Acta Physiologica Sinica, December 25, 2007, 59 (6): 759-764
http://www.actaps.com.cn

Research Paper

Effect of lysophosphatidic acid on differentiation of embryonic neural stem cells into neuroglial cells in rats *in vitro*

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Abstract: To study the effect of lysophosphatidic acid (LPA) on the differentiation of embryonic neural stem cells (NSCs) into neuroglial cells in rats *in vitro*, both oligodendrocytes and astrocytes were detected by their marker proteins galactocerebroside (Gal-C) and glial fibrillary acidic protein (GFAP), respectively, using double-labeling immunocytochemistry. RT-PCR assay was also used for analyzing the expression of LPA receptors in NSCs. Our results showed that: (1) LPA at different concentrations (0.01-3.0 μmol/L) was added to culture medium and cell counting was carried out on the 7th day in all groups. Exposure to LPA led to a dose-dependent increase of oligodendrocytes with the response peaked at 1.0 μmol/L, with an increased percentage of 32.6% (P<0.01) of total cells as compared to that of 8.5% in the vehicle group. (2) LPA showed no effect on the differentiation of NSCs into astrocytes. (3) RT-PCR assay showed that LPA_1 and LPA_3 receptors were strongly expressed while LPA_2 receptor expressed weakly in NSCs. These results suggest that LPA at low concentration might act as an extracellular signal through the receptors in NSCs, mainly LPA_1 and LPA_3 receptors, to promote the differentiation of NSCs into oligodendrocytes, while it exhibits little, if any, conceivable effect on the differentiation of NSCs into astrocytes.

Key words: differentiation; neuroglial cells; neural stem cells; lysophosphatidic acid; lysophosphatidic acid receptors

溶血磷脂酸对离体培养的大鼠胚胎神经干细胞向神经胶质细胞分化的影响

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摘 要：本研究用免疫细胞化学荧光双标技术观察了溶血磷脂酸(lysophosphatidic acid, LPA)对大鼠胚胎神经干细胞(neural stem cells, NSCs)分化为少突胶质细胞(galactocerebroside-positive，Gal-C 阳性)和星形胶质细胞(glial fibrillary acidic protein-positive，GFAP 阳性)的影响，并且用 RT-PCR 技术对 NSCs 可能表达的 LPA 受体进行分析。结果显示：(1)加入不同浓度(0.01~3.0 μmol/L) LPA，第 7 天进行检测时，少突胶质细胞数量呈明显的剂量依赖性增加，峰值出现在 1.0 μmol/L LPA 组，少突胶质细胞所占百分比从对照组的 8.5% 增加到 32.6%；(2)星形胶质细胞的分化几乎不受 LPA 的影响，第 7 天时各 LPA 处理组星形胶质细胞百分比与对照组相比均无显著性差异；(3) RT-PCR 结果显示，大鼠胚胎 NSCs 的 LPA_1 和 LPA_3 受体表达明显，而 LPA_2 受体表达很弱。以上结果表明，较低浓度的 LPA 可能作为细胞外信号，通过 LPA_1 和 LPA_3 受体促进大鼠胚胎 NSCs 向少突胶质细胞分化和生成，但对星形胶质细胞的分化过程无明显影响。

关键词：分化；神经胶质细胞；神经干细胞；溶血磷脂酸；溶血磷脂酸受体
中图分类号：Q254；Q189

Neural stem cells (NSCs) are the most immature progenitor cells in the neurogenesis. The progeny of NSCs can be oriented towards three main neural phenotypes: neurons, oligodendrocytes and astrocytes[1-3]. Oligodendrocytes are myelin-forming cells that allow the rapid conduction of neuronal impulses along axons and are lost in demylinating diseases such as multiple sclerosis[4]. Astrocytes are star-shaped cells that provide important structural, metabolic

Received 2006-12-21 Accepted 2007-05-21
This work was supported by the Youth Science Foundation of Shanxi Province, China (No. 20031062).
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and trophic supports to neurons, such as forming the blood-brain barrier, maintaining extracellular ion homeostasis, and secreting growth factors, cytokines, and the components of extracelluar matrix[5,6]. Lysophosphatidic acid (LPA) is a phospholipid mediator and is rich in brain[7]. The level of LPA increases significantly following brain injuries[7-9]. As we have demonstrated in rats recently[10] that exposure to LPA at low concentrations promoted the proliferation of NSCs and the differentiation of NSCs into neuronal progenitors, including cholinergic-committed neurons. It is interesting for us to observe the possible effect of LPA on the genesis of two kinds of neuroglial cells from NSCs that might be useful either in establishing *in vitro* resources of neuroglial cells or in predicting the fate of NSCs following their transplantation into the brain in different microenvironments for therapeutic aims.

1 MATERIALS AND METHODS

1.1 *Primary culture of embryonic NSCs and neurosphere passaging*

E14 rat embryos were obtained from timed-pregnant Wistar rats (from the Animal Center of Shanxi Medical University) with the morning of vaginal plug designated as embryonic day 0 (E0). Cultures were produced essentially as described before[10-13]. Briefly, the cortex was dissected out and dissociated. The dispersed cells were plated at 1×10^4 cells/cm^2 in the growth medium composed of 1:1 mixture of DMEM and F12 nutrient (Life Technologies, MD, USA), 20 ng/mL bFGF (Pepro Tech, NY, USA.), 10 ng/mL EGF (Pepro Tech), 0.025 mg/mL insulin (Sigma, USA), and 1×B27 supplement (Gibco-BRL, USA). All cells were incubated at 37 ºC in 95% air-5% CO_2. Cells grew rapidly to form neurospheres. The neurospheres were passaged at least twice to eliminate short-term dividing precursors and bulk cultures could be generated prior to use in further studies.

1.2 *Identification of NSCs by immunocytochemistry*

For identification of NSCs, neurospheres were plated in poly-*L*-lysine-coated glass coverslips, and grew for 1 d in growth medium. Then they were fixed with 4% paraformaldehyde in PBS for 20 min. Primary anti-nestin antibody (1:1 000, mouse monoclonal, BD Biosciences, USA) was applied overnight at 4 ºC, biotinylated secondary IgG (Bioster, China) was applied for 1 h and streptavidin-Cy3 (Bioster) was applied for 30 min. For the purity analysis of NSCs, dissociated cells were then incubated with Hoechst 33342 (Sigma) to visualize the nuclei of all cells other than of NSCs only.

1.3 *Differentiation of neuroglial cells from neurospheres induced by fetal bovine serum(FBS)*

The neurospheres at the third passage were transferred onto 35 mm diameter dishes with poly-*L*-lysine-coated glass coverslips. The culture medium was composed of DMEM/F12, 10% FBS (Gibco-BRL) and compound of interest without FGF-2 and EGF. FBS-induced differentiation[13,14] of NSCs was observed in the following days by immunocytochemistry and Hoechst 33342 staining.

1.4 *Identification of neuroglia by immunocytochemistry*

The double-labeling immunocytochemical procedure was performed as described previously[13] with a few modifications. In brief, cells on coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, and then washed with PBS before permeabilization in 0.4% Triton for 30 min. After washed with PBS, cells were blocked in PBS containing 20% goat serum for 60 min. For double-labeling experiments, the primary antibodies [rabbit monoclonal anti-galactocerebroside (Gal-C) antibody, 1:50, Sigma; and mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody, 1:100, BioGenex, USA] were diluted in PBS containing 10% normal goat serum and 0.3% Triton X-100. Coverslips were incubated for 2 h at 37 ºC and then washed three times with PBS as above. Biotinylated-conjugated secondary antibodies to mouse were added. Cells were incubated for 30 min at 37 ºC. After washed with PBS for 5 min three times, coverslips were incubated with streptavidin-Cy3 and FITC-conjugated goat anti-rabbit (Bioster) for 2 h at 37 ºC. The coverslips were washed again three times. Finally, for unspecific staining of nuclei of all cells in cultures, Hoechst 33342 (1 mg/mL) was added for 15 min at room temperature, followed by two more PBS rinses for 5 min each. A rapid water wash preceded the mounting on glass slides with glycerol. Then, fluorescence was detected with an Olympus BX51 microscope and photographed with Coolsnap CCD of this system. The figures were treated by image analysis software IPP 4.5.

1.5 *RT-PCR for detecting mRNAs encoding LPA receptors*

RT-PCR assays were used for detecting the possible presence of three different LPA receptors in NSCs. Total RNA was isolated from neurospheres at 20 day *in vitro* (DIV) using TRIzol reagent (Gibco-BRL) and quantified spectrophotometry. The synthesis of single strand of three related cDNAs was performed separately. Specific oligonucleotide PCR primers were designed to amplify the

regions of coding sequence. Primer sequences were as follows: LPA_1 (edg-2): 349 bp, 5'-TCT TCT GGG CCA TTT TCA AC-3' (forward), 5'-TGC CTG AAG GTG GCG CTC AT-3' (backward); LPA_2 (edg-4): 805 bp, 5'-CCT ACC TCT TCC TCA TGT TC-3' (forward), 5'-TAA AGG GTG GAG TCC ATC AG-3' (backward); LPA_3 (edg-7): 382 bp, 5'-GGA ATT GCC TCT GCA ACA TCT-3' (forward), 5'-GAG TAG ATG ATG GGG TTC A-3' (backward); β-actin: 750 bp, 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3' (forward), 5'-GAT CTT GAT CTT CAT GGT GCT-3' (backward). Cycle condition consisted of 5 min at 94 ºC, 30 s at 94 ºC, 30 s at 56 ºC, and 2 min at 72 ºC. The amplifications were carried through 35 cycles[16]. RT-PCR kit was from TaKaRa, China.

1.6 *Statistical analysis*

Each experiment repeated at least three times. Data were expressed as percentages of the control (mean±SD). The percentages of neuroglia expressing two different antigens were calculated, respectively, by their number against the total number of cells as obtained from 20 fields (on each coverslip, at least 200 Hoechst 33342-positive cells were counted and the total number was set arbitrarily as 100%). Data were submitted to one-way ANOVA followed by Dunnet's *post hoc* test. Differences were considered statistically significant at $P<0.05$.

2 RESULTS

Twenty-four hours after the cells isolated from the cortex of E14 rat embryo were cultured in growth medium, small-sized neurospheres composed of several cells appeared with many dead cells and cell debris. At 3-4 DIV, the number of neurospheres increased and the size became larger composed of more cells. After two passages (at 15-20 DIV), there were no attaching cells and dead cells but neurospheres with phase-bright suspended in growth medium. By immunocytochemical examination at 20 DIV, cells and neurospheres composed of the cells were strongly positive for nestin, identifying that these cells were NSCs in nature[10,16]. The purity of nestin-positive cells at 20 DIV was about 96%.

Oligodendrocytes (Gal-C-positive) and astrocytes (GFAP-positive) were detected by double-labeling immunocytochemical techniques and counterstained by Hoechst 33342 that revealed the nucleus specificity of all cells on coverslip, and thus the number of oligodendrocytes or astrocytes as well as the total number of cells on coverslips could be counted for calculating the percentages of two kinds of neuroglial cells in cultures under different conditions.

To observe whether LPA affected the emergence of two kinds of neuroglial cells, NSCs were treated with FBS in the presence of vehicle only or 1.0 μmol/L LPA and neuroglial cells were examined by immunocytochemistry in the following days. 1.0 μmol/L of LPA had previously been proved to be the optimal concentration for promoting the differentiation of NSCs into neuronal progenitors[10]. As shown in Table 1, although oligodendrocytes and astrocytes were first seen on the 5th day in both the vehicle and LPA groups, it was obvious that 1.0 μmol/L of LPA exhibited promotive effect on the differentiation of NSCs into oligodendrocytes only. There was a robust increase of oligodendrocytes in LPA group compared to that in the vehicle group as examined on the 5th or 7th day, while there existed only a small but not significant increase of astrocytes in LPA group as examined on the 5th or 7th day. Figures 1 and 2 present representative microphotographs taken on the 7th day, showing that exposure to LPA promoted the generation of oligodendrocytes (Fig.1), but had no effect on the generation of astrocytes (Fig.2).

In addition, we noticed that in Fig.1 the specially-stained oligodendrocytes were stellate in shape with elaborate processes in the absence of LPA (Fig.1*A*), whereas with less processes in the presence of LPA (Fig.1*C*). These changes indicated the possible effect of LPA on the morphology of oligodendrocytes during their differentiation from NSCs.

To further clarify the action of LPA on the differentiation of NSCs into neuroglial cells, different concentrations of LPA (0.01-3.0 μmol/L) were added into 10% FBS-containing culture and the percentage changes of two kinds of neuroglial cells were calculated on the 7th day by immuno-

Table 1. Effect of LPA on the generation of oligodendrocytes and astrocytes from NSCs following addition of fetal bovine serum

	Number of oligodendrocytes			Number of astrocytes		
	4 d	5 d	7 d	4 d	5 d	7 d
Vehicle	0	0.4±0.1	1.7±0.6	0	3.6±0.5	6.6±1.9
1.0 μmol/L LPA	0	2.0±0.7*	6.4±0.8*	0	4.6±1.6	7.2±2.2

Neuroglial cells were counted and averaged from randomly selected 60 microscopic fields of 3 independent cultures. *$P<0.05$ *vs* vehicle. mean±SD, $n=3$.

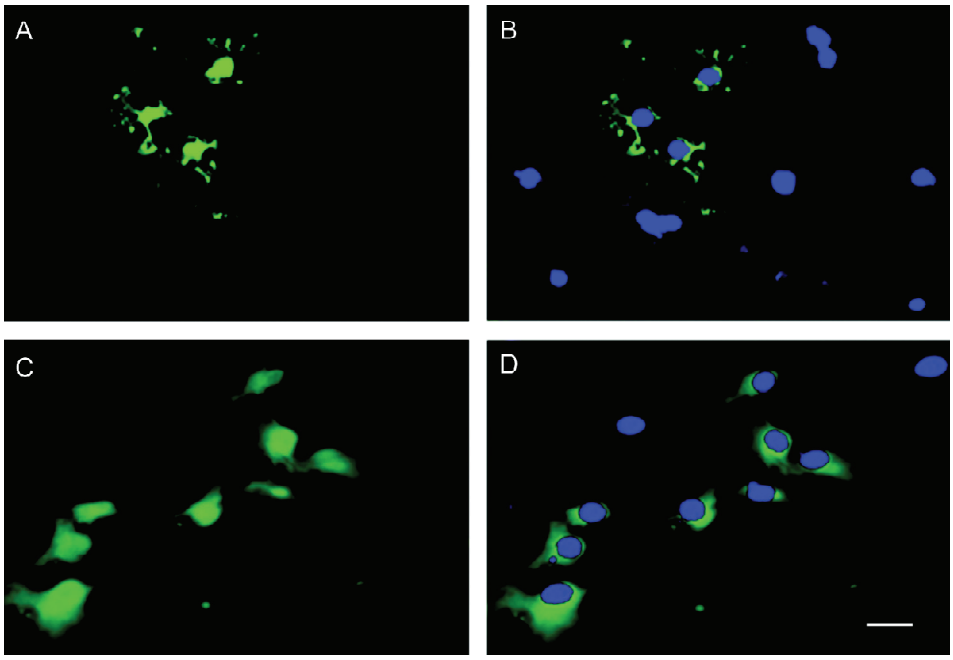

Fig.1. Representative microphotographs showing that LPA promoted the differentiation of NSCs into oligodendrocytes. *A*: Immunocytochemical staining of Gal-C-positive (green-stained) oligodendrocytes 7 d after the culture was treated with FBS and vehicle. *B*: The same field in *A* after it was counterstained by Hoechst 33342, showing the total number of cells (by counting blue-stained nuclei) and the small number of Gal-C-positive oligodendrocytes in the field. *C*: Immunocytochemical staining of Gal-C-positive oligodendrocytes 7 d after the culture was treated with FBS and 1.0 μmol/L LPA. *D*: The same field in *C* after it was counterstained by Hoechst 33342, showing the total number of cells and the increased number of oligodendrocytes compared to that in *B*. Scale bar, 30 μm.

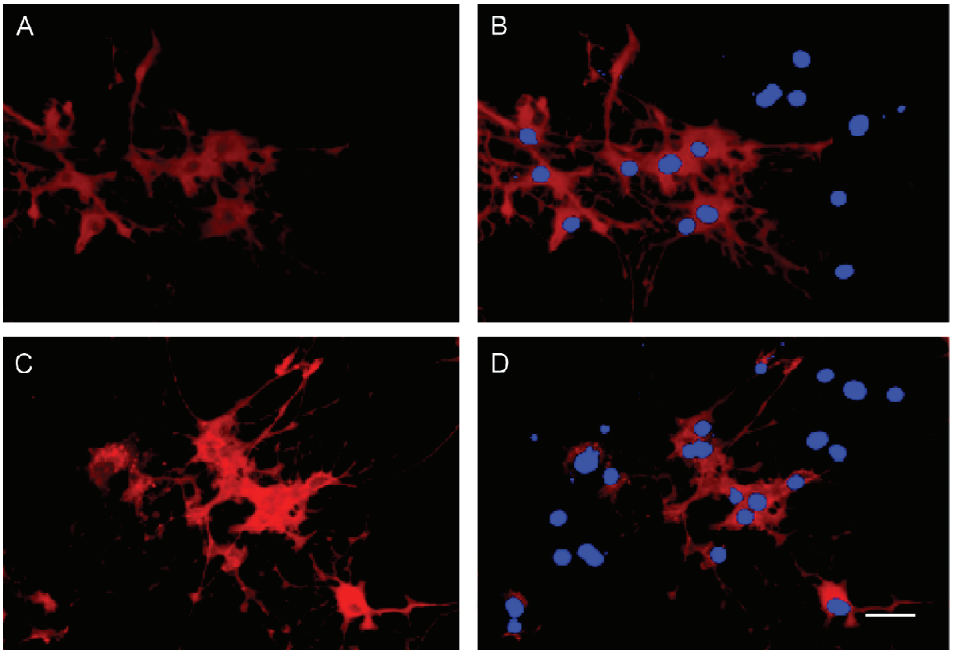

Fig. 2. Representative microphotographs showing that LPA did not affect the differentiation of NSCs into astrocytes. *A*: Immunocytochemical staining of GFAP-positive (red-stained) astrocytes 7 d after the culture was treated with FBS and vehicle. *B*: The same field in *A* after it was counterstained by Hoechst 33342, showing the total number of cells (by counting blue-stained nuclei) and the small number of GFAP-positive astrocytes. *C*: Immunocytochemical staining of GFAP-positive astrocytes 7 d after the culture was treated with FBS and 1.0 μmol/L LPA. *D*: The same field in *C* after it was counterstained by Hoechst 33342, showing the total number of cells in the field and the nearly similar percentage of GFAP-positive astrocytes to that in *B*. Scale bar, 30 μm.

cytochemical techniques. The percentage of Gal-C-positive oligodendrocytes increased dose-dependently when concentration of LPA exceeded 0.05 μmol/L, and peaked at 1.0 μmol/L LPA, showing an increased percentage of 32.6% (P<0.01) of the total cells as compared to that of 8.5% in the vehicle group; then it declined slowly, but was still higher than that in the vehicle group, when LPA at 1.0-3.0 μmol/L was added. On the other hand, different concentrations of LPA had no significant effect on the differentiation of NSCs into GFAP-positive astrocytes, namely, whatever concentration of LPA (0.01-3.0 μmol/L) was used, the percentages of astrocytes were only detected about 37.5% of the total cells in all groups, which were similar to that in the vehicle group.

Figure 4 showed the results obtained from RT-PCR assays for detecting the possible presence of receptor proteins related to the action of LPA in NSCs. Out of three well-known LPA receptors (LPA_1, LPA_2 and LPA_3 receptors), cDNAs against mRNAs related to LPA_1 and LPA_3 receptors were markedly expressed, with a much faint expression of cDNA for LPA_2 receptor in embryonic NSCs.

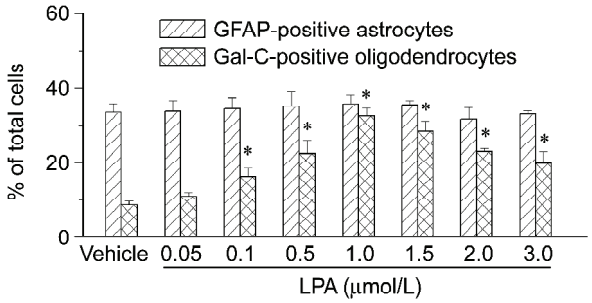

Fig. 3. Percentage changes of oligodendrocytes and astrocytes in total cells (set arbitrarily as 100%). FBS and different concentrations of LPA were added at the same time, then Gal-C-positive oligodendrocytes and GFAP-positive astrocytes were counted 7 d later, respectively, by double-labeling immunocytochemical and Hoechst-staining techniques. $^*P$<0.05 *vs* vehicle.

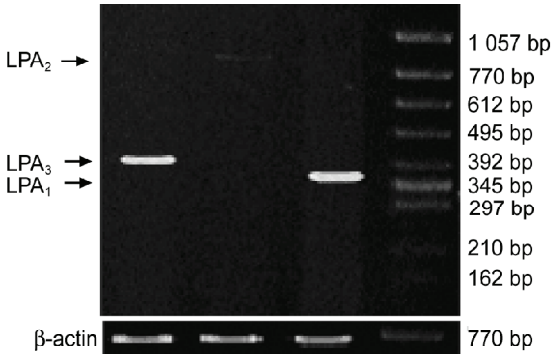

Fig. 4. Expression of mRNAs related to LPA receptors (LPA_1, LPA_2 and LPA_3 receptors) in cultured rat embryonic NSCs by RT-PCR analysis.

3 DISCUSSION

The present study has clearly demonstrated for the first time that LPA at lower concentration promotes FBS-induced differentiation of NSCs into oligodendrocytes *in vitro*. The concentration of 1.0 μmol/L might be the optimal dosage of LPA for promoting the relevant processes. Meanwhile, our results have also shown that LPA seems not, if any, to affect the natural differentiation of NSCs into astrocytes under the similar experimental conditions. Facing these diverse results in respect to two kinds of neuroglial cells, the central problem should be why LPA addition dramatically increases the percentage (and possibly the real number) of oligodendrocytes while has little effect on the differentiation of NSCs into astrocytes. Some investigations *in vitro* carried out on oligodendrocytes from adult or postnatal animals give some helpful clues. Yu *et al*.[17] reported recently that cultured rat cortical oligodendrocytes express mRNAs encoding LPA_1 and LPA_3 receptors and Dawson *et al*.[18] also reported LPA_1 receptor is expressed in oligodendrocyte precursor cells and mature oligodendrocytes in postnatal rat brain. To date, at least three G protein-coupled receptors have been identified for mediating the diverse cellular responses of LPA, named as LPA_1, LPA_2, and LPA_3 receptors in one nomenclature system[19,20]. We have demonstrated that rat embryonic NSCs express LPA_1 and LPA_3 receptors, so it is easily to envisage that the early-developed oligodendrocyte progenitors also possess LPA_1 and LPA_3 receptors as undifferentiated NSCs. Therefore, we would like to propose that LPA might activate these receptors and enhance the differentiation processes of oligodendrocyte progenitors at their early and intermediate stages, and thus accelerate the ripeness and expand the number and percentage of mature oligodendrocytes. Moreover, in Fig.1, the shape of oligodendrocytes also changed in the presence of LPA with a decreased number of processes. This reminds us of a previous report that treatment with platelet-derived growth factor (PDGF) could change the shape of oligodendrocyte progenitor cells into bipolar[21]. It seems that many extracellular active agents might affect the number and morphology during the differentiation of NSCs into neuroglial cells.

It is urgent to explain why the same process was not shown in astrocytes. The possible explanation is that, for some unknown reasons, the newly-developed premature astrocyte progenitors might stop expressing LPA receptors.

Some studies support our feasible proposal. Tigyi *et al*.[15] have reported that there are some cell types, including primary astrocytes, in which LPA is ineffective in altering their proliferation. Weiner *et al*.[22] have indicated that cells expressing vzg-1 (i.e., LPA_1) receptors in ventricular zone of postnatal rat co-express mRNA encoding proteolipid protein, a marker of mature oligodendrocytes, but not GFAP, a marker of astrocytes. These facts imply that astrocytes may lose the ability of expressing LPA receptors during the development from NSCs. At the same time, we have noticed a newly-published report[23], showing that LPA stimulates neuronal differentiation through the LPA_1-$G_{i/o}$ pathway in a neurosphere culture system while it reduces the proportion of oligodendrocytes in neurospheres. Evidently, these problems and contradictions in the experiments await to clarify further.

Taken together, our results suggest that LPA accelerates the differentiation of embryonic NSCs into oligodendrocytes through some related receptor pathways, while it exhibits little effect on the differentiation of embryonic NSCs into astrocytes in the same system.

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