

Growth factor pre-treatment differentially regulates phosphoinositide turnover downstream of lysophospholipid receptor and metabotropic glutamate receptors in cultured rat cerebrocortical astrocytes

Tadimeti S. Rao*, Karen D. Lariosa-Willingham, Fen-Fen Lin, Naichen Yu, Chui-Se Tham, Jerold Chun¹, Michael Webb

Merck Research Laboratories—San Diego, 3535 General Atomics Court, Bldg 1, San Diego, CA 92121, USA

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Abstract

Reactive gliosis is an aspect of neural plasticity and growth factor (GF) stimulation of astrocytes in vitro is widely regarded as a model system to study astrocyte plasticity. Astrocytes express receptors for several ligands including lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), agonists for the G-protein-coupled lysophospholipid receptors (lpRs). Activation of lpRs by LPA or S1P leads to multiple pharmacological effects including the influx of calcium, phosphoinositide (PI) hydrolysis, phosphorylation of extracellular receptor regulated kinase (ERK), release of arachidonic acid, and induces mitogenesis. Treatment of astrocytes in vitro with a growth factor cocktail (containing epidermal growth factor [EGF], basic fibroblast growth factor [bFGF] and insulin) led to a marked attenuation of lpR-induced PI hydrolysis. In contrast, under identical conditions, GF treatment led to marked potentiation of PI hydrolysis downstream of activation of another abundantly expressed G-protein coupled receptor, mGluR5. Quantitative gene expression analysis of GF-treated or control astrocytes by TaqMan RT-PCR indicated that GF treatment did not change gene expression of *lpa1* and *s1p1*, but increased gene expression of *s1p5* which is expressed at very low levels in basal conditions. These results suggest that GF differentially affected PLC activation downstream of mGluR5 versus lpR activation and that the changes in mRNA levels of lpRs do not account for marked attenuation of agonist-induced phosphoinositide turnover.

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1. Introduction

Lysophosphatidic acid (1-acyl-2-lyso-*sn*-glycero-3-phosphate; LPA) and sphingosine-1-phosphate (S1P) are agonists for the G-protein-coupled lysophospholipid receptors (lpRs; Goetzl and An, 1998; Kluk and Hla, 2002; Moolenaar, 1999). This receptor family is comprised of *lpA* receptors (LPA1, LPA2 and LPA3) and *s1p* receptors (S1P1, S1P2, S1P3, S1P4 and S1P5), respectively (Contos et al., 2000; Chun et al., 1999). Rat cerebrocortical astrocytes express mRNA for lpRs with the following relative abundance: $s1p3 > s1p1 > lpa1 = s1p2 = lpa3 \gg s1p5$ (Rao et al.,

2003). Activation of lpRs either by LPA and S1P results diverse signaling events (Goetzl and An, 1998) such as phosphoinositide hydrolysis, arachidonic acid release, increase in intracellular calcium levels, phosphorylation of extracellular signal regulated kinase 2 (ERK 2; Spiegel and Milstein, 2000; Siehler and Manning, 2002; Rao et al., 2003), release growth factors and proliferation of astrocytes (Tabuchi et al., 2000; Yamagita et al., 2003; Olivera and Spiegel, 1993; Sato et al., 1999). Changes in receptor expression and function are hallmarks of plasticity and reflect cellular responses to injury or signaling. Growth factors (GF), neurotrophins and cytokines released by glia or neurons can act on neighboring cells and affect cellular plasticity. GF treatment of astrocytes induces mitogenesis and alters signaling of G-protein coupled receptors (GPCRs) such as the metabotropic glutamate receptor 5 (mGluR5; Miller et al., 1995). It is also known that GF acting via receptor tyrosine kinases (RTK) can affect signaling via GPCRs through “transactivation” (Zwick et al., 1999).

* Corresponding author. Present address: Kalypsys Inc, 10420 Wateridge Circle, San Diego, CA 92121, USA. Tel.: +1-858-754-3331; fax: +1-858-754-3301.

E-mail address: trao@kalypsys.com (T.S. Rao).

¹ Present address: Scripps Research Institute, Department of Molecular Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

Since astrocytes are routinely exposed to GF and cytokines during differentiation or injury, GF treatment of astrocytes has been used a model of astrocyte plasticity (Miller et al., 1995). In the present study, we compared the influence of GF treatment on lpR and mGluR5 signaling in astrocytes with a special emphasis on activation of the phospholipase C pathway.

2. Materials and methods

2.1. Materials

Stock solutions of LPA (10 mM), SIP (500 μ M; both from Avanti Polar Lipids Inc, Alabaster, AL) were prepared using 1 mg/mL solution of fatty acid free bovine serum albumin (FAFBSA; Sigma Chemical Co, St. Louis, MO) in distilled water as the vehicle, stored at -70°C until use and diluted into tissue culture medium (DMEM; GIBCO, Gaithersburg, MD) with glutamine, 2 mM) containing 1 mg/mL FAFBSA; herein after referred to as TCM). At these concentrations, the LPA stock solution was clear while the stock solutions of both SIP and DHS remained cloudy. All stock solutions were diluted into TCM prior to application to astrocyte cultures. Appropriate vehicle controls were included in each experiment.

2.2. Secondary astrocyte cultures: growth factor treatment

Growth factor treatment of astrocytes was conducted as described by Peavy and Conn (1998) and Miller et al. (1995). Astrocyte-enriched cultures (>97% GFAP positive cells) were prepared as described previously (Rao et al.,

2003) and were used in all biochemical experiments within 3–5 days after re-plating. Twenty four to thirty six hours after re-plating, growth medium was replaced with DMEM containing growth factor supplement (G-5; Gibco, Gaithersburg, MD) containing epidermal growth factor (10 ng/mL), basic fibroblast growth factor (5 ng/mL), insulin (5 μ g/mL), biotin (1 μ g/mL), human transferrin (50 μ g/mL) and hydrocortisone (360 ng/mL) and grown for 4–6 days. G-5 supplement is a chemically defined, 100 \times concentrate of Bottenstein supplement widely used in neural cultures. Twenty four hours before the experiments, cells were washed twice and incubated overnight in a serum-free medium (DMEM with 2 mM Gln) containing [^3H]-myo-inositol (1 μ Ci/well). Radioactive tracers for biochemical experiments were added at the time of changing the medium. For mRNA analysis, cells were collected after an overnight incubation in serum-free medium ((DMEM with 2 mM Gln). Control cells underwent all of the media changes except for the inclusion of G-5 supplement.

2.3. Distribution of lpR mRNA in secondary astrocytes: TaqMan PCR analysis

The influence of growth factor treatment on lpR mRNA expression was evaluated by TaqMan PCR analysis of total RNA isolated from rat astrocytes was isolated using RNeasy[®] Protect Mini kit (QIAGEN). One μ g of total RNA was reverse transcribed using RETROscript[™] kit (Ambion). In each reverse transcription reaction, a reaction omitting reverse transcriptase was included for the assessment of genomic DNA contamination. Cloning, Construction and DNA Sequencing of mini genes for lpR and β -actin, PCR quantitation were conducted as per previously described

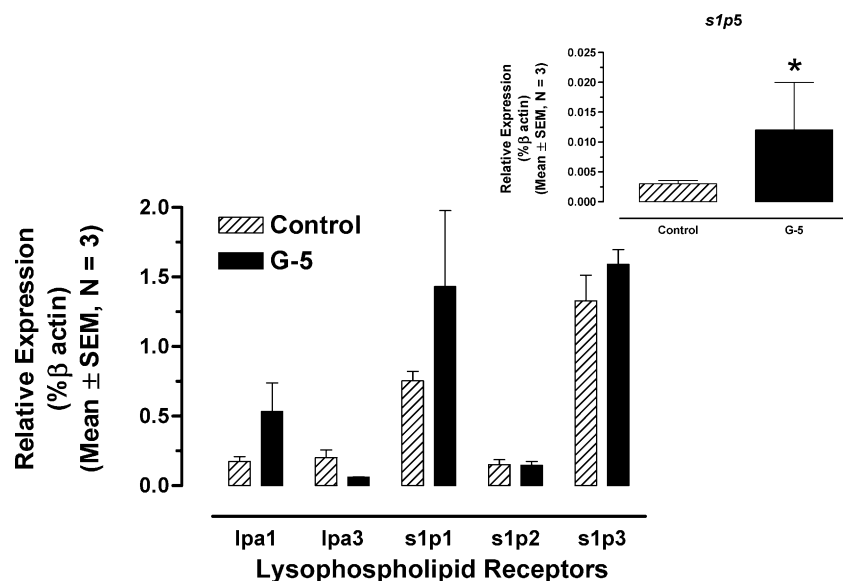


Fig. 1. Quantitative analysis of mRNA transcripts for lpR in rat cortical astrocytes by TaqMan PCR technique. Data represent expression of individual lpR mRNA relative to β -actin whose expression was normalized to 100. Values represent means \pm S.E.M., $n = 3$. * $P < 0.05$ vs. control (t -test).

procedure (Rao et al., 2003; Tham et al., 2003; Yu et al., 2004). All measured PCR products were normalized to the amount of β -Actin cDNA, and all data were normalized to β -Actin and expressed as percent β -Actin using software Prism 3.0.

2.4. PI hydrolysis

LPA or SIP induced PI hydrolysis in cultured astrocytes was measured using ion exchange separation protocols by the methods described previously (Rao et al., 2003).

2.5. Statistics

All values are expressed as mean \pm standard error of mean (S.E.M., $n = 3$ –4 individual experiments each with two to three replicates). Data were analyzed by analysis of variance (ANOVA) followed by post-hoc analysis (Neuman–Keuls test; Prism 3.0; GraphPad, San Diego, CA) or t -test as appropriate and statistical significance determined at a $P < 0.05$.

3. Results

3.1. Distribution of *lpR* mRNA in astrocytes: influence of growth factor treatment

Quantitative analysis by TaqMan PCR techniques indicated *s1p3* mRNA to be the most abundant *lpR* species in naïve rat astrocytes, followed by *s1p1* > *lp1* = *s1p2* = *lp3* (Fig. 1). Following growth factor treatment, there was a trend for an increase in the gene expression of *lp1*, *s1p1* and *s1p5*, and a trend for a decrease in *lp3* mRNA. However, only the changes in *s1p5* only reached statistical significance.

3.2. Activation of PLC pathway: PI hydrolysis

Both LPA and SIP increased PI hydrolysis in a concentration-dependent manner demonstrating that *lpR* activation triggers stimulation of the PLC pathway. Interestingly, in growth factor treated astrocyte cultures, the *lpR*-induced PLC signaling was substantially blunted with

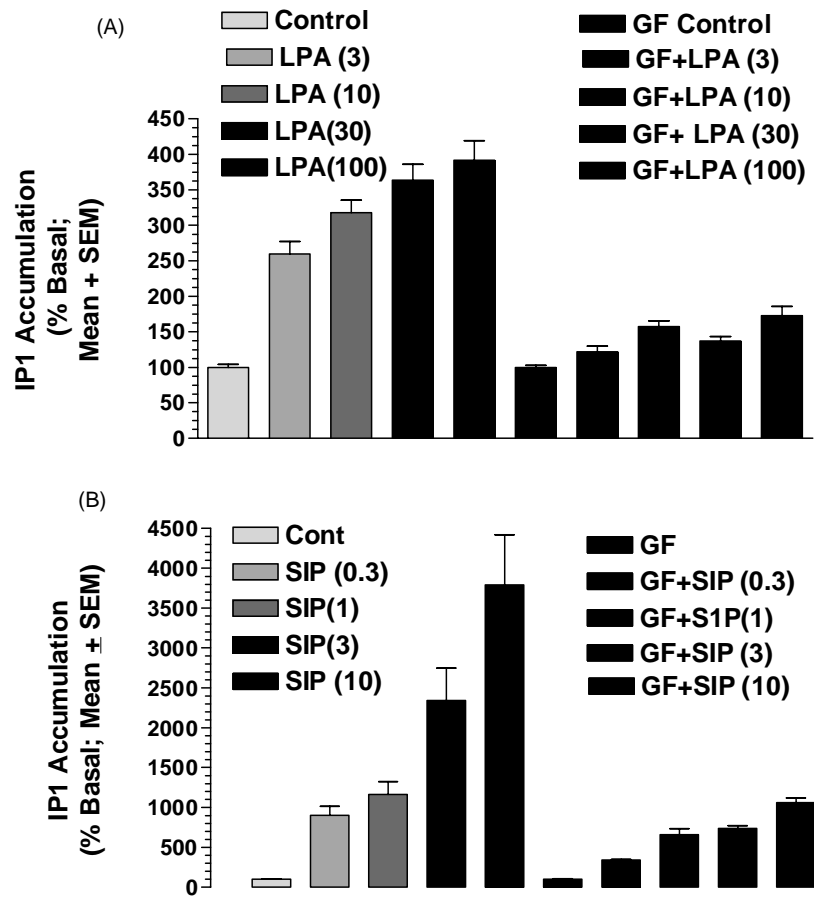


Fig. 2. Concentration-dependent increases in phosphoinositide hydrolysis in cortical astrocytes following activation of *lpR* with LPA or SIP. Astrocytes were incubated overnight with [3 H]-myoinositol to label lipid pools, washed, pre-incubated in lithium containing buffer prior to exposure to agonists in lithium-containing buffer for 1 h. Values represent PI hydrolysis expressed as a percentage of basal IP1 accumulation seen under control conditions (mean \pm S.E.M.; $n = 3$ –4 experiments each with two to three replicates). Values in parenthesis refer to concentrations in μ M.

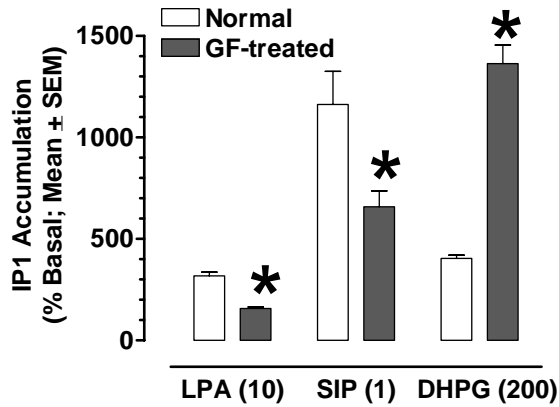


Fig. 3. LPA (10 μ M)-, SIP (1 μ M)- and DHPG (200 μ M)-induced PI hydrolysis in astrocytes in control and growth factor treated astrocytes. Data represent fractional IP1 accumulation observed with LPA, SIP or DHPG expressed as a percentage of IP1 accumulation seen with respective control response (mean \pm S.E.M., $n = 3$ –4 experiments each with three replicates), * $P < 0.05$ vs. control (t -test). Under identical conditions, PI hydrolysis response to saturating concentration of carbachol (CCh; 1 mM) was not affected. Basal conditions: values in (mean \pm S.E.M., $n = 3$), control, 100 ± 10 and CCh (1 mM) = 300 ± 25 ; GF control, 100 ± 12 , GF + CCh (1 mM) = 290 ± 20 .

a dramatic decrements in maximal responses (Fig. 2; Panels A and B).

In order to determine whether GF treatment globally affected PLC signaling down stream of G-protein coupled receptor activation, we compared downstream consequences of lpR signaling to those mediated via mGluR5 receptors, another GPCR widely expressed in astrocytes (Miller et al., 1995; Peavy and Conn, 1998). In naïve astrocytes, 3,5-Dihydroxyphenylglycine (DHPG), a group I mGluR agonist (Peavy and Conn, 1998), increased PI hydrolysis in a concentration-dependent manner with an approximate EC_{50} value of 30 μ M and saturating responses were seen at 200 μ M, both in naïve and GF-treated astrocyte cultures (data not shown). However, GF-treatment markedly potentiated the magnitude of DHPG-induced PI response as compared to its response in naïve astrocytes. In the same experiment, PI response to both LPA and SIP was greatly attenuated in GF treated cultures (Fig. 3). GF treatment did not affect magnitude of the PI hydrolysis response to a saturating concentration of carbachol (1 mM), an agonist of muscarinic acetylcholine receptors widely expressed in astrocytes (data not shown).

4. Discussion

Generation of growth factors by glia is one of the key responses of the central nervous system to Injury. Astrocytes, the primary glia, are a key player in this response and undergo phenotypic changes leading to scar formation (Ridet et al., 1997). Interestingly, astrocytic lpR activation results in growth factor gene expression and release of growth

factors (Tabuchi et al., 2000; Yamagita et al., 2003; Olivera and Spiegel, 1993; Sato et al., 1999). Exposure of astrocytes to growth factors is a widely used as a model system to study the plasticity of glia. Within this framework, the goal of this investigation is to examine influence of growth factor treatment on signaling down stream of lpR activation.

Previous studies indicate that rat cortical astrocytes grown in culture express lpR in the following order of relative expression: $s1p3 > s1p1 > lpa1 > s1p2 = lpa3 \gg s1p5$ (Rao et al., 2003). This contrasts with the expression pattern of $S1p1 > s1p2 = lpa1 \gg s1p3 = s1p5$ in microglia (Tham et al., 2003) or $lpa1 > s1p5 > s1p1 = s1p2 = lpa3 > s1p3$ in oligodendrocytes (Yu et al., 2004). A very low expression of $s1p5$ was seen in cultured astrocytes, an observation that is consistent with an exclusive distribution of $s1p5$ in oligodendrocytes (Yu et al., 2004). Since the TaqMan PCR technique can detect very low level gene expression, detection of $s1p5$ in astrocytes may reflect trace contamination of glial cells of oligodendroglial origin. Treatment with growth factors lead to a small, but statistically significant increase in $s1p5$ mRNA without changes in mRNA levels for other lpRs. We have considered the possibility that trace contamination of oligodendrocytes could contribute to lpR-evoked PI hydrolysis response in astrocyte culture. However, in highly enriched oligodendrocyte cultures as described by Yu et al. (2004), lpR activation was minimally effective at increasing PI hydrolysis as compared to the level of stimulation of this pathway in astrocytes. These results taken together suggest that GF treatment does not alter expression of lpRs in a global manner.

Interestingly, GF treatment of astrocytes led to marked down regulation of PLC signaling downstream of lpR activation. However, GF-treatment lead to significant increase in magnitude of PLC signaling downstream of activation of mGluR5, another GPCR widely distributed in astrocytes. These results argue GF treatment does not result in non-specific down regulation of the PLC pathway. The potentiation of mGluR5 mediated PLC pathway following growth factor treatment is consistent with literature reports (Miller et al., 1995; Peavy and Conn, 1998). The decrements in lpR-induced PI hydrolysis in the face of modest changes in lpR expression suggest that receptor gene expression changes alone are unlikely to explain marked reduction in lpR-induced PLC activation. While the GF treatment induces proliferation of astrocytes and increases mGluR5 expression, these are unlikely to explain changes in PI hydrolysis following agonist stimulation, as the IP1 accumulation data were normalized to total incorporation of the radiolabel and expressed as stimulation over basal levels. Stimulation of phosphoinositide-hydrolyzing phospholipase C (PLC) is a cellular biochemical response downstream of a stimulation of a large number of GPCRs by ligands such as LPA, SIP and DHPG as well as stimulation of RTKs by ligands such as EGF and PDGF. GPCRs and RTKs activate distinct PLC isozymes; GPCRs via PLC β isozyme and RTKs via PLC γ isozyme. Short term treatment with GPCR agonists such as

LPA is known to markedly potentiate PI hydrolysis response to a subsequent RTK stimulation (Schmidt et al., 2000) suggesting a cross talk between GPCR- and RTK-mediated PLC activation pathways. Conversely, emerging evidence suggests “transactivation” of RTK by GPCR signaling to be a common cellular mechanism (Zwick et al., 1999; Peavy et al., 2001). In astrocytes, mGluR5-induced phosphorylation of extracellular signal-regulated kinase in astrocytes depends on transactivation of the epidermal growth factor receptor (Peavy et al., 2001). While this transactivation process takes place on a short time scale (<15 min), prolonged treatment with GF may have longer-lasting consequences on GPCR signaling in receptor-specific manner. The present findings suggest that prior RTK stimulation markedly down regulate lpR responses in a highly selective manner while up-regulating mGluR5-mediated responses. The GF cocktail contained containing epidermal growth factor (10 ng/mL), basic fibroblast growth factor (5 ng/mL), insulin (5 µg/mL), biotin (1 µg/mL), human transferrin (50 µg/mL) and hydrocortisone (360 ng/mL). Although it is unclear which of these factors alone are responsible, preliminary studies indicated that a combined overnight treatment with epidermal growth factor and epidermal growth factor led to significant blunting of lpR-induced PI hydrolysis (data not shown). Further studies are needed to clearly delineate mechanisms underlying changes in lpR signaling in astrocytes in response to growth factors. In addition, further studies are also needed to explore whether growth factor treatments globally affect lpR signaling or specifically target PLC pathway down stream of lpR activation.

The precise roles of astrocytic lpRs in vivo physiology are unclear. Astrocytes not only provide nutrient support to neurons, but emerging evidence points to an active role of astrocytes in CNS repair following injury (Ridet et al., 1997). In addition, astrocytes, in concert with endothelial cells, form the blood brain barrier. CNS injury results in expression and release of growth factors which in turn influence neural plasticity. The results described here suggest that growth factors differentially affect GPCR signaling in astrocytes. Blunting of S1P signaling pathway as a response to GF may reflect a transition of astrocytes from an acute response to injury characterized by active damage, blood brain barrier breach, release of S1P from platelets to a secondary phase representing down regulation of S1P signaling to support or modify tissue repair and remodeling.

References

Chun, J., Contos, J.J., Munroe, D., 1999. A growing family of receptor genes for lysophosphatidic acid (LPA) and other lysophospholipids (LPs). *Cell Biochem. Biophys.* 30, 213–242.

- Contos, J.J., Ishii, I., Chun, J., 2000. Lysophosphatidic acid receptors. *Mol. Pharmacol.* 58, 1188–1196.
- Goetzl, E.J., An, S., 1998. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* 12, 1589–1598.
- Kluk, M.J., Hla, T., 2002. Signaling of sphingosine-1-phosphate via S1P/Edg—family of G-protein-coupled receptors. *Biochem. Biophys. Acta* 1582, 72–80.
- Miller, S., Romano, C., Cotman, C.W., 1995. Growth factor upregulation of a phosphoinositide-coupled metabotropic glutamate receptor in cortical astrocytes. *J. Neurosci.* 15, 6103–6109.
- Moolenaar, W.H., 1999. Bioactive lysophospholipids and their G protein coupled receptors. *Exptl. Cell Res.* 254, 230–238.
- Olivera, A., Spiegel, S., 1993. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* 365, 557–560.
- Peavy, R.D., Conn, P.J., 1998. Phosphorylation of mitogen-activated protein kinase in cultured rat cortical glia by stimulation of metabotropic glutamate receptors. *J. Neurochem.* 71, 603–612.
- Peavy, R.D., Chang, M.S., Sanders-Bush, E., Conn, P.J., 2001. Metabotropic glutamate receptor 5-induced phosphorylation of extracellular signal-regulated kinase in astrocytes depends on transactivation of the epidermal growth factor receptor. *J. Neurosci.* 21, 9619–9628.
- Rao, T.S., Lariosa, K., Lin, F.-F., Palfreyman, E.L., Yu, N., Chun, J., Webb, M., 2003. Pharmacological characterization of lysophospholipid receptor signal transduction pathways in rat cerebrocortical astrocytes. *Brain Res.* 990, 182–194.
- Ridet, J.L., Malhotra, S.K., Privat, A., Gage, F.H., 1997. Reactive astrocytes: cellular and molecular cues to biological functions. *Trends Neurosci.* 20, 570–577.
- Sato, K., Ishikawa, K., Ui, M., Okajima, F., 1999. Sphingosine 1-phosphate induces expression of early growth response-1 and fibroblast growth factor-2 through mechanism involving extracellular signal-regulated kinase in astroglial cells. *Brain Res. Mol. Brain Res.* 72, 182–189.
- Schmidt, M., Frings, M., Mono, M.-L., Guo, Y., Oude, W., Evelin, S., Han, L., Jakobs, K., 2000. G protein-coupled receptor-induced sensitization of phospholipase C stimulation by receptor tyrosine kinases. *J. Biol. Chem.* 275, 32603–32610.
- Siehler, S., Manning, D.R., 2002. Pathways of transduction engaged by sphingosine-1-phosphate through G-protein-coupled receptors. *Biochem. Biophys. Acta* 1582, 94–99.
- Yu, N., Lariosa, K.D., Lin, F.-F., Palfreyman, E.L., Webb, M., Rao, T.S., 2004. Characterization of lysophosphatidic acid and sphingosine-1-phosphate-mediated signal transduction in rat cortical oligodendrocytes. *Glia* 45, 17–27.
- Spiegel, S., Milstein, S., 2000. Functions of a new family of sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* 1484, 107–116.
- Tabuchi, S., Kume, K., Aihara, M., Shimizu, T., 2000. Expression of lysophosphatidic acid receptor in rat astrocytes: mitogenic effect and expression of neurotrophic genes. *Neurochem. Res.* 25, 573–582.
- Tham, C.S., Lin, F.F., Rao, T.S., Yu, N., Webb, M., 2003. Microglial activation state and lysophospholipid receptor expression. *Int. J. Dev. Neurosci.* 21, 431–443.
- Yamagita, K., Tagami, M., Torri, Y., Takenaga, F., Tsumagari, S., Itoh, Y., Yamori, Y., Nara, Y., 2003. Sphingosine-1-phosphate induces glial cell line-derived neurotrophic factor production and cellular proliferation in astrocytes. *Glia* 41, 199–206.
- Zwick, E., Hackel, P.O., Prenzel, N., Ullrich, A., 1999. The EGF receptor as central transducer of heterologous signaling systems. *Trends Pharmacol.* 20, 408–412.