reports supported a role for lysophospholipids as not simply lytic, but as potential extracellular effectors, most notably through affecting blood pressure.[2] Akira Tokumura identified LPA as one such effector in 1978. As researchers studied these molecules further, a number of theories emerged on how they worked. Most compelling was a report by Moolenaar in 1989,[5] which drove much of our thinking that lysophospholipid effects likely involved a G protein-coupled receptor.

However, lysophospholipids were and are difficult to study: They are sticky, binding to many surfaces or freely entering membranes, and can be rapidly degraded. These properties hampered classical receptor-binding approaches for finding a receptor; when cells were exposed to lysophospholipids, the lipids immediately dispersed throughout the cell membrane, lost activity, or stuck to utilized glass or plastic ware. These qualities vastly complicated any proof that LPA acted through a specific receptor.

Our paper identifying VZG-1 as a receptor for LPA was submitted to a prominent journal and after a while, it was rejected. When the *Journal of Cell Biology* finally accepted it in 1996, our results were greeted with healthy (perhaps overly healthy) skepticism. A paper announcing a completely different GPCR as an LPA receptor (called PSP24) was published soon after ours, while skeptical views on our data emerged in the published literature.[6,7] I remember one researcher from a pharmaceutical company standing up at a conference where I spoke about our results, and betting a case of beer that I was wrong. (Though by now our results have been confirmed many times over, she hasn't yet made good on the bet; my lab would still happily put it to good use).

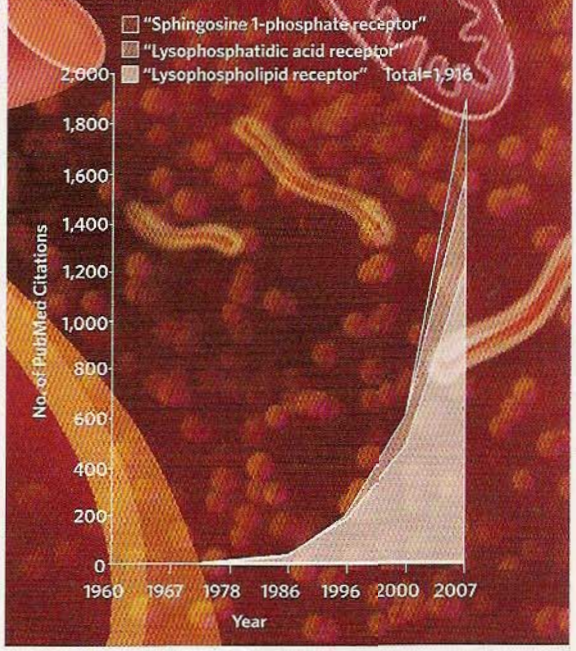
In retrospect, we could have done some things better. One accurate criticism was ▶

Lysophospholipid receptor knockouts in mice and their major phenotypes

Deleted Receptor(s)	Major Phenotypes of Null Mutants
LPA_1	• Smaller size and 50% perinatal mortality • Cerebral cortical development influences • Abnormal facial bone formation and olfaction defects • Increased schwann cell death • Inhibition of cell migration and adipocyte differentiation • Loss of neuropathic pain responses
LPA_2	No obvious phenotype
LPA_3	Reduced female reproduction: mediates implantation timing by regulating the COX pathway.
LPA_4	Unknown
LPA_5	Unknown
$LPA_{1,2}$	Lpa1 null phenotype; defective response to LPA signaling in embryonic cerebral cortex involved in neurogenesis and surface folding
$S1P_1$	• Embryonic lethality: vascular maturation. • Defective lymphocyte egress
$S1P_2$	• Normal general appearance • Spontaneous seizures and epilepsy-like symptoms • Hyper-excitable neurons • Hepatic wound healing • Inner ear function (hearing and balance)
$S1P_3$	• Normal appearance • Defective immune cell regulation (DC migration, spleen) • Reduced S1P-regulated vasodilitation • Loss of S1P-regulated bradycardia • Myocardium protection • Inner ear function (with s1p2)
$S1P_4$	Unknown
$S1P_5$	Normal general appearance signaling defects in young oligodendrocytes
$S1P_{2,3}$	• Reduced litter size and perinatal lethality • Loss of myocardium protection hearing and balance loss associated with inner ear hair cell loss
Triple $S1P_{1,2,3}$	Embryonic development: lethality in early stages, more severe vascular defects

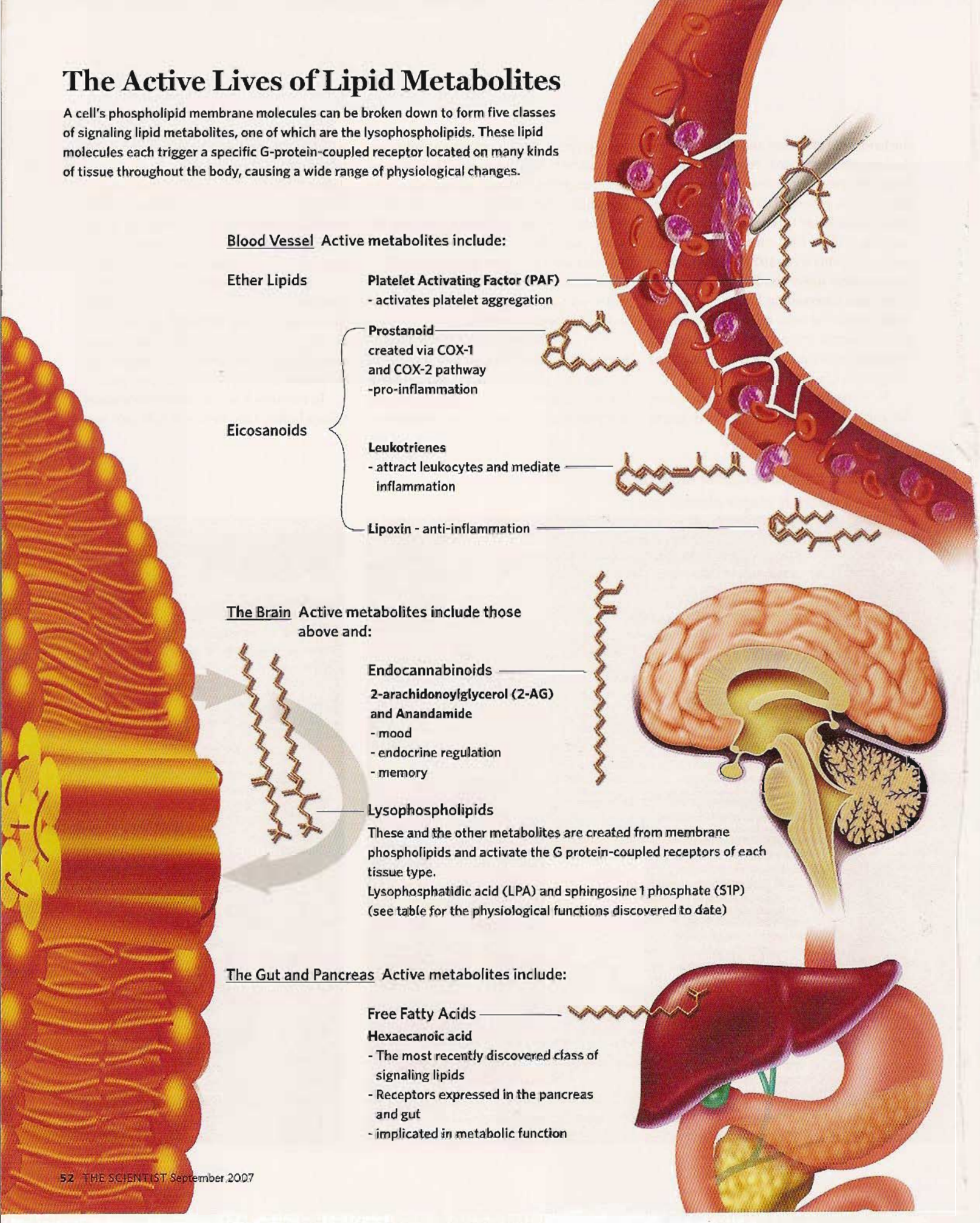
Publication History

"The cumulative number of PubMed citations in the years preceding and after our 1996 paper is one indication of the increased research activity."



The Active Lives of Lipid Metabolites

A cell's phospholipid membrane molecules can be broken down to form five classes of signaling lipid metabolites, one of which are the lysophospholipids. These lipid molecules each trigger a specific G-protein-coupled receptor located on many kinds of tissue throughout the body, causing a wide range of physiological changes.



<u>Blood Vessel</u> Active metabolites include:

Ether Lipids

Platelet Activating Factor (PAF)
- activates platelet aggregation

Eicosanoids

Prostanoid
created via COX-1 and COX-2 pathway
-pro-inflammation

Leukotrienes
- attract leukocytes and mediate inflammation

Lipoxin - anti-inflammation

<u>The Brain</u> Active metabolites include those above and:

Endocannabinoids

2-arachidonoylglycerol (2-AG) and Anandamide
- mood
- endocrine regulation
- memory

Lysophospholipids

These and the other metabolites are created from membrane phospholipids and activate the G protein-coupled receptors of each tissue type.
Lysophosphatidic acid (LPA) and sphingosine 1 phosphate (S1P)
(see table for the physiological functions discovered to date)

<u>The Gut and Pancreas</u> Active metabolites include:

Free Fatty Acids

Hexaecanoic acid
- The most recently discovered class of signaling lipids
- Receptors expressed in the pancreas and gut
- implicated in metabolic function

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that we had used overexpression, with a cell line that naturally expressed the receptor. Until we cloned the receptor into a cell line that didn't naturally express it (called heterologous expression) the criticism remained, and a difficult one at that, since all cell lines seemed to respond to LPA. That was quite a stressful time, as I was an assistant professor facing the tenure gauntlet, and a new dad as well.

One of the great things about science is that experiments can (and must) be repeated by others. The following year, Edward Goetzl's group at UCSF confirmed our basic conclusion. We also managed to identify two cell lines (one from liver, another from a neuroblastoma) that lacked VZG-1 gene expression, and LPA responses, allowing heterologous expression, which nailed down the receptor's identity. Importantly, other groups began examining receptors that had entered the databases as homologous sequences. When we first cloned VZG-1, its only close homologues were the melanocortin and cannabinoid receptors, along with an orphan receptor called Edg-1 that Timothy Hla (now at the University of Connecticut) had identified in 1990. In the intervening years preceding LPA receptor identification, more Edg homologues entered the databases – including Edg-2, which is the same as VZG-1 – and making it probable that all homologous genes were lysophospholipid receptors. It was thus reassuring that S1P was found to interact with other related receptors by Timothy Hla and Sarah Speigel (Virginia Commonwealth) as well as Goetzl, Moolenaar, ourselves, and numerous other laboratories around the globe that contributed to this effort.

The increasing level of research activity and eventual acceptance of our data led to a renaming of the receptors based on their preferred ligands; thus there are now 5 LPA receptors (LPA 1–5) and five S1P receptors (S1P 1–5). The cumulative number of PubMed citations in the years preceding and after our 1996 paper is one indication of the increased research activity.

The identification and cloning of lysophospholipid receptors opened the field to powerful genetic approaches through the construction of mice in which one or more receptors had been deleted. By 2000, work from our lab and Richard Proia at the National Institutes of Health resulted in the first LPA and S1P receptor knockouts, respectively. Molecular genetics approaches by our lab and an increasing number of groups in academia and industry have revealed a rich and expansive range of organismal influences, many having medical relevance to the nervous, immune, reproductive, cardiovascular, respiratory, and hepatic systems, as well as to cancer and a range of other pathologic settings.[8] We're still in the early days of understanding the important influences of these lipid signals.

Despite the contribution to lipidology, traditional neurobiologists have been generally unimpressed with lysophospholipids. There are just so many agents that can affect the brain, and even more cellular phenomena to consider, which makes yet another complex network of ligands and receptors less than exciting. However, our research has been pointing to roles for lysophospholipids in brain development,[9] where a host of effects has been observed, ranging from cell biologic influences on cell shape and electrophysiology to survival, proliferation, myelination, brain organization, and behavior – including clear effects on developing neurons (as we initially surmised).

Intriguing diseases with developmental etiologies, such as schizophrenia and autism, may also involve lysophospholipid receptors, as well as diseases for which proof of concept has emerged by pharmacologically targeting lysophospholipid receptors. Since, as a class, GPCRs represent one of the most successful targets for human medicines (antihistamines, cardiovascular medicines, neuropsychiatric medicines, etc.), lysophospholipid receptors could themselves be important for disease treatment, and this is especially true for multiple sclerosis (MS). This prevalent demyelinating disease has no cure, and is characterized by a relapsing-remitting presentation of impairment that includes muscle weakness, visual problems, fatigue, depression, and other symptoms that can progress to a debilitating neurodegenerative state.

Current MS therapeutic regimens require injection, so an oral treatment that could help prevent recurring attacks would be of great benefit to patients with MS. Novartis is currently testing such a compound, called FTY720, in human clinical trials.[10] Once phosphorylated, it looks like the lysophospholipid S1P. Phosphorylated FTY720 interacts with four of the five known S1P lysophospholipid receptors. It is thought to mediate immunosuppression by causing lymphocytes to remain in the lymph nodes rather than trafficking to sites of inflammation. While the immunosuppressive properties initially suggested its use in organ transplantation, researchers at Novartis tried using FTY720 for MS. Their Phase II trial showed encouraging results, with a 50% reported decrease in relapse over placebo. The drug has recently entered Phase III clinical trials.

In view of the widespread expression of S1P lysophospholipid receptors, it remains unclear as to how precisely FTY720 is working in the treatment of MS. Researchers from Novartis in Basel, Switzerland, recently published data showing that FTY720 activated astrocytes, important glial cells in the brain, thus stimulating their migration. Other cell types shown to be activated included endothelial cells and lymphocytes. Work from many laboratories will explore these ideas, along with the validity of this approach – targeting lysophospholipid receptors – in treating diseases. The coming years will no doubt be rich with new insights, and, it is hoped, therapeutic success.

Myriad questions remain in the field of lysophospholipid signaling. Are there more receptors? The recent identification by Takao Shimizu's lab at the University of Tokyo of another unique LPA GPCR, along with our work and that of others, underscores the probability that more receptors may yet be identified. Detailed studies of how receptor-mediated signaling occurs in each organ system will occupy individual fields for many years. In view of the many organ systems that are influenced, there must also be coordination between systems to allow homeostasis, which has not been examined in-depth.

Moreover, work in the early stages involves identifying the range of endogenous and exogenous influences that can regulate lysophospholipids in the body, including important ongoing work on synthetic and degradative enzymes from many labs worldwide. The complex process of creating medicines makes it difficult to predict which, if any, lysophospholipid receptors or related enzymes can be targeted for medicinal benefit, yet the current data on FTY720 shows promise for success.

Finding new effects of lysophospholipids in the brain would be most intriguing as well as poetically just: Vauquelin first identified phospholipids from the brain in the early 1800s.[4] And, it is no trivial coincidence that Thudichum discovered sphingolipids and phospholipids (lysophospholipid precursors) in the brain – a fitting achievement for the father of neurochemistry.[1] The brain remains an enormously rich source of lysophospholipids, and most of the known receptors are expressed there. It would thus be no surprise if the coming years reveal novel functions for lysophospholipid signaling, allowing a better understanding of the brain in both its normal and pathologic states. ■

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Have a comment? E-mail us at mail@the-scientist.com

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