The Rho/ROCK pathway is essential to the expansion, differentiation and morphological rearrangements of human neural stem/progenitor cells induced by lysophosphatidic acid.

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ABSTRACT

We previously reported that lysophosphatidic acid (LPA) inhibits the neuronal differentiation of human embryonic stem cells (hESCs). We extended these studies by analyzing LPA’s effects on the expansion of neural stem/progenitor cells (NS/PCs) derived from hESCs and human induced pluripotent stem cells (iPSCs) and assessed if data obtained on the neural differentiation of hESCs were relevant to iPSCs. We showed that hESCs and iPSCs exhibited comparable mRNA expression profiles of LPA receptors and producing enzymes upon neural differentiation. We demonstrated that LPA inhibited the expansion of NS/PCs of both origins, mainly by increased apoptosis in a Rho/Rho-associated kinase (ROCK)-dependent mechanism. Furthermore, LPA inhibited the neuronal differentiation of iPSCs. Lastly, LPA induced neurite retraction of NS/PC-derived early neurons through Rho/ROCK and is accompanied by myosin light chain phosphorylation. Our data demonstrates the consistency of LPA effects across various sources of human NS/PCs, rendering hESCs and iPSCs valuable models for studying lysophospholipid signaling in human neural cells. Our data also highlights the importance of the Rho/ROCK pathway in human NS/PCs. As LPA levels are increased in the central nervous system (CNS) following injury, LPA-mediated effects on NS/PCs and early neurons could contribute to the poor neurogenesis observed in the CNS following injury.

Keywords: neural stem/progenitor cell; human embryonic stem cell; induced pluripotent stem cell; lysophosphatidic acid; differentiation; Rho pathway.
INTRODUCTION

Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that induces pleiotropic effects in many cell types. LPA mainly acts through binding to its specific G-protein-coupled receptors LPA₁₋₅ which can couple to Gᵢ, Gᵣ, G₁₂, and possibly Gₛ to modulate specific downstream signaling pathways (see (1) for review). LPA can also activate the purinergic receptors LPA₆/P2Y₅ (2), GPR87 (3) and P2Y₁₀ (4), the transient receptor potential vanilloid receptor 1 cation channel, TRPV₁ (5) and the intracellular peroxisome proliferator-activator receptor (PPAR) γ (6). LPA receptors are expressed in various types of stem cells, and demonstrate a differential expression profile across various cells and tissues (7, 8). LPA can be synthetised both intracellularly and extracellularly by activation of different enzymes (1) and it is not yet entirely clear if/how intracellular LPA contributes to extracellular signaling. Although LPA can be synthetised extracellularly by secreted phospholipases A, in particular by the secreted PLA₂ group IIA (sPLA₂), a major source of extracellular LPA in the central nervous system (CNS) most probably arises from the activity of the secreted lysophospholipase D enzyme autotaxin (ATX), as this enzyme is found to be expressed in various CNS regions, during development or adulthood and its activity is modified following various physiopathological events (1). In the CNS, LPA can target most cell types and plays roles in a variety of developmental and pathological processes, including neurogenesis, neuropathic pain, neural injury, schizophrenia, epilepsy and memory impairment (1).

Neural stem/progenitor cells (NS/PCs) have been extensively studied with the aim of using endogenous and/or donor NS/PCs to replace neurons and restore circuitry
in a neurodegenerative microenvironment. In theory, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs) are a great source of cells to generate NS/PCs and progeny, which could potentially be used for transplantation but also to provide insight into human neurogenesis. Although not identical, hESCs and iPSCs seem very similar, but the extent of the variations and similarities between the two types of cells remains open (9). Hence, comparing their differentiation potentials and response to specific signaling molecules is still required to allow drawing conclusion on whether or not hESCs and iPSCs show critical differences. It was previously shown in hESCs that the bone morphogenetic protein inhibitor noggin induces neuroectodermal differentiation, as shown by the expression of SOX2, paired box protein 6, and nestin, and a lack of expression of early mesoderm or endoderm markers (10). Once dissected, these colonies are propagated in suspension in neural basal media (NBM) supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) where they aggregate and form a spherical-like cluster named neurosphere, which consists of a heterogeneous population of NS/PCs (10). Neurospheres can be differentiated to give rise to neurons and glia when plated onto laminin or fibronectin substrates respectively. Hence, this differentiation protocol allows the progressive neural patterning of human pluripotent stem cells (hPSCs, noggin stage), efficient generation and expansion of NS/PCs (neurosphere stage) and subsequent differentiation into early neurons and glial cells (11). Other protocols of differentiation have been established for hESCs (12) including protocols that maintain NS/PCs as a monolayer instead of a neurosphere but are less defined. This overall method allows to precisely divide the whole differentiation process into defined stages and to efficiently generate human neural progenitors and early neurons, rendering this
technique robust and well defined. These features make this protocol highly useful for the study of fundamental signaling mechanisms involved in NS/PC multipotency and expansion. Unraveling these mechanisms may allow for better and more efficient techniques to use human NS/PCs, either endogenous or exogenous, to treat neurodegeneration and during inflammation of the CNS, by characterizing for instance how the cellular environment modifies NS/PC fate, in term of survival and differentiation.

LPA’s effects on NS/PCs and neuroblasts seem to vary depending on the origin of the cells (8). These differences might be the consequences of discrepancies in terms of cell source (different lines and differentiation stages), heterogeneity of cell populations, species, LPA receptor expression profiles, LPA concentration used and the culture conditions of the cell lines. In rodents, LPA was reported to stimulate, inhibit or not affect NS/PC proliferation (13-16). Further, LPA has been shown to be a survival factor, a pro-apoptotic agent or a pro-differentiation factor of NS/PCs (16-18). Comparably, LPA has also been described as a proliferative, survival or pro-differentiation factor in some neuroblasts but not all (8). It was recently shown that LPA can induce fetal hydrocephalus in the mouse, by an aberrant activation of LPA1 on NS/PCs during development (19). LPA also acts through the Rho pathway to induce morphological rearrangements in neuroblasts and neurons (20-24), including actin polymerization (21) which leads to the formation of retraction fibers, neurite retraction (21, 25-32), cell rounding (26, 29, 33, 34), cluster compaction (35-38) and growth cone collapse (21, 26, 27).
The study of LPA in human NS/PCs and neurons is still extremely limited. Although we already briefly reported that LPA inhibits the ability of hESC-derived NS/PCs to form neurospheres, we did not attempt to characterize this biological effect and the signaling pathways associated (39). We also previously showed that when two-weeks-old hESC-derived neurospheres were plated onto laminin or fibronectin, LPA inhibited their neuronal differentiation through the Rho/ROCK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (39). This effect was linked to an anti-differentiation effect of LPA as no modification in apoptosis or proliferation could be detected on these plated neurospheres (39). Hurst and colleagues however reported that LPA stimulates proliferation and cell-rounding of hESC-derived neuroepithelium cell line (NEP), a stable line enriched in hESC-derived NS/PCs and grown under adherent conditions (40, 41). These variations may be due to culture conditions or cell origin.

Here and given the potential differences of hESCs and human iPSCs, we dissected LPA’s effects on the progressive neural differentiation on both types of human pluripotent stem cells (hPSCs), thus allowing us to directly compare LPA signaling in hESCs and human iPSCs. Our differentiation protocol allows us to assess effects of LPA on NS/PCs, during their neural differentiation and on NS/PC-derived neurons. While our previous study concentrated on the impact of LPA on the neuronal and glial differentiation of hESC-derived NS/PCs (39), this current study assessed the effects of LPA at an earlier stage of neuralization, i.e. the expansion of NS/PCs, from both hESCs and human iPSCs. Further, we assessed if the data obtained on the neuronal and glial differentiation of hESCs were relevant to human iPSCs, hence allowing us to draw conclusion on the similarity of LPA’s effects across these two different cell types.
Finally, we assessed LPA’s effects on the morphology of early human neurons derived from NS/PCs. This study thus provides a comprehensive assessment of the role of LPA in these various differentiation stages on hESCs and human iPSCs. As LPA is released upon inflammation and is involved in neurotrauma and various CNS diseases (1), appreciating its role on neurogenesis and understanding its impact specifically on NS/PCs and progeny is relevant to transplantation work. LPA might be the environmental cue that is able to modify the behaviour of NS/PCs and their derivatives during inflammation after neurotrauma.
MATERIALS AND METHODS

Ethics. All experiments were approved by the Human Research Ethics committees of the University of Melbourne (Approvals 0605017 and 0830010).

Reagents. Dilutions of LPA (Sigma-Aldrich, Castle Hill, Australia) were made in 0.1% fatty acid-free bovine serum albumin (BSA, final concentration, 0.01% BSA; Sigma-Aldrich). Cells were treated with LPA (up to 10 µM), LPA₁/LPA₃ antagonists Ki16425 (Sigma-Aldrich, 10 µM), LY294002 (Promega, 10 µM), Y27632 (Biomol, 1 µM), GW9662 (Cayman Chemicals, Ann Arbor, 1 µM), cell permeable C3 Transferase (Cytoskeleton, 1 ng/mL, pretreatment 4 hours prior to LPA), Pertussis toxin (PTX, Biomol, 10 ng/mL, pretreatment 18 hours). Unless otherwise specified, inhibitors were added to cells 30 min prior to addition of LPA to the culture medium on the first incubation.

Reverse transcription (RT) - quantitative real time polymerase chain reaction (PCR). mRNA were isolated from undifferentiated hPSCs, noggin-treated hPSCs, NS/PCs in neurospheres and monolayers of NS/PCs using Dynabeads Oligo (dT)25 (Dynabeads®). RT-PCR were conducted using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA), following the manufacturer’s instructions. A negative control (-RT) consisting in the absence of reverse transcriptase was performed to check the absence of genomic DNA. qPCR was carried out using TaqMan Universal master mix (Applied Biosystems, Foster City, CA) and the 7900HT Fast Real-Time PCR system (Applied Biosystems) and TaqMan gene expression assays for LPA₁, LPA₅, and ATX (Applied Biosystems). TaqMan gene expression assays for LPA₁, LPA₅, and ATX were used.
(Hs00173500_m1), LPA2 (Hs00173704_m1), LPA3 (Hs00173857_m1), LPA4 (Hs00271072_s1), LPA5 (Hs01051307_m1), ATX (Hs00196470_m1), PLA2-g group IIA (Hs00179898_m1), ROCK1 (Hs01127699_m1) and ROCK2 (Hs00178154_m1) were used (Applied Biosystems). The relative quantitation was achieved by applying the comparative CT method (ΔΔCT) whereby the mRNA levels were normalized against the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (TaqMan gene expression assay Hs99999905_m1) or beta-actin (Hs99999903_m1) with LPA5 used as the reference. Q-PCR reaction of non-reverse-transcribed sample was also conducted for each Taqman probe above to check the genomic DNA contamination in the sample and the specificity of the probes.

**Immunofluorescence.** Following fixation with PFA 4% for 10 minutes, cells were blocked in a 10% fetal calf serum-PBS 0.1% tween 20 (PBT, 60 minutes) and immunostained with the following primary antibodies: neurons with mouse anti- βIII-tubulin (Millipore); glial with rabbit anti glutamate aspartate transporter (GLAST, Millipore); rabbit anti-glial fibrillary acidic protein (GFAP, DAKO); mouse anti-A2B5 (Millipore); rabbit anti-nestin (Millipore); rabbit anti-doublecortin (DCX, Abcam); Mouse Phospho-Myosin Light Chain 2 (Ser19) and Rabbit Phospho-Cofilin (Ser3) (77G2) (Genesearch). Cells were then immunostained with the appropriate conjugated secondary antibodies (Alexa Fluor 555 or 488, Cy3, Molecular probes-Invitrogen). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich). Specificity of the staining was verified by the appropriate negative control immunoglobulin fraction (Fig. 4G, H). For monolayer NS/PCs, cells were permeabilized with PBS-Tween/0.3% Triton X-100 following PFA fixation.
Cell culture and neural induction of hPSCs. The iPS (Foreskin) 4 clone 1 and clone 2, abbreviated iPS1 and iPS2, (42) and the hESC line ENVY (ES Cell International) were cultured as previously described (43, 44). Neuronal induction by noggin (500 ng/mL, R&D) was performed as described in (11). Noggin-treated cells were dissected after 14 days and were further subcultured in suspension in NBM together with bFGF (Millipore) and EGF (R&D, 20 ng/mL each) to form neurospheres (45).

NS/PC monolayer culture. The monolayer culture of NS/PCs was generated as previously reported (46) with some modifications. Briefly, two week-old neurospheres cultured with NBM supplemented with bFGF and EGF were collected and dissociated by disaggregation with Trysin-EDTA (Invitrogen). The enzymatic reaction was stopped using soybean trypsin inhibitor (Sigma). The dissociated cells were seeded in NBM supplemented with growth factors onto pre-coated tissue culture dishes with laminin 1µg/cm² (Sigma-Aldrich) to generate a monolayer of NS/PCs. The medium was changed every second day. NS/PCs were further passaged with accutase (Sigma). Medium was changed every second day and cells were cultured for 3 weeks. All experiments were consistently conducted on less than passage 5 monolayered NS/PCs.

Neurosphere formation assay (39). Noggin-treated cells were harvested after 14 days by dissecting and further subcultured as neurospheres, in suspension in NBM together with bFGF and EGF (20 ng/mL each), in the presence or in the absence of LPA (complete with any inhibitor used) with medium change every second day. Each condition was performed in at least 8 individual wells and repeated in at least 3
independent experiments. Assessment of neurosphere formation was performed after 7 days.

**Neuronal and glial differentiation of neurosphere** (39). After 2 weeks in suspension, neurospheres cultivated in NBM with bFGF and EGF were plated as previously described (11) onto poly-l-lysine/laminin (Sigma, 10 and 5 µg/mL respectively) or poly-l-lysine/fibronectin (Millipore, 10 µg/mL) -coated dishes in NBM lacking growth factors to induce neuronal and glial differentiation respectively, allowed to attach and incubated in the presence or in the absence of LPA (complete with any inhibitor used) for 5 days. For each type of experiment, after attachment, medium was changed every second day. Pictures were taken of each individual well (inverted microscope Olympus IX71 and CellIR software) following 5 days in culture. The cells were then fixed with 4% paraformaldehyde in phosphate buffered saline (PFA/PBS).

**Quantification of neuron-forming spheres.** (39) Quantification was performed by counting the number of spheres from which neuronal outgrowth was observable. In some cases neurospheres failed to attach, independently of the treatments, and these floating neurospheres were excluded from quantification.

**Cell morphology assays.** 6-7 day neurospheres cultured on poly-l-lysine/laminin coated slides were placed on the heated stage of an inverted microscope (Olympus) equipped with phase-contrast optics and temperature control. During time-lapse recording, the plated neurospheres were maintained in 25mM HEPES-buffered NBM (pH 7.4) at 37°C and observed continuously using a camera connected to time-lapse software, Axiovision.
(Carl Zeiss, Jena, Germany). Various concentrations of LPA and/or selected inhibitors were applied during time-lapse recording. To observe reversibility of LPA’s effect, LPA was withdrawn from the culture medium and replaced by normal 25mM HEPES-buffered complete medium, followed by time-lapse recording as indicated times. Images were acquired using 5-second interframe intervals. The pictures were then assessed for morphological modifications of plated neurosphere and neurite retraction. At least 3 independent experiments were performed for each treatment.

**Apoptosis and proliferation assays.** Cell apoptosis was quantified by measuring numbers of condensed nuclei with terminal transferase dUTP nick end labeling (TUNEL) immunocytochemistry. TUNEL analysis was performed using the In Situ Cell Death Detection Kit (Roche) following the manufacturer’s instruction. Proliferation was assessed by staining with Ki67 (Clone SP6, Thermo Scientific). Briefly, 7-day dissociated neurospheres were collected, manually dissociated, centrifuged onto glass slides (4 min at 1000 rpm, Shandon Cytospin 4, ThermoFisher Scientific), air dried, fixed with 4% PFA and permeabilized with 0.1% Triton X-100 before immunostained with a TMR Red-conjugated TdT enzyme, or Ki67 respectively. Apoptosis and proliferation were also assessed on laminin-plated 2-week old neurospheres treated with or without LPA (10 µM, 18 hours) as described in (39). Cell nuclei were counterstained with DAPI. Specificity of the staining was verified by the absence of staining in negative controls without the TdT enzyme or negative isotype. Apoptosis and proliferation were respectively quantified by manually counting TUNEL positive cells and Ki67 positive cells as a percentage of total cell number, which were counting at least 1000 cells per treatment by using Image J (NIH software).
**RhoA activation assay.** Active RhoA was measured using the G-LISA RhoA activation assay biochem kit (colorimetric assay; Cytoskeleton, Inc.) according to the manufacturer's instructions. Briefly, monolayered NS/PCs were cultured for 2 days in NBM supplemented with bFGF and EGF (20 ng/mL) until they reached 30-50% confluency. Concentration of bFGF and EGF was reduced to 10 ng/mL for 1 day and then removed overnight prior to treatment. Cells were treated or not with LPA 10 µM for 1, 3, 5, 15, 30 minutes. Following treatments, cells were rinsed twice with cold PBS, rapidly scraped and lysed in a cold premixed lysis buffer with protease inhibitor on ice and centrifuged (1000 g, 4°C, 1 minute). Supernatants were collected and snap-frozen in liquid nitrogen. Some aliquots were taken for protein concentration measurement. Following adjustment of protein concentration, G-LISA was then processed according to manufacturer’s kit instruction. The optical density (OD) was read at 490 nm using a 96-well microplate reader (Biorad).

**NS/PC monolayer differentiation.** To induce neuronal and astrocytic differentiations, the monolayer NS/PCs were re-plated onto poly-l-ornithine/laminin treated wells at 1-5 × 10⁴ cells/cm². Medium was changed every second day and cells were cultured for 3 weeks.

**LPA treatment in the maintenance of monolayer NS/PCs culture.** NS/PCs were seeded onto laminin pre-coated 24 well in NBM supplemented with growth factor (bFGF and EGF, 20 ng/mL each). After 2 days, medium was changed and supplemented with LPA and fixed in PFA 4% 18 hours later.
Cell morphology assays of the differentiated neurons derived from monolayer NS/PCs culture. Neurons cultured for 3 weeks on poly-l-lysine/laminin coated slides were placed on the heated stage of an inverted microscope (Olympus) equipped with phase-contrast optics and temperature control. During time-lapse recording, the plated neurons were maintained in 25 mM HEPES-buffered NBM (pH 7.4) at 37°C and observed continuously using a camera connected to time lapse software, Axiovision, Olympus IX71. LPA at different concentrations (0.1, 1, 10 μM) was applied during time lapse recording. Images were acquired using 5-second interframe intervals.

siRNA knockdown of ROCK. Monolayer NS/PCs were passaged into complete NBM media without antibiotic one day before transfection at 2.5-5x10⁵/well in six-well plates. Knockdown of ROCKI and/or ROCKII were performed using Dharmacon SMART pool ON-TARGETplus ROCK1 siRNA (L-003536-00-0005) and ON-TARGETplus ROCK2 siRNA (L-004610-00-0005) which were already demonstrated to be specific in hESC (47). Control for transfection was done using ON-TARGETplus Non-Targeting Pool (D-001810-10-05). Specific siRNA (25 nM) for each pool was mixed with Dharmafect II, following Dharmacon siRNA Transfection’s protocol. Measurement of knockdown efficiency and survival were respectively performed at 48 hours and 72-96 hours following transfection. Quantification of ROCKI and ROCKII mRNA level were determined by qPCR. Expression level of corresponding genes were normalized to the housekeeping gene beta-actin and expressed as the percentage level over the control. At 48 hours post transfection, cells were passaged onto laminin-coated chamber slides. 72 hours post transfection, LPA (10 μM) was added for 18 hours prior to TUNEL assay.
**Statistical analysis.** All sets of experiments were performed at least three times in triplicates, unless specified (n refers to the number of independent experiments performed on different cell cultures). Data-sets were expressed as mean ± standard error of the mean (SEM). Significance of the differences was evaluated using the t-test or the one and two-way ANOVA followed by the Newman-Keuls test for multiple comparisons. Statistical significance was established at *p< 0.05, **p< 0.01 and ***p< 0.001.
RESULTS

Neural differentiated hPSCs express LPA_{1-5} and autotaxin mRNA.

We performed qPCR analysis of hPSCs at the different stages of progressive neural differentiation (undifferentiated cells, noggin–treated cells and neurospheres) to characterize their expression profile (Fig. 1). All of the undifferentiated and the differentiated hPSCs expressed LPA_{1-5}, ATX and sPLA_{2} mRNA (Fig. 1), with LPA_{2} and LPA_{4} mRNA being the most abundant. LPA_{5} mRNA was expressed at very low levels in neurospheres obtained from all lines relative to LPA_{1-4} (Fig. 1A-C). To examine the expression profile of LPA receptors, ATX and sPLA_{2} at each differentiation stage, the mRNA expression levels of each gene were presented in comparison to their corresponding levels in undifferentiated hPSCs (Fig. 1D-F). Fig. 1D provides an illustration of the data obtained with iPS1. Temporal upregulation of LPA_{1} mRNA expression was found during early differentiation (noggin-treated stage), followed by a downregulation during later differentiation (neurosphere stage). Similarly, an increase of ATX mRNA expression was observed in both noggin-treated cells and neurospheres. LPA_{3} and LPA_{5} mRNA were down regulated upon neural differentiation and no significant modulation was observed for LPA_{2} and LPA_{4}. Very low levels of sPLA_{2} were observed at all stages of differentiation. Similar trends were observed in the other lines tested (Fig. 1E-F). Given the similar trend of expression of LPA_{1-5}, ATX and sPLA_{2} mRNA in neurospheres from the two clones of iPSCs, we assessed most biological effects of LPA in iPS1 and compared with hESCs.

LPA inhibits neurosphere formation through activation of the Rho/ROCK pathway.
We previously reported that one dose of LPA (10 µM) significantly inhibits neurosphere formation of hESC-derived NS/PCs, without further description of this effect (39). Here, we observed a similar effect in two clones of iPSCs and characterize this in both iPSCs and hESCs (Fig. 2, 3). Interestingly, LPA-mediated inhibition of neurosphere formation is dose-dependent in iPSC1 and hESCs and shows a similar trend in iPSC2 although not statistically significant (Fig. 2C, Fig 3A, F). As exemplified in Fig. 2A-C in iPSC1, LPA strongly inhibited sphere formation in a dose-dependant manner (LPA (10 µM): 10.4 ± 3.8 % of sphere formation compared to control: 59.4 ± 7.5 %, n>3, p<0.001, Fig. 2A-C). This effect was associated with a decrease in proliferation (36.11 ± 10.1 % of Ki67 positive cells in LPA-treated cells versus 64.67 ± 5.84% in control conditions, n>3, p<0.01, Fig. 2C-D) as well as an increase in apoptosis (LPA: 69.86 ± 9.1% of cells were TUNEL positive versus 40.03 ± 5.9 % in control, n>3, p<0.01, Fig. 2D). A similar trend was observed in hESCs, although the decreased proliferation was not found to be statistically significant (Fig. 3J).

Quantifying neurosphere formation frequency does not discriminate between apoptosis and proliferation but provides a reliable and robust measurement of NS/PC expansion and was used here to identify the signaling mechanisms used by LPA during this process. The impact of LPA on neurosphere formation was found not to be mainly through its extracellular receptors LPA₁/₃, as pre-treatment with Ki16425, at a dose that is specifically antagonistic to LPA₁/₃ (48), did not modify LPA-mediated inhibition of sphere formation in the iPSCs but significantly reduced the effect of LPA on hESC sphere formation (Fig. 2E, Fig. 3B, G). It is likely that the blockage of these receptors is compensated by the other LPA receptors present in NS/PCs. Due to the lack of
commercially available and specific LPA receptor antagonists, the involvement of the other receptors was not assessed in this study. Further, we decided not to use siRNA in these experiments because of the poor transfection efficiency due to the 3-dimensional structure of the sphere. Instead, siRNA knockdown of ROCKI/II were performed on monolayered NS/PCs as explained below. In the presence of the selective PPARγ antagonist GW9662 (49), the effect of LPA was not modified, suggesting that LPA does not act through PPARγ to inhibit neurosphere formation (Fig. 2F).

In all cell lines, the effect of LPA on sphere formation was abolished by pre-treatment with either the specific Rho inhibitor C3 exoenzyme (50) (Fig. 2G, Fig. 3C, H), or with the p160 ROCK inhibitor Y27632 (used at a dose specific to p160ROCK inhibition (51), Fig. 2H, Fig. 3D, F) which alone have no marked effect, indicating that LPA acts through the Rho/ROCK pathway to inhibit neurosphere formation. Furthermore when cells were incubated with PTX, which ADP-ribosylates αi proteins, LPA’s effect was maintained (Fig. 2I, Fig. 3E, I), hence suggesting that LPA’s effect is not G_{i/o} mediated and is consistent with a G_{12/13} -Rho mediated mechanism. This data was confirmed by measuring apoptosis and proliferation of NS/PCs in the presence of LPA and Y27632 (Fig. 2D, Fig. 3J). The sole application of Y27632 did not modify basal proliferation or apoptosis (Fig. 2D, Fig. 3J). As shown in Fig. 2 in iPS1, LPA-induced apoptosis and LPA-reduced proliferation were abolished by Y27632 (Fig. 2D). Thus, this data indicates that LPA acts through the Rho/ROCK pathway to inhibit neurosphere formation, at least by increasing cells apoptosis and also by decreasing proliferation in iPS1.
**LPA induces RhoA activation**

To confirm that LPA modulated NS/PC expansion by activation of the Rho/ROCK pathway, we measured Rho activity in NS/PCs by using an adherent culture of human NS/PC-derived from dissociated neurospheres. This protocol was favored over spheres as the monolayer NS/PC culture ensures an even exposure of LPA to all cells at the same time, which cannot be controlled in 3-dimensional neurospheres. The adherent culture expressed NS/PC markers (Fig. 4A, B), could be sub-cultured for several passages, reformed neurospheres in suspension culture (Fig. 4C), could be differentiated into neurons and glial cells (Fig. 4D-F) and also express mRNA for LPA receptors and producing enzymes (Fig. 4I). Similar trends in LPA-mediated effects were observed between suspension culture and adherent culture of NS/PCs, thus allowing paralleled conclusions to be made between the two culture systems (Fig. 4J-M). Adherent NS/PCs were cultured in the presence or absence of LPA, followed by Rho activation measurements by ELISA. A basal level of Rho activation was detected on control NS/PCs. As shown in Fig. 4N, LPA induced a rapid increase of active RhoA (GTP-Rho) in NS/PCs, which was biphasic with an elevation that peaked at 1 min post-exposure followed by a sustained but lower activity for at least 30 min. This result directly demonstrates that LPA stimulates Rho in NS/PCs and that this activation critically modulates NS/PC expansion.

To exclude potential off-targets of the ROCK kinase inhibitor Y27632 and despite its high specificity, we further confirmed our results by a molecular approach consisting of knocking down ROCKI and ROCKII by siRNA in monolayered NS/PCs, individually or together (Fig. 4O, P). Following 48 hours post transfection, single siRNA treatment for either ROCKI or ROCKII specifically knocked down their
corresponding gene while their dual knocking down resulted in 75.6 ± 7.0% and 76.2 ± 4.3% downregulation of ROCKI and ROCKII mRNAs (Fig. 4O). When monolayered NS/PCs were knock downed for both ROCKI/ROCKII, the apoptosis induced by LPA was abolished hence demonstrating the involvement of ROCK in LPA’s effect (Fig. 4P).

LPA inhibits the neuronal differentiation of iPSCs through the Rho/ROCK and PI3K/Akt pathways

LPA did not modify glial differentiation of iPSC-derived neurospheres but inhibited their neuronal differentiation (Fig. 5A-G). This effect was dose dependent, ROCK- and PI3K/Akt- dependent (Fig. 5H, I). As shown in Fig. 5I, LPA’s effect on human iPSC-derived NS/PCs was partially abolished by the sole application of Y27632 or LY294002 but was abolished in the presence of both inhibitors. These effects were observed in both iPSC lines tested. As previously observed with hESC-derived neurospheres (39), we confirm here that LPA acts through an inhibition of differentiation, rather than by modifying proliferation or apoptosis of these 2-week old neurospheres. Indeed, neurospheres plated onto laminin in the presence of LPA (10 µM, 18 hours) did not show modification of Ki67 or TUNEL when compared to control conditions (no LPA, Fig. 5J).

LPA induces morphological rearrangements of hPSC-derived early neurons through the Rho/ROCK pathway

After 6 days of plating, neurospheres had already given rise to βIII-tubulin positive early neurons, which radially migrate out from the edges of the neurospheres
(Fig. 6). When incubated with LPA, these early neurons underwent rapid process and neurite retractions leading to cell rounding (occurs within minutes, Fig. 6A-D, Suppl. Video 1). These effects were dose-dependent, starting at 1 µM and reversible (Table 1, Fig. 6E-G). The reversibility took longer (within days) when compared to the rapid retraction observed in the presence of LPA, but suggest that the neurite retraction was not the result of cell death.

LPA-induced morphological rearrangements could be prevented by pre-incubation with C3 exoenzyme or Y27632 (Table 1, Fig. 6H-K, Suppl. Video 1), indicating that LPA acts through the Rho/ROCK pathway to induce neurite retraction in early neurons derived from hPSCs. PTX and LY294002 had no effect on LPA-induced neurite retraction (Table 1), indicating that this mechanism is Gαi and PI3K/Akt independent. Similar data were observed in early neurons derived from monolayered NS/PC cultures (Fig. 4K, L). In order to further elucidate LPA’s role in neural development, we analysed the impact of LPA on the actin-myosin cytoskeleton, assessing coflin and myosin light chain (MLC) respectively, as these proteins are downstream effectors of ROCK (52). These experiments were performed on monolayered NS/PCs, by immunohistochemistry to assess localization of phospho-cofilin and phospho-MLC. As shown in Fig. 6L-O, while we did not observe an effect of LPA on phospho-cofilin, LPA induced the phosphorylation of MLC, hence suggesting that it induces morphological rearrangements through modification of myosin.
DISCUSSION

LPA is bioactive lipid known to affect most cell types of the nervous system. Limited studies have addressed LPA’s role in the human CNS and in human neural cells. We previously described that LPA inhibits the neuronal differentiation of hESC-derived NS/PCs and briefly reported that LPA inhibits neurosphere formation of hESCs (39). Here we established a comprehensive in vitro system to assess the role of LPA at multiple stages of human neural differentiation using both hESCs and human iPSCs. We assessed whether these two different sources of human NS/PCs are equivalent in term of LPA’s effects upon neuralization, by describing $LPA_{1-5}$, $ATX$ and $sPLA_2$ mRNA expression and by assessing if effects previously observed with hESCs were retrieved in human iPSCs. We also characterized how LPA modifies NS/PC expansion and the morphology of early human neurons. We observed a similar pattern of effects of LPA throughout the neural differentiation of hESCs and iPSCs. The three lines tested expressed LPA receptors, $ATX$ and $sPLA_2$ mRNA with a similar profile. In all NS/PCs tested, LPA increases cell death, inhibits neuronal differentiation without modifying glial differentiation. Furthermore, LPA induces morphological rearrangements in early neurons differentiated from NS/PCs. In iPSC-derived NS/PCs, LPA also significantly decreases proliferation, while a similar trend is observed in hESC-derived NS/PCs but without reaching statistical significance. Hence our data shows generally consistent results across different iPSC and hESC lines, with some minor interline differences in responsiveness to LPA, indicating that the mechanisms mediated by LPA are fundamental in neural differentiation and are not influenced by other variables often observed between different hESC/iPSC lines (53).
Our results show that both undifferentiated hPSCs and neural differentiated hPSCs express \( LPA_{1-5} \) mRNA, confirming our previous RT-PCR data (8, 39, 54) and show that \( LPA_2 \) and \( LPA_4 \) are the most abundant mRNA in undifferentiated hPSCs and neurospheres. Modulation in the receptor expression profile is observed following neural differentiation with an upregulation of \( LPA_1 \) mRNA during the early neural commitment of hPSCs (noggin-treated stage), followed by its downregulation during later differentiation (neurosphere stage). The mRNA expression profile in hPSC-derived neurospheres share some similar trends to the profile observed in hESCs derived-NEP (41) and in the monolayer NS/PCs, with \( LPA_{1, 2, 4} \) being the most expressed mRNA. The low level of \( sPLA_2 \) expression and the presence of \( ATX \) mRNA following neural differentiation, as exemplified in this data, is consistent with the fact that \( ATX \) expression is associated with neurogenesis (55).

As \( LPA_1 \) shows its highest expression level at the noggin stage, we assessed its involvement in neurosphere formation, as readout of the impact of LPA on NS/PC expansion. Using the \( LPA_{1/3} \) antagonist Ki16425, we observed no significant effect of this treatment on LPA’s effect in iPSCs and a partial inhibition in hESCs, suggestive that other receptors are involved in the inhibition of NS/PC expansion by LPA. This is consistent with the fact that \( LPA_{2, 4} \) are the most abundant mRNAs in NS/PCs from all cell lines. LPA receptors are coupled to multiple G proteins with \( LPA_{1-5} \) coupled to \( G_q \), \( LPA_{1,2,4,5} \) coupled to \( G_{12} \), \( LPA_{1,4} \) coupled to \( G_i \) and \( LPA_{4,5} \) also coupled to \( G_s \) (1). PTX did not affect LPA’s effect on sphere formation, suggesting that LPA acts independently of \( \alpha_i \), either through \( G_{12} \) and/or \( G_q \) and/or the \( \beta\gamma \) subunits of G proteins. This is highly likely given that the main receptors present either at the noggin stage or neurosphere
stage (LPA$_{2,4>1}$) signal through G$_q$ and G$_{12}$. Additionally, as LPA’s effect on NS/PC expansion is Rho/ROCK mediated, it is also probable that the mechanism is G$_{12}$ dependant (56). Further, we showed that LPA inhibits neuronal differentiation of iPSC-derived NS/PCs through the PI3K/Akt and Rho/ROCK pathways, which are likely to be mediated by $\beta_\gamma$ and G$_{12}$ respectively (56) and which is consistent with our previous data obtained with hESCs (39). Lastly, we describe that LPA induces morphological rearrangements of early neurons in a Rho/ROCK manner, presumably mediated by G$_{12}$. Altogether, this data demonstrates the importance of LPA receptor-mediated signaling along the whole neural differentiation process.

We found that LPA promotes apoptosis of early human NS/PCs, as revealed by neurosphere formation assay, which is consistent with some NS/PC and neuroblast studies performed in rodents (16, 17, 38, 57, 58) but differs with others in which LPA improves cell survival (16, 38) or proliferation (13, 35, 59). In human cells, we previously showed that LPA does not modify apoptosis of older hESC-derived NS/PCs (39) as when two-weeks-old hESC-derived neurospheres plated in condition allowing differentiation were incubated with LPA for 18 hours, no modification in apoptosis or proliferation was detected by TUNEL and BrdU assays respectively (39). Here we confirmed this data using human iPSC-derived neurospheres. Further, others demonstrated that LPA increases growth of hESC-derived NEP (41), an effect observed at a concentration of up to 0.1 µM. In this study, that concentration already inhibited sphere formation but did not significantly affect monolayered NS/PCs. Similarly, LPA inhibits neuronal differentiation but not glial differentiation of human iPSCs which is in agreement with our previous study using hESCs (39) and with data obtained in rodents.
(60); although other studies found opposite effects in various NS/PCs and neuroblasts (18, 38, 58, 59, 61, 62).

Even though using similar protocols of maintenance of NS/PCs as monolayers, it is interesting to note that we observe some variations with data obtained on hESC-derived NEP (41). In particular, as shown in Fig. 4, we did not observe the growth effect of low doses of LPA on monolayers of NS/PCs described by others (41) but demonstrate that LPA induces apoptosis of the monolayer NS/PCs at a dose that was not previously tested in hESC-NEP. Our data, using two different sources of human NS/PCs and two different protocols for maintenance (neurospheres and monolayers), indicates that the observed pro-apoptotic effects of LPA are consistent, regardless of the NS/PC origin. It is thus likely that the variations between our data and those of Hurst and colleagues in term of cell growth at low concentrations of LPA are due to a difference in the populations of human cells studied. In particular, the main difference between the protocols for generating a monolayer of NS/PCs is in the derivation of the NS/PCs themselves. We use dissociated neurospheres to obtain a monolayered culture while the NEP used by Hurst and colleagues were directly obtained from hESCs without going through a neurosphere stage (41, 46). This variation in protocol might account for the discrepancies in LPA-mediated effects between the studies, which could reflect a variation in the developmental state (either more or less restricted) of the human NS/PC populations in use.

LPA has been extensively studied for its prominent effect in inducing cytoskeletal rearrangements. In particular, LPA is shown to induce neurite retraction
and cell rounding through the Rho/ROCK pathway in rodent immortalized neuroblasts and PC12 (26, 63). We observe a similar pattern of morphological changes in early human neurons, which, following exposure to LPA, undergo immediate and rapid retraction, cell rounding and migration to form cell clusters and compaction in a Rho/ROCK dependent manner. Interestingly, in hESC-NEP, LPA induced ROCK-dependent morphological rearrangements but these were observed to be slow, peaking around five hours (41).

The importance of the Rho/ROCK pathway in stem cell survival is now established. The ROCK inhibitor Y27632 significantly increases hESC single cell survival (64, 65) and NS/PCs following neurosphere dissociation (66). Further, upon dissociation to single cells, the Rho/ROCK pathway is activated and responsible for modulating cell-cell contact-induced apoptosis in hESCs (67), NS/PCs (66) and neurons (68), an effect associated to MLC phosphorylation in hESC (47). Moreover, in neurons, Rho/ROCK activation induces apoptotic membrane blebbing by stimulating MLC phosphorylation (68) and has associations to neuronal death following spinal cord injury (69). Other data suggests that ROCK inhibition may not act directly by reducing apoptosis, but rather by desensitizing cells to their environment and thus limiting apoptosis (70).

Our data showing LPA’s activation of RhoA is in line with data obtained by others in other stem and progenitor cells (1) and is the first demonstration in human derived NS/PCs at various stages of differentiation. A large body of evidence show that LPA acts through the Rho pathway to modify cell survival, apoptosis and proliferation.
in diverse types of cells by mediating cell shape/focal adhesion alteration (38, 71, 72). Our current data demonstrates that LPA activates RhoA in human NS/PCs and implicates this pathway in LPA-induced apoptosis, decreased proliferation and, together with the PI3K/Akt pathway, LPA-inhibition of neuronal differentiation of human NS/PCs. Furthermore, activation of this pathway is also essential to LPA-induced morphological rearrangements in human NS/PC-derived early neurons.

Adult NS/PCs are present in the CNS, predominantly in neurogenic regions such as the subventricular zone and hippocampus. They have been reported to migrate to sites of injury and tumours (73), effects likely to be linked to the repair of damaged tissue. Furthermore, evidences suggest that NS/PCs contribute to neurogenesis in the adult mouse and human following stroke (74, 75). Similar data were observed following brain injury in the juvenile rat (76). Following CNS injury, ischemia or events which damage the blood brain barrier, LPA-like activity is increased within the CSF, levels of LPA within the CNS would increase up to 10 µM (77-80) and levels of ATX increase within astrocytes neighbouring a lesion of the adult rat brain (81). Our data using human cells suggests that the presence of LPA in regions of neurogenesis within the CNS may modify NS/PC survival and differentiation. Our data also suggest that high levels of LPA have the ability to be pro-apoptotic on human NS/PCs, to bias their differentiation towards glial cells and to induce neurite retraction and cell rounding of early neurons and thus limit the regenerative responses to injury. This is relevant to endogenous neurogenesis and might explain low levels of neurogenesis observed following trauma, with LPA being a limiting factor of genesis. This finding is also relevant to transplantation of human stem or progenitor cells within the CNS, as the inflammation
present or generated by the transplantation procedure itself may induce increased levels of LPA which could thus limit neurogenesis and repair. Our data also suggest that neurogenesis may be enhanced by blockers of LPA signaling, such as LPA antibodies which we recently demonstrated are able to abolish LPA’s effect on human NS/PCs in vitro and improve neuronal survival and functional recovery following spinal cord injury in vivo (82). Such mechanism has already been proven with sphingosine-1-phosphate, as blocking its signaling enhances endogenous NS/PC migration to injury site following trauma (83).
CONCLUSION

LPA plays a broad role in human NS/PCs and their neural derivatives, regulating expansion, differentiation and morphology. We showed that in hPSC-derived NS/PCs, LPA increases apoptosis, decreases proliferation, inhibits neuronal differentiation and induces rapid morphological rearrangements in early neurons. All these effects are at least Rho/ROCK dependant. We observed that the effects of LPA on NS/PCs are insensitive to PTX treatment, indicating no involvement of \( \alpha_i \) but suggestive of the involvement of \( \alpha_{12}, \alpha_q \) or \( \beta/\gamma \) in these biological effects. LPA-induced activation of Rho/ROCK is likely to be \( G_{12} \) mediated, as this is the common upstream G protein for Rho and has been reported to be involved in the pro-apoptotic effect of LPA through LPA\(_{1,2,4}\) (16). Thus, our data demonstrates the consistency in the effect of LPA across various sources of human NS/PCs, rendering hESCs and human iPSCs valuable in vitro models for studying lysophospholipid signaling in human neural cells. Our data also highlights the importance of the Rho/ROCK pathway in human NS/PC expansion and differentiation. It is thus possible that blocking LPA signaling in vivo, which levels increase following trauma, would enhance neurogenesis.

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AUTHOR CONTRIBUTIONS. F.F. & A.P.: concept and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; D.C., M.D. & Y.G: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript.
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FIGURE LEGENDS

Figure 1. \( LPA_{1-5}, ATX \) and \( sPLA_2 \) gene expression profile in neural differentiated hPSCs. (A-C) mRNA expression (\( 2^{-\Delta\Delta Ct} \)) profile of \( LPA_{1-5}, ATX \) in neurosphere relative to LPAs, in iPS1 (A), iPS2 (B) and hESCs (C). (D-F) mRNA profile of undifferentiated and progressively differentiated into NS/PCs (noggin-treated and neurosphere) relative to undifferentiated iPS1 (D), iPS2 (E) or hESCs (F). The mRNA expression levels were normalized against the level of \( GAPDH \) mRNA (\( \Delta Ct \)) with the level of \( LPA_{5} \) (A-C) or \( LPA_{1-5}, ATX \) and \( sPLA_2 \) of the undifferentiated hPSCs (D-F) used as the reference genes (\( \Delta\Delta Ct \)). Data were obtained from at least three independent experiments and expressed as means \( \pm \) SEM of triplicate of each sample. The statistical analysis was established by one-way ANOVA, \( p^*<0.05; **<0.01; ***<0.001 \).

Figure 2. LPA inhibits neurosphere formation of hPSC-derived NS/PCs. Representative images of neurosphere in the absence (A) or presence of LPA (10 \( \mu M \), B) for 7 days. (C) Quantification of neurosphere formation in the absence (Control) or presence LPA at various concentrations. (D) Quantification of proliferation (Ki67) and apoptosis (TUNEL) in neurospheres treated or not (Control) with LPA (10 \( \mu M \)) and/or Y27632 (1 \( \mu M \)) for 7 days. (E-I) Quantification of neurosphere formation in the absence (Control) or presence of LPA (10 \( \mu M \)) and/or Ki16425 (10 \( \mu M \), E), GW9662 (1 \( \mu M \), F), C3 (1 ng/mL, G), Y27632 (1 \( \mu M \), H), PTX (10 ng/mL, I). The specific inhibitors were pre-incubated as specified in the Materials and Methods prior to LPA addition and maintained in the culture medium for the entire differentiation period. Data are expressed as Mean \( \pm \) SEM from at least three independent experiments. The
statistical analysis was established by one way-ANOVA analysis (C-I); p *<0.05; **<0.01; ***< 0.001. Data presented were obtained with iPS1. Scale bar: 200 µm.

**Figure 3. LPA inhibits neurosphere formation of iPS2- and hESC-derived NS/PCs.** Quantification of neurosphere formation in the absence (Control) or presence of LPA at various concentration in iPS2 (A-E) and hESCs (F-I), with or without Ki16425 (10 µM, B, G), C3 (1 ng/mL, C, H), Y27632 (1 µM, D, F), PTX (10 ng/mL, E, I). (J) Quantification of proliferation (Ki67) and apoptosis (TUNEL) in hESC-neurospheres treated or not (Control) with LPA (10 µM) and/or Y27632 (1 µM) for 7 days. The specific inhibitors were pre-incubated as specified in the Materials and Methods prior to LPA addition and maintained in the culture medium for the entire differentiation period. Each panel represents a pool of at least three independent experiments and data are expressed as Mean ± SEM. The statistical analysis was established by one way-ANOVA analysis; p *<0.05; **< 0.01; ***< 0.001.

**Figure 4. Characteristics of adherent NS/PC in culture.** (A-F) Representative images of plated NS/PCs, showing brightfield (A) and immunostaining for nestin (red) and DAPI (blue, B). (C) Representative brightfield image of a neurosphere formed from plated NS/PCs. (D-F) Representative immunostainings of NS/PCs differentiated into neurons with βIII-tubulin (green, D), DCX (red, E) and glial cells with GFAP (red, F) and DAPI counterstain (blue). (G) Rabbit and (H) mouse negative isotype controls. (I) mRNA expression (2^ ΔΔCt) profile of LPA1-5, ATX and sPLA2 in NS/PCs. For LPA1-5 and ATX, mRNA expression levels were normalized against the level of GAPDH mRNA (ΔCt) with the level of LPA5 used as the reference gene (ΔΔCt), sPLA2 was expressed
compared to undifferentiated cells to show its very low level of expression. (J) Quantification of proliferation (Ki67) and apoptosis (TUNEL) in plated NS/PCs treated or not (Control) with various doses of LPA (0.1 to 10 µM) for 18 hours. (K, L) Representative images of early neurons from monolayered NS/PCs cells prior to treatment (K) and treated with LPA (10 µM) for 20 min (L) showing morphological rearrangements. (A-H, K, L) Data are representative pictures of at least three independent experiments. Scale bars are indicated within each image. (M) Quantification of apoptosis (TUNEL) in plated NS/PCs treated or not (Control) with LPA (10 µM) and/or Y27632 (1 µM) for 18 hours. (N) Time course of activated RhoA (GTP Rho) by LPA measured at 490 nm by ELISA in monolayered NS/PCs. (O) mRNA expression profile of ROCKI and ROCKII following knockdown of ROCKI and/or ROCKII by siRNA for 48 hours. mRNA expression levels were normalized against the level of β-actin mRNA and are expressed as % of control. (P) Quantification of apoptosis (TUNEL) in siRNA control pool- (Control, LPA) or ROCKI and ROCK II-treated- monolayer NS/PCs, subsequently incubated in the absence or presence of LPA (10 µM) for 18 hours. (I, J, M-P) Data were obtained from at least three independent experiments and are expressed as means ± SEM of triplicate of each sample. The statistical analysis was established by one-way ANOVA, p *<0.05; p **< 0.01; ***< 0.001.

**Figure 5.** LPA inhibits neuronal differentiation of human iPSCs without modifying apoptosis and proliferation. (A-C) Immunostaining of glial differentiation of neurospheres plated onto fibronectin and incubated in the presence of LPA (5 days, 10 µM) with GLAST (A), GFAP (B) or A2B5 (C) antibodies with DAPI counterstain
(blue). (D, E) βIII-tubulin immunostaining (red) and DAPI counterstain (blue) of neurospheres plated onto laminin and incubated in the presence of LPA (5 days, 10 µM, D) or in its absence (E). (F) Mouse and (G) rabbit negative isotype controls. Data are representative pictures of at least three independent experiments. Scale bars: A-C, F-G: 20 µm; 3D-E: 100 µm. (H, I) The percentage of neuron-forming neurospheres was quantified in the presence of various concentrations of LPA (H) or in the absence (Control) or presence of LPA (10 µM) and/or Y27632 (1 µM) and/or LY 294002 (10 µM) (I). (J-L) Proliferation and apoptosis by Ki67 and TUNEL quantification respectively of 2-week old neurospheres plated onto laminin in the absence (Control) or in the presence of LPA (10 µM) for 18 hours in iPS1 (J), iPS2 (K) and hESCs (L). (H-L) Data are expressed as Mean ±SEM of at least three independent experiments. The statistical analysis was established by one way-ANOVA analysis (H, I) or t-test (J-L); p *<0.05; **< 0.01; ***< 0.001. (A-J) Data presented were obtained with iPS1.

Figure 6. LPA induces morphological rearrangements in early neurons derived from hPSCs. Representative images of cells prior to treatment (0, A, E) and treated with LPA (10 µM) for 1, 5, and 10 min (1’, 5’, 10’, C-D); or treated with LPA (10 µM) for 10 min (F) and then incubated with normal medium for 2 days (G); or pre-incubated with C3 (1 ng/mL, H) or Y27632 (1 µM, J), prior to addition of LPA (10 µM, I, K) for 10 min. Data presented were obtained with iPS1. Square shape: example of an area showing retractions. Scale bar: 100 µm. (L-O) Representative immunostaining images of early neurons in control or following LPA treatment (10 µM, 15 min) with phospho-cofilin (L, M) or phospho-MLC antibody (N,O) and DAPI counterstain (blue).
### Table 1. Neurite retraction in early neurons derived from hPSCs. LPA’s effect in neurite retraction among tested agents in human NS/PCs.

Early neurons differentiated from hPSCs were incubated or not with LPA and/or Y27632 (1 µM, 30 min pre-incubation), C3 Transferase (1 ng/mL, 4 hours pre-incubation), PTX (10 ng/mL, 18 hours pre-incubation), LY 294002 (10 µM, 30 min pre-incubation). (+): retraction; (-): no retraction. Each treatment assessment is representative from at least three independent experiments.

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<tr>
<th>Tested Agents</th>
<th>Neurite Retraction (+/-)</th>
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<td>Control</td>
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<td>LPA 0.1 µM</td>
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