

Role of Lysophosphatidic Acid on Corneal Nerve Regeneration

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To my dear husband, Mohammad

﴿ إِنَّ مَعَ الْعُسْرِ يُسْرًا ﴾

“Indeed, With Hardship Comes Ease”

Qur'an, Surah Al-Inshirah, verse 6

I said: What about my eyes?

He said: Keep them on the road.

I said: What about my passion?

He said: Keep it burning.

I said: What about my heart?

He said: Tell me what you hold inside it?

I said: Pain and sorrow.

He said: Stay with it, "The wound is the place where the Light enters you."

Jalaluddin Rumi

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Abbreviation

Ach	Acetylcholine
AH	Aqueous Humor
ALS	Amyotrophic Lateral Sclerosis
AMEED	Amniotic Membrane Extract into Eye Drops
AMT	Amniotic Membrane Transplantation
ATP	Adenosine Triphosphate
BAK	Benzalkonium Chloride
BAX	BCL2 Associated X
<i>BCL2</i>	B-cell lymphoma 2
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium ion
CaCCs	Ca ²⁺ -activated Cl ⁻ Channels
CaCl ₂	Calcium chloride
<i>Calca</i>	Calcitonin-related Polypeptide Alpha
cAMP	Cyclic Adenosine Monophosphate
CAS3	Caspase 3
cDNA	Complementary DNA
<i>Cdc42</i>	Cell Division Cycle 42
CGRP	Calcitonin Gene-Related Peptide
CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
<i>CK12</i>	Cytokeratin 12
<i>CK15</i>	Cytokeratin 15
CNS	Central Nervous System
<i>CNTF</i>	Ciliary Neurotrophic Factor
CO ₂	Carbon Dioxide
SC	Schwann Cells
CT	Cycle Threshold
DALK	Anterior Lamellar Keratoplasty
DAPI	4',6-diamidino-2-phenylindole
DED	Dry Eye Disease

DEPC	Diethyl dicarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal Root Ganglion
ECM	Epithelial Cell Medium
EGF	Epidermal Growth Factor
<i>ENPP2</i>	Ectonucleotide Pyrophosphatase/Phosphodiesterase 2
<i>ERK</i>	Extracellular signal-Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
5-FU	5-Fluoro-2'-Deoxyuridine
<i>GAP43</i>	Growth Associated Protein 43
GCL	Ganglion Cell Layer
<i>Gapdh</i>	Glyceraldehyde 3-Phosphate Dehydrogenase
<i>Gdnf</i>	Glial cell-Derived Neurotrophic Factor
G3P	Glycerol-3-Phosphate
GPAT	Glycerol-3-Phosphate Acyltransferase
GPCRs	G-protein-coupled receptors
HCE	Human Corneal Epithelium
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	High Power Field
hLESCs	Human Limbal Epithelial Stem Cells
IGF	Insulin-like Growth Factor
INL	Inner Nuclear Layer
KCl	Potassium chloride
JNK	c-Jun N-terminal kinase
LASIK	Laser in-Situ Keratomileusis
LDL	Low-density Lipoprotein
LPA	Lysophosphatidic Acid
LPAR1–6	Lysophosphatidic Acid Receptor 1 to 6
<i>Lpar2</i> -KD	Lysophosphatidic Acid Receptor2 gene Knockdown
LPC	Lysophosphatidyl-choline

LPE	Lysophosphatidyl-Ethanolamine
LPI	Lysophosphatidyl-Inositol
LPS	Lysophosphatidyl-Serine
MAGK	Monoacylglycerol kinase
<i>Mapk</i>	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MgCl ₂	Magnesium chloride
MMC	Mitomycin-C
MUC	Membrane-bound Mucins
mRNA	messenger Ribonucleic Acid
NaCl	Sodium chloride
<i>Ngf</i>	Nerve Growth Factor
NPY	Neuropeptide Y
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PA	Phosphatidic acid
<i>PAX6</i>	Paired Box 6 gene
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PGAs	Prostaglandin Analogs
PKP	Penetrating Keratoplasty
PLA1/A2	Phospholipase A1 or A2
PNI	Peripheral nerve injuries
PNS	Peripheral Nervous System
<i>PRG</i>	Plasticity-Related Gene
PVR	Proliferative Vitreoretinopathy
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
<i>Pi3k</i>	Phosphatidylinositol-4,5-bisphosphate 3-Kinase
<i>Plc</i>	Phospholipase C
<i>Rac</i>	Ras-Related C3 Botulinum Toxin Substrate
<i>Ras</i>	Rat Sarcoma Virus

<i>RAGs</i>	Regeneration Associated Genes
RGCs	Retinal Ganglion Cells
<i>RhoA</i>	Ras Homolog Family Member A
ROCK1 (2)	Rho-Associated Kinase 1(2)
ROCKi	ROCK inhibitor
ROI	Region of Interest
ROP	Retinopathy of Prematurity
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelial
RPM	Revolutions Per Minute
RT	Room Temperature
SCG	Superior Cervical Ganglion
SCI	Spinal Cord Injury
scr	Scramble siRNA
SEM	Standard Error of the Mean
siRNA	Small Interfering RNA
SP	Substance P
STAT3	Signal Transducer and Activator of Transcription 3
<i>Tac1</i>	Tachykinin Precursor 1
T3	Triiodo-L-thyronine
tSC	terminal Schwann Cells
TE	TRIS-EDTA
TG	Trigeminal Ganglion
TGF- β	Transforming Growth Factor beta
TNF- α	Tumor Necrosis Factor alpha
VIM	Vimentin
VIP	Vasoactive Intestinal Peptide
h	Hours
mm	Mili Meter
ng	Nano Gram
μ m	Micro Miter
°C	Degree Celsius

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Summary

Neurotrophic keratopathy (NK) is a rare, degenerative disease characterized by the loss of corneal innervation, resulting in persistent epithelial defects and corneal ulceration/perforation. Corneal nerves and epithelial cells produce trophic factors and neuropeptides that are critical for maintaining ocular surface homeostasis. Upon nerve damage, these factors are reduced, which disturbs epithelial integrity and can cause persistent epithelial defects, stromal ulceration, scarring, and eventual corneal perforation. Conversely, epithelial breakdown also impairs corneal nerve function, establishing a self-perpetuating vicious cycle. Despite improvements in NK treatment modalities over the last decades, most treatments target the restoration of the tear film, and the symptom, instead of focusing on the pathophysiology of the damaged nerves. Furthermore, the underlying pathology of NK and the pathophysiology of corneal nerve regeneration are not yet clearly understood. To overcome the gap of current therapy strategies, this thesis explores the role of lysophosphatidic acid (LPA), a bioactive phospholipid, in modulating the regeneration of corneal nerves.

LPA has been demonstrated to exert diverse cellular effects via its six G-protein-coupled receptors (LPAR1–6), modulating processes including proliferation, differentiation, neurite retraction, and growth cone collapse. Preliminary data from our lab showed that inhibition of the downstream effector Rho-associated kinase (ROCK) promotes nerve regeneration following corneal injury *in vitro* and *in vivo*. Despite LPA acting upstream of ROCK, it was critical to determine whether LPA has a role in corneal nerve dysfunction or in promoting regeneration, and which specific LPA receptor subtypes are involved.

Primary cultures of dorsal root ganglion (DRG) neurons were used as a model for sensory neurons innervating the cornea. The primary objective of this thesis was to investigate the effect of LPA on neurite outgrowth. In the first experimental setups, it was shown that LPA treatment significantly inhibited neurite outgrowth and reduced fiber length in both injured and non-injured DRG neurons. Moreover, this inhibition was reversed by co-treatment of LPA with a

ROCK inhibitor Y-27632 (ROCKi), which confirmed the involvement of the LPA–ROCK signaling pathway in promoting nerve regeneration. These findings provided the first direct evidence that elevated levels of LPA in the cornea, as found in a range of ocular diseases, can hinder nerve regeneration by activating ROCK.

The second aim of this study was to identify the specific LPA receptors that are responsible for the previously found effect of LPA on nerve regeneration. For this, a small interfering RNA (siRNA) -mediated knockdown approach of each single LPA receptor (LPAR1, 2, and 4) in DRG neurons was performed. Among these siRNA approaches, LPAR2 knockdown (*Lpar2*-KD) was found to significantly promote fiber outgrowth, especially when combined with LPA treatment. Additionally, the peripheral neurons revealed elevated expression levels of neurotrophic factors, including *Calca* (calcitonin-related polypeptide alpha), *Tac1* (Tachykinin Precursor 1), *Gdnf* (glial cell-derived neurotrophic factor), and *Cntf* (ciliary neurotrophic factor), which indicated that *Lpar2*-KD attenuates the inhibitory effect of LPA on neurite regeneration. Knockdown of the other receptors (*Lpar1*-KD and *Lpar4*-KD) produced no significant effects. This suggests that LPAR2 plays a central role in regulating LPA's inhibitory effect on corneal sensory neurons.

To achieve a more physiologically comparable model, the third part of the thesis established a co-culture system involving mouse DRG neurons and primary human limbal epithelial stem cells (hLESCs). This *in vitro* model mimics the interaction of the limbal epithelial cells with the nerve fibers in the cornea. Co-cultures of DRG neurons with healthy hLESCs showed synergistic regenerative effects when treated with ROCKi. Despite this, LPA suppressed the neuronal fiber length again, even in the presence of hLESCs (only at 48h). Interestingly, hLESCs delayed the inhibitory effect of LPA on neurite outgrowth, most notably by maintaining neurite length at 24h. This may be due to their secretion of neurotrophic factors that promote neuronal stability and growth. Notably, *Lpar2*-KD in DRG neurons co-cultured with hLESCs restored regenerative capacity under LPA treatment.

Gene expression profiling of the co-cultured hLESCs with *Lpar2*-KD neurons following injury revealed that not only hLESCs maintain their stem cell nature but also provide trophic factors such as *GDNF*, *NGF*, *CNTF*, and *TAC1* that naturalized LPA-induced growth inhibition, leading to improved nerve fiber regrowth and increased expression of neurotrophic and neurogenic genes compared to neuron monocultures. In contrast, LPA treatment alone did not increase trophic factor expression and even reduced *CALCA* and *TAC1* levels in co-cultured hLESCs.

In conclusion, this study identifies LPA as an inhibitor for corneal nerve regeneration and demonstrates that its negative effects are largely mediated through LPAR2 in sensory neurons. The results provided a novel understanding of the mechanisms by which elevated LPA levels in ocular disease may impair corneal healing. In addition, a target therapy against LPAR2 could be a promising therapeutic approach to facilitate nerve regeneration in NK.

Zusammenfassung

Die neurotrophe Keratopathie (NK) ist eine seltene degenerative Erkrankung, die durch den Verlust der Hornhautinnervation gekennzeichnet ist und zu persistierenden Epitheldefekten sowie zu Ulzerationen bzw. Perforationen der Hornhaut führt. Hornhautnerven und Epithelzellen produzieren trophische Faktoren und Neuropeptide, die für die Aufrechterhaltung der Homöostase der okulären Oberfläche entscheidend sind. Nach einer Nervenschädigung werden diese Faktoren exprimiert, was die epitheliale Integrität stört und persistierende Epitheldefekte, stromale Ulzerationen, Vernarbungen und letztlich eine Hornhautperforation verursachen kann. Umgekehrt beeinträchtigt auch der Verlust der epithelialen Integrität die Funktion der Hornhautnerven, was einen sich selbst verstärkenden Teufelskreis in Gang setzt. Trotz therapeutischer Fortschritte in den letzten Jahrzehnten zielen die meisten Behandlungsansätze auf die Wiederherstellung des Tränenfilms und damit auf die Linderung von Symptomen, ohne die Pathophysiologie der geschädigten Nerven direkt zu adressieren. Zudem sind die zugrunde liegende Pathogenese der NK und die Mechanismen der Regeneration von Hornhautnerven bislang nicht vollständig geklärt.

Zur Schließung dieser Lücke untersucht die vorliegende Arbeit die Rolle der bioaktiven Phospholipidverbindung Lysophosphatidinsäure (LPA) bei der Modulation der Regeneration von Hornhautnerven. LPA entfaltet über ihre sechs G-Protein-gekoppelten Rezeptoren (LPAR1–6) vielfältige zelluläre Effekte, darunter Proliferation, Differenzierung, Neuritenretraktion und Wachstums kollaps von Wachstumskegeln. Vorläufige Daten aus unserem Labor zeigten, dass die Hemmung der nachgeschalteten Rho-assoziierten Kinase (ROCK) die Nervenregeneration nach Hornhautverletzungen *in vitro* und *in vivo* fördert. Da LPA vorgeschaltet von ROCK wirkt, war es entscheidend zu klären, ob LPA zur Schädigung der Hornhautnerven beiträgt oder deren Regeneration unterstützt und welche Rezeptorsubtypen daran beteiligt sind.

Primärkulturen von Dorsalwurzelganglien-(DRG-)Neuronen dienten als Modell für sensorische Neuronen der Hornhaut. Ziel des ersten Experimentteils war es, den Einfluss von LPA auf das Neuritenwachstum zu untersuchen. Dabei zeigte sich, dass LPA sowohl in verletzten als auch in unverletzten DRG-Neuronen das Neuritenwachstum signifikant hemmte und die Faserlänge reduzierte. Diese Hemmung wurde durch die gleichzeitige Behandlung mit LPA und dem ROCK-Inhibitor Y-27632 (ROCKi) aufgehoben, was die Beteiligung des LPA-ROCK-Signalwegs an der Nervenregeneration bestätigte. Diese Ergebnisse liefern den ersten direkten Nachweis dafür, dass erhöhte LPA-Spiegel, wie sie bei verschiedenen Augenerkrankungen auftreten, die Nervenregeneration durch Aktivierung von ROCK behindern können.

Das zweite Ziel dieser Arbeit bestand darin, die spezifischen LPA-Rezeptoren zu identifizieren, die für die hemmende Wirkung von LPA auf die Nervenregeneration verantwortlich sind. Dazu wurde in DRG-Neuronen eine siRNA-vermittelte Knockdown-Strategie für jeden einzelnen LPA-Rezeptor (LPAR1, 2 und 4) durchgeführt. Unter diesen siRNA-Ansätzen zeigte sich, dass der *Lpar2*-Knockdown (*Lpar2*-KD) das Faserauswachsen signifikant förderte, insbesondere in Kombination mit der LPA-Behandlung. Darüber hinaus zeigten die peripheren Neuronen erhöhte Expressionsniveaus neurotropher Faktoren, darunter *Calca* (calcitonin-related polypeptide alpha), *Tac1* (Tachykinin Precursor 1), *Gdnf* (glial cell-derived neurotrophic factor), und *Cntf* (ciliary neurotrophic factor), was darauf hinweist, dass *Lpar2*-KD die hemmende Wirkung von LPA auf die Neuritenregeneration abschwächt. Die Runterregulation der anderen Rezeptoren (*Lpar1*-KD und *Lpar4*-KD) zeigte keine oder nicht signifikante Effekte. Diese Ergebnisse weisen LPAR2 eine Schlüsselrolle bei der LPA-vermittelten Hemmung sensorischer Hornhautneuronen zu.

Um ein physiologisch relevanteres Modell zu etablieren, wurde im dritten Teil der Arbeit ein Kokultur-System aus Maus-DRG-Neuronen und primären humanen limbalen epithelialen Stammzellen (hLESCs) entwickelt. Dieses *in-vitro*-Modell bildet die Interaktion von limbalen

Epithelzellen mit Hornhautnervenfasern nach. Kokulturen von DRG-Neuronen mit gesunden hLESCs zeigten bei Behandlung mit ROCKi synergistische regenerative Effekte. Dennoch hemmte LPA erneut die Faserlänge, selbst in Gegenwart von hLESCs (nach 48h). Auffällig war, dass hLESCs die hemmende Wirkung von LPA verzögerten und das Neuritenwachstum nach 24h stabil hielten. Dies ist vermutlich auf die Sekretion neurotropher Faktoren zurückzuführen, die neuronale Stabilität und Wachstum fördern. Bemerkenswerterweise stellte *Lpar2*-KD in DRG-Neuronen, die mit hLESCs kokultiviert waren, die Regenerationsfähigkeit unter LPA-Behandlung wieder her.

Die Genexpressionsanalyse der hLESCs nach Kokultur mit *Lpar2*-KD-Neuronen und Verletzung zeigte, dass hLESCs ihre Stammzeleigenschaften bewahren und zugleich trophische Faktoren wie *GDNF*, *NGF*, *CNTF* und *TAC1* bereitstellen, die die LPA-bedingte Wachstumshemmung ausgleichen. Dadurch kam es zu einer verbesserten Nervenfaserverregeneration und einer gesteigerten Expression neurotropher bzw. neurogener Gene im Vergleich zu reinen Neuronenkulturen. Im Gegensatz dazu führte die alleinige LPA-Behandlung nicht zu einer vermehrten Expression trophischer Faktoren und senkte sogar *CALCA*- und *TAC1*-Spiegel in den kokultivierten hLESCs.

Zusammenfassend identifiziert diese Arbeit LPA als Inhibitor der Hornhautnervenregeneration und zeigt, dass seine negativen Effekte überwiegend über LPAR2 in sensorischen Neuronen vermittelt werden. Die Ergebnisse liefern neue Einblicke in die Mechanismen, durch die erhöhte LPA-Spiegel bei Augenerkrankungen die Hornhautheilung beeinträchtigen können. Darüber hinaus könnte eine gezielte Hemmung von LPAR2 einen vielversprechenden therapeutischen Ansatz darstellen, um die Nervenregeneration bei NK zu fördern.

1

1. General Introduction

The cornea, the transparent outer layer of the eye, is a specialized tissue that plays a key role in both eye function and health [1]. Although the cornea is an avascular tissue, it receives its nutrition from both the aqueous humor (AH) and the tear film [2, 3]. An extensive network of nerve fibers in the cornea makes it one of the most highly innervated tissues in the human body [4]. Nerves from the trigeminal ganglia (TG) enter the cornea at the limbus area and extend through the corneal stroma. Eventually, they form a compact subbasal plexus located under the corneal epithelium, playing a pivotal role in maintaining corneal sensitivity as well as triggering the tear reflex, such as blinking and tearing in response to irritation [5, 6]. Any injury or malfunction of these nerves can result in decreased sensitivity, impaired healing of the corneal epithelium, or increased susceptibility to infection [7]. Restoring corneal nerve function after an injury still presents an important challenge. Recent research has identified potential therapeutic targets for promoting nerve regeneration through inhibition of the Rho Kinase (ROCK) *in vitro* and *in vivo* [8], or modulation of intracellular signaling such as lysophosphatidic acid receptors (LPARs), especially LPAR2, highlighting the importance of intracellular signaling pathways in corneal nerve repair [9].

1.1 Corneal anatomy and nerve structure

As the cornea is among the most sensitive structures in the human body, nerve fibers originating from the TG are responsible for this sensitivity. Thus, most of the sensible corneal innervation is served via the first trigeminal branch, called the ophthalmic nerve (V1). In some cases, a small part of the inferior cornea can be innervated by the second trigeminal branch, known as the maxillary nerve (V2), which mostly innervates the midface [10, 11]. The third

trigeminal branch innervates the lower face, named the mandibular nerve (V3) (Figure 1) [12–14]. The ophthalmic branch (V1) is responsible not only for corneal sensibility but also carries two reflex arches, which drive the motor regulation of the eyelid closure and the autonomous regulation of the meibomian and lacrimal glands [12, 15].

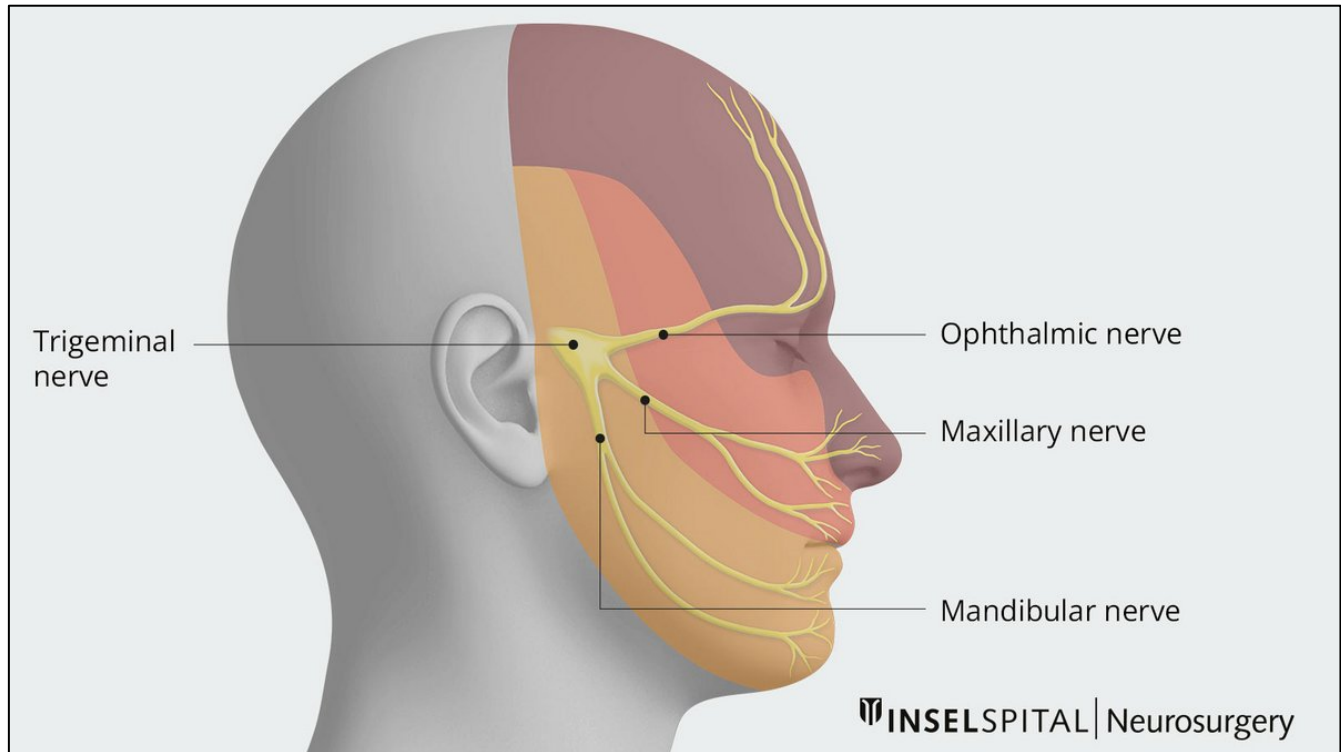


Figure 1: Anatomy of the trigeminal nerves (Cranial Nerves V).

The trigeminal nerve divides into three major branches: the ophthalmic (V1), maxillary (V2), and mandibular (V3) nerves. V1 provides sensory innervation to the forehead, upper eyelid, and cornea via branches like the frontal and lacrimal nerves. V2 innervates the midfacial region, including the cheeks and upper lip. V3 has both sensory and motor functions, innervating the lower face, jaw, and muscles of mastication through branches such as the buccal, lingual, and mylohyoid nerves [14]. The figure adapted from University Department of Neurosurgery, Inselspital Bern, “Trigeminal Neuralgia,” © Inselspital Bern, licensed under [CC BY-NC 4.0](https://creativecommons.org/licenses/by-nc/4.0/) [16].

1.1.1 Overview of corneal anatomy

The cornea has five layers. They include epithelium, the Bowman's membrane, the stroma, the Descemet's membrane, and the endothelium [17]. The tear film is a multi-layered structure on the surface of the eye. It plays an important role in the maintenance of corneal hydration,

surface smoothness, and visual acuity. The tear film is composed of an outer lipid layer (oil), a middle aqueous layer (water), and an inner mucin layer (mucus), each produced by specific glands and cells, and contributes to the preservation of tear stability and protection of the ocular surface and corneal epithelium from desiccation, mechanical stress, and microbial infection [18]. The outer layer of the cornea is made of a stratified squamous, non-keratinized epithelium [6, 19], which can regenerate from human limbal epithelial stem cells (hLESCs) located at the corneal periphery and can regenerate the epithelial surface within the first 12 hours, whereas complete regeneration of the epithelial layer typically requires about 7-14 days, depending on the size of the defect. The epithelium layer is composed of 5-6 cell layers and three different cell types (basal, wing, and superficial squamous cells) that protect the eye and maintain a smooth surface [20]. The epithelium is placed upon an acellular layer of collagen fibers and mucoproteins known as the Bowman's membrane [6]. Corneal stroma (which comprises 90% of the corneal thickness) contains keratocytes that produce extracellular matrix. This extracellular matrix consists predominantly of type I collagen fibers of uniform diameter, which, together with the relative dehydration of the stroma, maintain corneal transparency and homeostasis [13, 21]. The stroma also contains resident immune cells, predominantly dendritic cells (DCs) and macrophages, which maintain immune surveillance [22, 23]. A basal lamina, known as Descemet membrane, which consists of elastic fibers, underlies the inner layer of the cornea [21]. This inner layer is formed by a single layer of tight junction-connected endothelial cells, facilitating an effective diffusion barrier against the AH [6]. The endothelium plays a role in maintaining the hydration of the corneal stroma by aquaporin-1 channels and active transport of ions such as Na^+ , K^+ , and HCO_3^- . This endothelium is not able to regenerate after injury. Defects are compensated by enlargement of the remaining endothelial cells, which leads to endothelial dysfunction and can progress to corneal decompensation [21]. The anatomy of the cornea and tear film is represented in Figure 2.

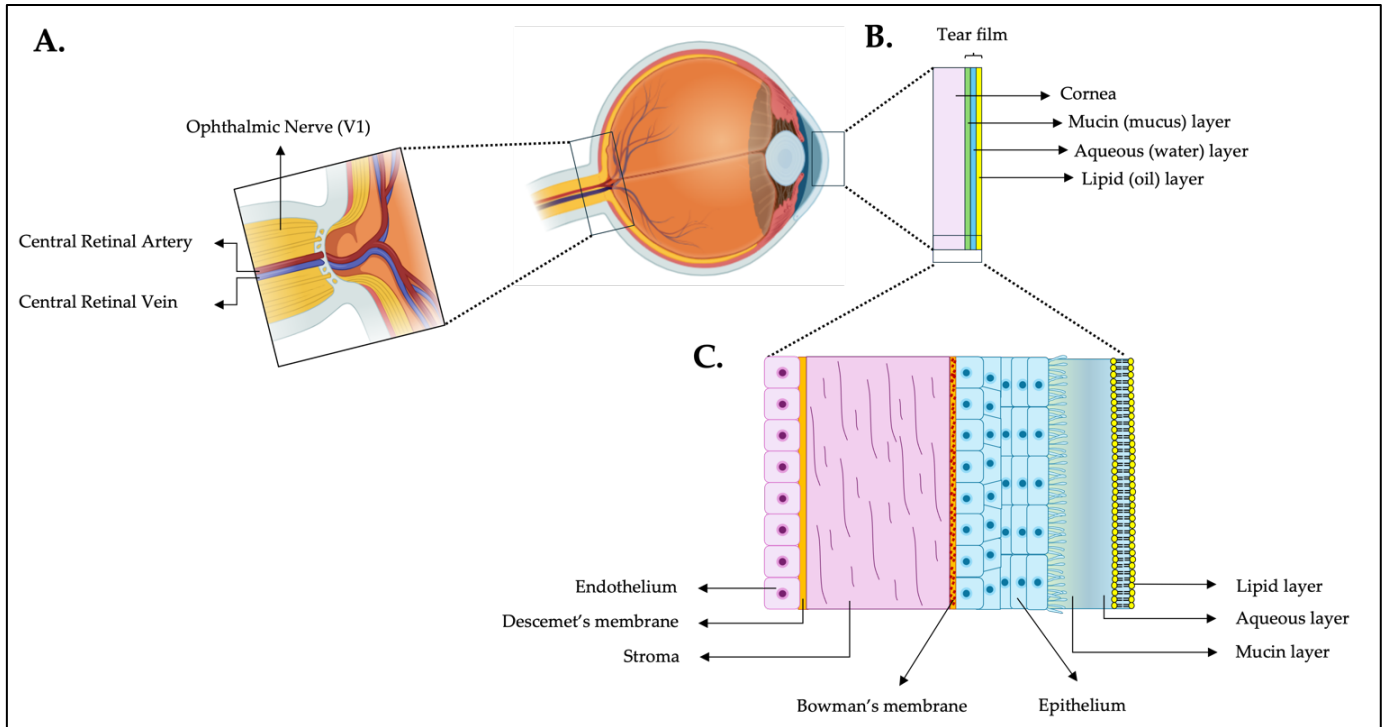


Figure 2: Anatomy of the cornea and tear film.

This illustration shows A) the optic nerve penetrating the sclera at the optic disc and entering the posterior segment of the eye, B) a cross-sectional representation of the eye illustrating the anterior segment, comprising the cornea and tear film, and C) the five layers of the cornea: the epithelium, Bowman's membrane, the stroma, Descemet's membrane, and the endothelium, along with the overlying tear film. The tear film is structured in three distinct layers: the outer lipid layer, the middle aqueous layer, and the inner mucin layer [24]. The figure was created with BioRender.com

1.1.2 Corneal nerves anatomy

The cornea is innervated via sensory and autonomic nerve fibers. From current literature, much more is known about the sensory fibers, while many aspects of corneal autonomic nerves mostly remain unknown [13]. Corneal sensory nerves consist of both myelinated A- δ fibers (20-30%) and unmyelinated C-fibers (70-80%), which lose their myelin sheaths after entering the cornea [25, 26]. Approximately 50-80 nerve bundles (each bundle containing 50-450 axons) enter the cornea around the limbus. The nerve fibers approach the cornea via the middle-peripheral stroma, reaching a depth of 300-400 μm from the surface, penetrating from the limbus in all directions [27]. The nerve bundles are spread all around the entire corneal

periphery at the corneoscleral junction, creating the limbal nerve plexus before entering the stromal tissue [28, 29]. From this stromal network, smaller nerve branches ascend anteriorly into the epithelium, becoming progressively finer as they approach the Bowman's membrane [30]. At the boundary of the Bowman's membrane and the epithelial membrane, stromal nerves create the subbasal nerve plexus [31, 32]. After penetrating the Bowman's membrane, stromal nerve fibers innervate into groups of up to 20 subbasal nerve fibers, which are referred to as "epithelial leashes" [26]. The epithelial leashes show intense anastomosis in the central cornea to form a continuous subbasal network, while less interconnected leashes occur in the peripheral regions [28]. From the subbasal plexus, fine intraepithelial nerve terminals extend vertically into the basal epithelial layer and, to a lesser extent, into superficial epithelial layers. The terminal nerve fiber density has been around 7000-40.000 nerve terminals per mm² in the central cornea of humans [33, 34]. The branching of nerve fibers is not equal in all parts of the cornea, with the highest nerve densities observed in the central cornea (stroma and epithelium), while peripheral regions show decreased nerve density near the limbus [29, 35].

The autonomic innervation of the cornea is via both sympathetic and parasympathetic pathways. Sympathetic fibers originate in the superior cervical ganglion (SCG), which is located near the internal carotid artery at the level of the second and third cervical vertebrae [36]. The sympathetic fibers join the long ciliary nerves directly or travel through the ciliary ganglion to reach the short ciliary nerves before entering the cornea. The parasympathetic innervation of the cornea is not significant, with few fibers arising from the ciliary ganglion, accompanying the ciliary nerves [37]. Both sympathetic and parasympathetic fibers reach the cornea through sensory nerve bundles, which predominate in the anterior one-third of the stroma and the limbal region [38, 39]. Autonomic fibers in both the human and various animal models account for only about 10% of the total corneal innervation [27].

1.1.3 Functions of corneal nerves

Corneal nociceptors consist of C-fibers and A- δ fibers, which are polymodal nociceptors capable of responding to mechanical, chemical, and thermal stimuli. Different types of nociceptors in the cornea are responsible for detecting the surrounding environments of the ocular surface, such as mechanical (mechano-nociceptors), thermal (thermal-nociceptors), and chemical signals (polymodal-nociceptors). Noxious stimulations activate these corneal nociceptors whose cell bodies are located in the trigeminal ganglion (TG) [27]. Another corneal nociceptor, named silent nociceptor, is suggested to be present in the intact cornea, which is not sensitive to mechanical, thermal, and chemical stimuli, however, it reacts to all these stimuli while a local inflammation occurs in the cornea [40, 41]. Sensory nerves also regulate corneal integrity by facilitating the blink reflex, tear production, and secretion, and promoting epithelial cell proliferation, migration, and differentiation, which are pivotal for maintaining corneal function [42]. Corneal nerves interact directly with resident immune cells of the corneal compartments, such as stromal DCs and [43] macrophages [22, 23], and epithelial Langerhans cells, to balance local immune responses to pathogens and injury [23, 44]. Moreover, corneal nerves contribute to this immune privilege by releasing neuropeptides that can suppress the activation of T-cells and other inflammatory cells [45, 46]. Additionally, neuropeptides released by corneal nerves stimulate epithelial and immune cells to increase the secretion of anti-inflammatory cytokines while inhibiting the release of pro-inflammatory cytokines and chemokines [22, 47]. Moreover, corneal nerves communicate with other cell types, such as keratocytes, epithelial cells, and endothelial cells, to maintain the clarity and function of the cornea [27, 48, 49].

1.1.4 Corneal nerves and epithelial interactions

To maintain the health and integrity of corneal epithelial cells as well as the stromal keratocytes, another important function of sensory corneal nerves is their trophic supply via release of

nutrients and multiple trophic neuromediators, which are secreted from some polymodal receptors of the terminal sensory nerve endings. Neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) serve as slow-release neurotransmitters for sensory nerves. They also support corneal homeostasis and overall corneal health by promoting epithelial cell proliferation, migration, adhesion, and differentiation [13, 19, 27, 50, 51]. Trophic support from sensory corneal nerves is highly critical, as the healthy cornea remains an avascular tissue. It is stated that intact corneal nerves continuously release SP and CGRP that help maintain epithelial cell viability and suppress the apoptosis process [52–54]. Conversely, corneal nerves rely on an intact corneal epithelium, as epithelial cells provide (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin 3, 4/5, Epidermal Growth Factor (EGF), and glial cell-derived neurotrophic factor essential for neurite survival and regeneration (GDNF) [6, 19, 55]. Overall, this shows the “epithelial-nerve cross-talk” [19] in which one supports the other and *vice versa* [13]. Regarding corneal epithelial cell mitosis, which is known to increase through sensory nerve support, epinephrine and norepinephrine, as sympathetic neuromediators, seem to decrease it [56, 57]. Thus, in animal studies, a reduction of epithelial changes following sensory nerve lesions was seen when cervical sympathetic nerves were denervated. Moreover, sympathetic nerves seem to be able to hinder sensory nerve reinnervation in the cornea and stimulate stromal inflammation [13].

1.2 Mechanisms of peripheral nerve injury and repair

The peripheral nervous system (PNS) shows excellent regenerative capacity upon nerve injuries, while the central nervous system (CNS) shows limited regenerative potential. The regenerative capacity of PNS relies on the severity of nerve damage, distance from the cell body, and the supportive environment created by Schwann cells (SCs) [58, 59]. There are two main classifications for nerve injury, which were postulated by Seddon in 1943 and Sunderland in 1992 [60, 61]. The Lundborg and Birch & Bonney classifications are also recognized in clinical

and experimental contexts [62, 63]. The classification by Seddon has three subgroups: neurapraxia, axonotmesis, and neurotmesis, while the classification by Sunderland uses a grading system (grades I to V) (Table 1). In neurapraxia (grade I in Sunderland classification), which is caused by myelin injury, the nerves are not able to transmit impulses while the nerve is intact. Neurapraxia nerve injury can recover and return to normal function after weeks to months. However, in axonotmesis (grades II, III, and IV in Sunderland classification), the axon is damaged and the continuity of the axon is lost, but the connective tissue remains. In grade II, while the endoneurium is intact, the recovery of the nerve, depending on the injury, can be expected in months. In grade III, the recovery is expected to be incomplete because the neuronal cell bodies are injured. Grade IV needs some surgery to recover, and there is only a rare chance of recovery because the only intact part is the epineurium. On the contrary, severe nerve injuries, which led to entire nerve disruption, are categorized in the neurotmesis group (Grade V), and most connective tissue is damaged [60, 61].

Table 1: Seddon and Sunderland classification of nerve injuries.

Seddon	Sunderland	Pathological features	Recovery prognosis
Neurapraxia	Grade I	Intact nerve with focal segmental demyelination [60]	Complete recovery (days up to few weeks) without surgical methods [60]
Axonotmesis	Grade II	Damaged axon and myelin (endoneurium, perineurium, and epineurium are intact) [61]	Complete recovery without surgery is possible but slower than grade I (2-3 mm/day) [61]
	Grade III	Axon, myelin sheath, and endoneurium are damaged [61]	Incomplete and slow recovery (1 mm/day), surgical methods maybe needed [61]
	Grade IV	Axon, myelin, endoneurium, and perineurium are damaged [61]	Incomplete and slow recovery, depend on the injury and surgical methos is needed (months up to years) [61]
Neurotmesis	Grade V	Axon, myelin, and all layers (endoneurium, perineurium, epineurium) are damaged (complete nerve transection) [60, 61]	Incomplete and unpredictable recovery with surgical methods [60, 61]

1.2.1 Common Causes of Peripheral Nerve Injury

Peripheral nerve injuries have a variety of causes, but when considering the eye, the focus shifts to damage affecting the trigeminal nerve and its corneal branches. Mechanical trauma, such as accidental damage to the cornea, contact lens, various surgical procedures, including refractive surgery, keratoplasty, and cataract operations, all with the ability to cause damage to corneal nerve fibers [27, 64]. Infections such as herpes simplex keratitis and herpes zoster ophthalmicus directly affect the TG and corneal nerves, often leading to chronic NK [19, 65]. Systemic diseases, such as diabetes mellitus, are another crucial cause that affects corneal nerves and corneal sensibility, and further prevents epithelial wound healing [66, 67]. Inflammatory disorders, including ocular surface inflammation, severe dry eye disease (DED), and ocular graft-versus-host disease, can further induce loss of corneal innervation [68, 69]. Neurotoxic drugs, such as excessive use of topical anesthetics or chemotherapeutic agents systemically, have been shown to induce damage to corneal nerves and worsening NK [50, 56].

1.2.2 Peripheral Nerve Regeneration Mechanisms

Although injured peripheral nerves can regenerate, the rate of axon regeneration is slow, which is estimated at 1-3 mm/day [70]. The capability of axon regeneration decreases along with the distance from the site of injury and time, due to reduced SCs support, which negatively impacts the functionality and quality of recovery [71]. Peripheral nerves are predominantly myelinated, while the smallest fibers remain unmyelinated [72–74]. Proximal damage to the cell body can cause neuronal death, which can often be regenerative in the case of distal injuries [74, 75]. In the distal part of the injury, the nerve stump degenerates to enable the remaining nerve to regenerate from the proximal. This process is referred to as Wallerian Degeneration [73–76]. At the site of injury, several key factors become activated such as cyclic adenosine monophosphate (cAMP), signal transducer and activator of transcription 3 (STAT-3), and extracellular-signal-regulated kinase (ERK). This results in the activation of regeneration associated genes (RAGs),

including GAP-43, neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP). This process is supported by macrophages, which are recruited inside the ganglia after injury [75, 77–82]. Following damage, surviving SCs lose axonal contact and dedifferentiate into a progenitor-like state. Damaged axons secrete neuropeptides such as CGRP and SP, inducing vasodilation of intraneural blood vessels. Simultaneously, SCs close to the damage release monocyte chemoattractant protein-1 (MCP-1), attracting resident macrophages, which start phagocytosing fragmented axonal and myelin leftovers. Macrophages, at the injury site, release vascular endothelial growth factor (VEGF)-A, causing the genesis of new blood vessels. Meanwhile, due to macrophage secretion of IL-1, SCs release NGF, BDNF, CNTF, and GDNF, stimulating the recruitment of new SCs. These recruited SCs are supplied with oxygen and nutrients from the new blood vessels and organize themselves along those vessels in lines called bands of Büngner. Ephrin B-expressing endoneurial fibroblasts trigger the upregulation and membrane localization of N-cadherin on SCs, promoting cell-cell adhesion necessary for band of Büngner. Bands of Büngner leading the regrowing axons from proximal to their target tissue. Axons stimulate themselves via the release of acetylcholine (Ach) and Adenosine Triphosphate (ATP) and are further stimulated by the earlier described neurotrophins, which are released by the SCs. Thus, the regrowing axons enter the bands of Büngner, follow them to the target tissue, and get directed to their definite innervation sites via terminal Schwann Cells (tSC). Following axonal reinnervation of target tissues, SCs differentiate into their mature phenotype, inflammation subsides, and remyelination of the regenerated axons begins [74, 75].

1.2.3 Corneal Nerve Regeneration

When corneal nerve damage is located before they lose their myelin sheath and therefore the SCs support, it is likely that mechanisms similar to those described above will be initiated. If corneal nerve damage results from trauma to the ocular surface, both epithelium and distal corneal nerves, without SCs support, require regeneration [13]. It has been found that the

corneal subbasal nerve plexus is a dynamic structure having potential for regeneration. Different types of ophthalmologic surgery and/or intervention have shown different rates of nerve regeneration [12, 64, 83–86]. For instance, following Penetrating Keratoplasty (PKP), stromal nerves were seen to regenerate 7 months post-surgery, followed by sub-basal reinnervation after 2 years [83, 86]. However, it has been reported that, even 12 years post-surgery, 48% of transplants remained free of sub-basal nerves. Moreover, in some cases, the nerves have been described to grow into the cornea in a disorientated manner, and thus not reaching the corneal epithelium, possibly leading to NK. Laser in-situ keratomileusis (LASIK) causes nerve fiber loss of up to 90%, resulting in insufficient nerve fiber density even 3 years post-surgery [12]. Moreover, complete reinnervation fails to occur even 30 years post-LASIK [12, 87]. Small Incision Lenticule Extraction (SMILE) produces initial corneal nerve damage comparable to LASIK. However, at least in animal studies, nerve regeneration improves by 4 weeks post-intervention [12], and a human study by Liu *et al.* showed better results for SMILE compared with LASIK in nerve regeneration, although neither surgery restored nerve status to normal even at 5.5 years [88]. The factors limiting corneal nerve regeneration after surgical injury are not well characterized and require further investigation.

1.3 Neurotrophic Keratopathy (NK)

A link between trigeminal transection and degenerative alterations of the cornea was first described in 1824 [19]. Until today, the NK has been specified and described several times, with all definitions classifying it as a degenerative disease following impaired corneal innervation [6, 50]. Additionally, in the latest definition, Dua *et al.* emphasized the correlation of impaired trophic and sensory corneal nerve function and the consecutive breakdown of the corneal epithelium, altering the health and integrity of the tear film, the epithelium, and the stroma [19].

1.3.1 Mechanisms of corneal nerve damage

Currently, NK is stated to be a rare condition with 5 or fewer affected per 10.000 people (ORPHA137596). However, current literature suggests that the prevalence of NK often remains underestimated, when occurring as a secondary condition or as the result of various underlying diseases. Another factor for underdiagnosed NK might be linked with the lack of routine testing of corneal sensitivity, which is considered one of the most important diagnostic tools for the recognition of NK [19]. NK can be subdivided into primary and secondary NK depending on etiological factors. Thereby, primary is stated as direct nerve damage, while in secondary NK, nerve damage follows impairment of the ocular surface [19, 89]. Any partial or total damage to the sensory nerves between the sensory cortex and the distal corneal nerve endings can lead to NK. Etiologic factors can be subcategorized into genetic, systemic, central nervous system, ocular, surgery, and other causes [13, 19]. Herpetic keratitis is the leading etiologic factor in developing NK. The second leading factor is neurosurgical surgery in the treatment of trigeminal neuralgia [6, 51]. Nevertheless, there is a lack of epidemiological data concerning the prevalence of NK and its diverse causes. These include systemic diseases (diabetes), medications (beta blockers), genetic disorders (familial dysautonomia), intracranial masses affecting the trigeminal nerve, ocular surface trauma, prolonged contact lens use, and ophthalmological and neurosurgical interventions [19, 90]. Usually, NK is unilateral; however, in systemic conditions, it can be found bilateral [19]. There is evidence that a decreased tear secretion is a consequence of deficient nerve innervation via the corneal sensitive reflex arc, as well as the tear composition concerning growth factors, antimicrobial peptides, cytokines, or ions. At the same time, regardless of the site of nerve damage on the trigeminal tract, the trophic function of the corneal nerve will be compromised. In either case, a lack of trophic factors on the ocular surface disturbs epithelial homeostasis, thereby affecting epithelial cell mitosis, maturation, and centripetal migration from the limbus. Several studies on animals showed a potential alteration of epithelial microvilli morphology, which could potentially contribute to decreased trophic absorption from the tear film. The natural epithelial cell tends towards

hyper-exfoliation, resulting in an accumulation of older cells in the center of the cornea. Those older cells are less capable of maintaining the tear film on the ocular surface, as they secrete a lower amount of membrane-bound mucins (MUC1, MUC4, MUC16) [19, 34, 91, 92]. An impaired corneal epithelium, together with reduced lacrimation, decreases the epithelium's physical resilience, causing physical and mechanical stress on the epithelial cells. This epithelial damage further compromises the already impaired corneal nerves, as epithelial cells normally provide essential neurotrophic factors and growth factors necessary for nerve survival and regeneration. Conversely, the damaged nerves lose their ability to release neuropeptides that support the integrity of the epithelial cells. Especially, the accumulated older epithelial cells at the center of the cornea become more vulnerable to damage during a blink, while the natural epithelial healing capacity becomes progressively insufficient, even in the case of minor damages. The loss of neural-epithelial interactions leads to continuous deterioration, since neither the neural nor the epithelial cells can properly support each other for healing. With continuous epithelial damage, tight junctions and zonula occludens between epithelial cells become disrupted, causing continuous instability in the epithelial cells. This increases the risk of infection. Epithelial cells produce NGF, BDNF, and GDNF, EGF, and insulin-like growth factor 1 (IGF1), that support neural cell survival, axon outgrowth, and neural functionality. On the other hand, corneal sensory fibers produce SP and CGRP which support epithelial cell growth, migration, proliferation, and wound healing. When either the epithelial or neural tissues are injured, the mutual support will become disrupted, causing a cascade effect in the mutual impairment. The lack of epithelial neurotrophic factors will cause progressive degeneration, while the lack of neural trophic factors will cause impairment to the epithelial dysfunction, delayed wound healing, and compromised barrier function. Moreover, the continuous epithelial damage also leads to a decrease in physical/structural support, leaving the nerve not only more vulnerable to damage but also to excessive stimulation [34, 91, 92]. This may contribute to neurogenic inflammation, which causes damage to the adjacent epithelial tissues, thereby initiating a self-reinforcing process [19].

1.3.2 Current treatment approaches and limitations

Despite the progress in treatment approaches for NK over the last decades, most therapies have been aimed at ocular surface homeostasis rather than addressing the underlying pathophysiology of NK. Furthermore, the main underlying mechanisms of NK and the underlying pathology of corneal nerve regeneration need to be understood to develop curative approaches [93]. Current treatments are categorized from conservative to more invasive procedures [56]. NK classification traditionally follows the Mackie classification system, consisting of three stages, and is still the most popular system used in clinical practice [19, 94]. Stage 1 is characterized by epithelial irregularities, punctate keratopathy, corneal edema, superficial stromal scarring, and neovascularization, with the corneal epithelium having a dry and cloudy appearance [56, 94]. Stage 2 is marked by persistent epithelial defects (PEDs), typically oval or circular in shape, most commonly located in the superior cornea, along with possible edema and characteristic smooth, rolled edges [19, 94]. Stage 3 is the most severe form, including corneal ulceration, perforation, and stromal melting [19, 56, 94]. Recently, the Neurotrophic Keratopathy Study Group (NKSG) has proposed a new classification in six stages for better staging accuracy and early diagnosis. The classification contains Stage 1: altered corneal sensation without visible keratopathy, Stage 2: punctate epithelial keratopathy without stromal haze, Stage 3: persistent or recurrent epithelial defects without stromal haze, Stage 4: epitheliopathy or persistent epithelial defects with stromal haze, Stage 5: persistent epithelial defects with corneal ulceration, and Stage 6: corneal perforation [95, 96]. Treatments can be prescribed for NK, depending on the stages classified according to the Mackie classification system, but for artificial tear use, there are no restrictions for any stages [65]. Lubricating eye drops and artificial tear ointments are the primary first-line treatments for the early stages of NK to keep the cornea moist and protect the epithelial surface. Eyelid taping and punctal plugs are reserved for advanced or refractory cases when initial treatments are insufficient [56, 65]. However, eye drops and ointments often cause iatrogenic ocular toxicity due to the presence of benzalkonium chloride (BAK) as a preservative compound. This situation is correlated with

the concentration, frequency, and duration of the drug administration [97]. Topical non-steroidal anti-inflammatory drugs (NSAIDs) can contribute to the development of severe corneal complications particularly corneal melting and perforation, especially in compromised corneas. Therefore, the use of NSAIDs in conditions of corneal hypoxia may not only result in a disappointing therapeutic result but also may lead to paradoxical inflammatory exacerbations [98]. Recently, a research group provided an amniotic membrane extract in eye drops (AMEED) and tested it on patients with chronic DED, limbal stem cell deficiency, and NK. While AMEED was safe and tolerable, the quality of the vision did not improve significantly; however, AMEED reduced severe symptoms such as epithelial defects, de-epithelialization, and corneal ulceration [99].

In stage 2, as the progression of NK is fast, the goal is to prevent the progression of NK to corneal ulcer. Damage or loss of the epithelial layer in the cornea is defined as corneal ulceration [100]. Here, the treatment approaches focus on promoting corneal healing and restoring sensitivity in situations characterized by persistent epithelial defects. Therapeutic contact lenses are often applied to protect the corneal surface, reducing irritation and promoting healing [101]. Blood-derived eye drops (serum, platelet-rich plasma, and plasma) from autologous or homologous sources are another type of approach for stage 2. These eye drops react like natural tears and promote epithelialization through different components such as EGF, fibroblast growth factor (FGF), transforming growth factor beta (TGF- β), NGF, platelet-derived growth factor (PDGF), and IGF. However, such a therapeutic approach has the risk of inducing circulating antibodies/pro-inflammatory mediators in patients [102]. Scleral lenses or soft contact lenses can offer epithelial healing, stabilize corneal thinning, and reduce the risk of corneal perforation [103]. Punctal plugs are an invasive treatment that blocks tear drainage, enhancing both the quality and quantity of the tear film and its retention on the ocular surface. Due to their invasive nature, they are typically reserved for advanced stages of NK when conservative treatments have failed [104, 105]. During epithelial defect, different growth factors such as EGF are required, which contribute to the healing process, although proteins

responsible for degradation, including metalloproteinases, cathepsins, plasminogen, and cytokines, complicate the healing process further [106]. However, after the healing process, the edge of the healing epithelium can become thick and tight, which suppresses the continuous growth of the epithelium. Debridement can enhance epithelial healing by creating a clear edge, thereby enhancing centripetal migration towards the epithelium[107]. Tarsorrhaphy partially or fully closes the eyelids through suturing, thereby reducing tear evaporation and increasing corneal healing. This technique can either be used temporarily by utilizing removable sutures or permanently through lateral canthal sutures. This, in turn, could inhibit the progression of epithelial damage from becoming NK. Partial tarsorrhaphy is beneficial since the vision for the patient is retained, and the risk of infectious keratitis can be reduced in several mechanisms, including decreased corneal exposure to airborne pathogens, improved tear film retention that maintains antimicrobial factors, and reduced epithelial defects that could facilitate bacterial invasion [108]. Botulinum toxin injection into the upper eyelid levator muscle (levator palpebrae superioris) is less invasive than tarsorrhaphy, an alternative approach to inducing temporary eyelid closure in NK patients. Botulinum toxin injection is less invasive in relation to tarsorrhaphy and does not require surgery, offering an alternative for temporary eyelid closure [109].

Stage 3 is the most severe and challenging stage of NK. It is marked by persistent epithelial defects, stromal ulceration, and corneal perforation. Treatment at this stage aims to induce corneal healing and prevent further deterioration of ocular surface integrity. The treatments that can be proposed for this stage include several medications/surgeries [19, 110]. Cenergermin (Oxervate; Dompé, Milan, Italy), a recombinant human nerve growth factor, has emerged as an advanced treatment option for stage 3. It enhances corneal healing by promoting epithelialization and nerve regeneration, and also prevents corneal perforation. The most common side effects associated with the use of cenergermin include eye pain, leading to decreased vision, conjunctival hyperemia, inflammation, tearing, corneal deposition, cataracts, and foreign body sensation [111]. Cenergermin is currently the only non-surgical curative

treatment available. Although cenergermin has shown promising results in corneal epithelial healing and nerve fiber regrowth, it has failed to improve corneal sensitivity significantly in patients (NGF0212/REPARO) [111]. Additionally, the relatively high cost of the treatment and the occurrence of side effects. Moreover, the Federal Joint Committee (Gemeinsamer Bundesausschuss (G-BA)) has withdrawn its approval since 2020 in Germany. Therefore, it is not used in countries like Germany anymore [112]. Amniotic membrane transplantation (AMT) is a simple surgical method that promotes corneal healing in patients with NK by providing anti-inflammatory and regenerative factors [65]. Conjunctival flap is a thin covering layer that prevents stromal lysis by blocking the access of tears, proteolytic enzymes, and proinflammatory factors to the ulcerated part of the cornea [113]. However, several side effects, such as iatrogenic limbal cell deficiency and the impairment of the possibility to measure the intraocular pressure, were described for this treatment [114]. Corneal transplantation, including PKP, lamellar keratoplasty (LK), and deep anterior lamellar keratoplasty (DALK), is applicable in the case of a large perforation size [115]. The cornea's lack of trophic factors leads to a reduction in the success outcome of the corneal transplantation, as corneal grafts are prone to developing ulcerative complications [116]. Corneal neurotization restores corneal sensation by transferring intact donor nerves (a healthy sensory nerve from a different anatomical location within the same patient) to promote the healing procedures of NK. Corneal neurotization provides a long-lasting outcome; however, it requires sophisticated surgical expertise and may not be appropriate for all patients. Additionally, the procedure can result in visible scarring on the forehead, which may be cosmetically unacceptable to some patients [117]. Some other treatments for NK are being evaluated and are in different clinical trial phases, such as varenicline nasal sprays [118, 119], Thymosin beta 4 (T β 4) [120, 121], Nexagon (a connexin43 antisense gel) [122], and ReGeneraTing Agent (RGTA), which have shown promising results in corneal healing [123, 124]. In addition, autologous adipose-derived mesenchymal stem cells have also been investigated more recently to promote corneal healing [125]. Current treatment approaches based on the NK stage are listed below, in Table 2.

Table 2: Current Treatments for NK and their Limitations.

Treatment	Stage	Purpose	Limitation
Artificial tears / Lubricating eye drops/ Ointment	All stages	Corneal moisturization	Preservative toxicity [126, 127]
AMEED	All stages	Reduce epithelial defects	Vision improvement limited [128–130]
Punctal plugs	Stage 1-2	Prevent tear drainage, enhance tear film	Invasive [131, 132]
Anti-inflammatory drops / NSAIDs	Stage 1-2	Reduce inflammation	Risk of corneal complications [133, 134]
Preservative-free drops	Stage 1-2	Avoid ocular toxicity	Limited availability [135, 136]
Therapeutic contact lenses	Stage 2	Protect surface, reduce irritation	Discomfort, risk of infection [137]
Blood-derived eye drops	Stage 2	Promote epithelialization	Risk of pro-inflammatory response [138, 139]
Debridement	Stage 2	Debridement	Requires clinical supervision [140, 141]
Tarsorrhaphy	Stage 2-3	Reduce tear evaporation	Cosmetic, visual obstruction [142, 143]
Botulinum toxin	Stage 2-3	Temporary eyelid closure	Reversible [144, 145]
Cenegermin (Oxervate)	Stage 3	Stimulate nerve regeneration	Eye pain, visual side effects, high cost [111, 146, 147]
Amniotic membrane transplantation	Stage 3	Anti-inflammatory, promote healing	Temporary effect [148, 149]
Conjunctival flap	Stage 3	Protect ulcer site	Limbal deficiency risk [113, 150]
Corneal transplantation	Stage 3	Repair large perforations	Low graft success rate [148, 149, 151]
Corneal neurotization	Stage 3	Restore sensation	Restore sensation [93, 117]
Autologous mesenchymal stem cells	Various	Corneal repair	Corneal repair [125, 152]

1.4 Lysophosphatidic Acid (LPA): Structure and function

The lysophospholipid family is a key group of lipids classified according to the structure of the polar head, and divided into several subclasses, namely, lysophosphatidyl-serine (LPS), lysophosphatidyl-ethanolamine (LPE), lysophosphatidyl-choline (LPC), lysophosphatidyl-inositol (LPI), and LPA [153]. Both cytoplasmic and extracellular lysophospholipids are involved in cellular functions such as migration, proliferation, apoptosis, invasion, and cell cycle progression [154]. LPA, as a bioactive lipid mediator, is an important molecular subgroup belonging to the lysophospholipid family, consisting of a fatty acid molecule, a glycerol, and a phosphate group [155]. Many studies were used to explore the biological significance and expressions of LPA in various cancers, proving that it exerts a promotional action on tumor growth through several proposed mechanisms, such as increasing cell growth, migration, and angiogenesis. Studies have shown that elevated levels of LPA are associated with increased metastatic index and poor prognosis in various cancer types [156–162]. However, LPA function on the ocular surface remains poorly understood and requires further investigation. Additionally, the underlying signaling mechanisms by which LPA is involved in nerve regeneration of the ocular surface are still uninvestigated. Thus, targeting LPA and its receptors suggests a novel therapeutic avenue for ocular surface diseases.

1.4.1 Chemical structure of LPA

LPA is a small glycerophospholipid with a simple bioactive structure consisting of a glycerol backbone, a single fatty acid chain, and a phosphate group [155]. LPA is capable of interacting with different G-protein-coupled receptors (GPCRs) known as LPARs, which comprise six well-defined subtypes (LPAR1-LPAR6) in mammals, each related to various cellular/biological activities. The fatty acid chains can also exhibit variations in the number of carbons, saturation, and unsaturation, for example, 16:0-, 18:0-, 18:1-, 18:2-, and 20:4-LPA, thereby influencing their specific biological activity (Figure 3) [163].

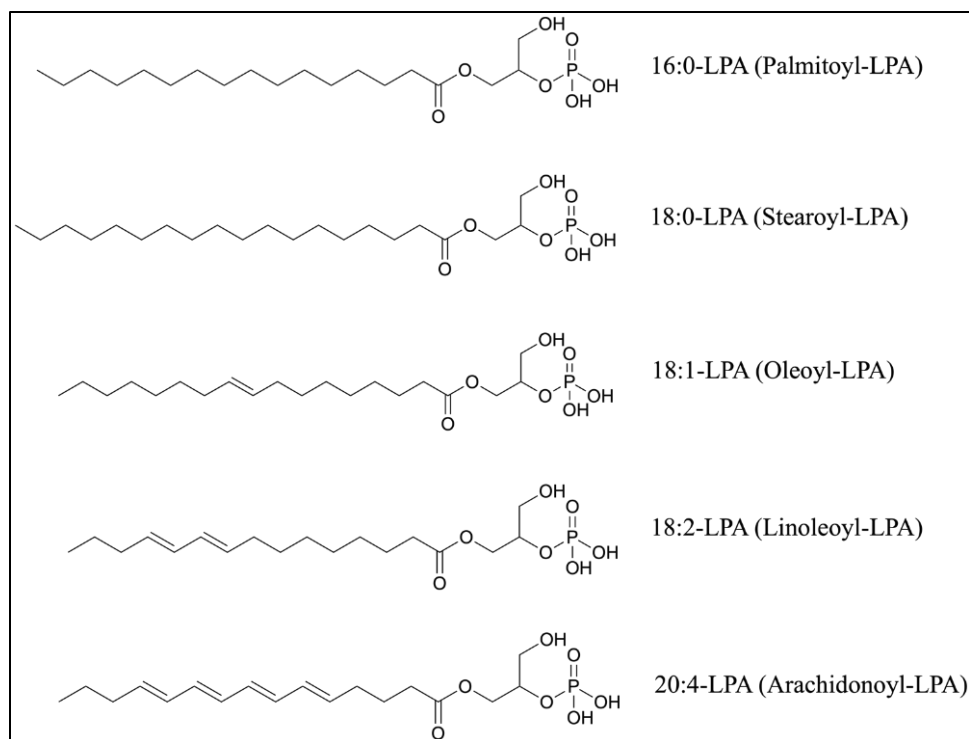


Figure 3: Chemical structures of LPA species.

This figure illustrates five common molecular species of LPA, which differ in their acyl chain composition and levels of unsaturation. These include saturated LPA (LPA 16:0), monounsaturated forms (LPA 18:1 and 20:1), and polyunsaturated forms (LPA 18:2 and 20:4). All share a common glycerol backbone with a phosphate group and a single fatty acid chain at the sn-1 position, which determines their biological activity and receptor specificity [163].

LPA is produced primarily through the extracellular enzymatic activity of autotaxin (ATX, coding by Ectonucleotide Pyrophosphatase/Phosphodiesterase 2 (*Enpp2*)), which converts LPC into LPA [164, 165]. Intracellularly, LPA can also be produced from phosphatidic acid (PA) by phospholipase A1 or A2 (PLA1/A2), or phosphorylation of monoacylglycerol by monoacylglycerol kinase (MAGK). In addition, oxidative modification of low-density lipoprotein (LDL) has been identified as a non-enzymatic source of LPA (Figure 4) [166, 167].

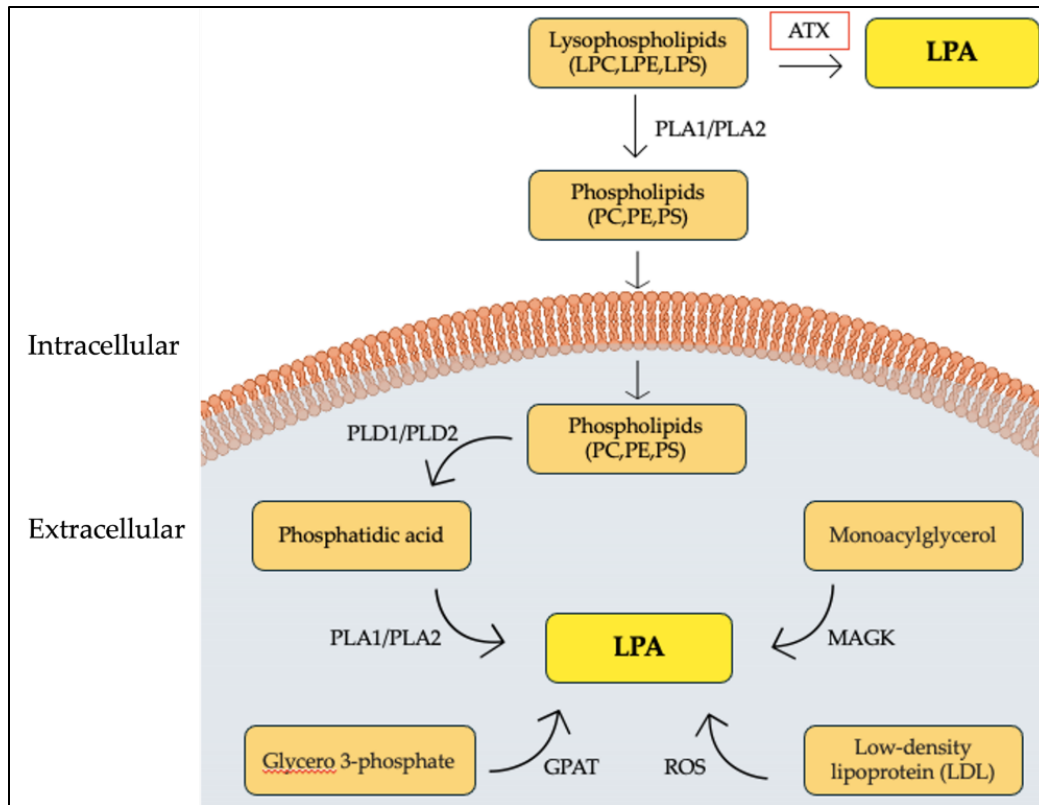


Figure 4: Biosynthesis and metabolic pathways of LPA.

LPA is generated via both extracellular and intracellular pathways by multiple lipid substrates and enzymes. In the extracellular space, lysophospholipids (LPC, LPE, and LPS) are converted into LPA by ATX. These lysophospholipids themselves originate from phospholipids (PC, PE, PS) through the action of phospholipase A1 or A2 (PLA1/PLA2). Intracellularly, phospholipids are hydrolyzed via phospholipase D (PLD1/PLD2) to produce PA, which can be further hydrolyzed into LPA by PLA1/PLA2. Other intracellular sources are the conversion of monoacylglycerol by MAGK, LDL via oxidative reactions, and Glycerol-3-Phosphate (G3P) through Glycerol-3-Phosphate Acyltransferase (GPAT) or reactive oxygen species (ROS) -mediated reactions [166, 167].

Recent research determined novel function for LPA in the pathophysiology of diseases, especially in cancer and fibrosis, since it can modulate cell motility and inflammation [168]. Studies are also determining the development of antagonists of the LPA receptors as therapeutic options for cancer, fibrosis, and cardiovascular diseases [169, 170]. LPAR1-6 are GPCRs that share the common structural features of the GPCR family, including seven transmembrane helices, an extracellular N-terminus, and an intracellular C-terminus [171]. The transmembrane helices are connected via alternating extracellular and intracellular loops,

which play an important role in ligand binding and receptor activation [172]. The interaction between LPA and its receptors initiates conformational changes in these helices, which trigger downstream signaling cascades through interaction with various G-proteins ($G\alpha$, $G\beta\gamma$ subunits). These helices form a binding pocket for LPA [173]. The extracellular N-terminal domain of LPARs, especially in LPAR1 and LPAR2, is the binding site of the receptor [174]. The intracellular C-terminal region and loops of LPA receptors are important for G-protein-coupling. The C-terminal domains also undergo other post-translational modifications, such as phosphorylation, which regulate receptor desensitization and internalization [175]. LPAR1-LPAR3 exhibit a high level of structural homology, particularly in the transmembrane regions, but display differences in their extracellular loops, which affect ligand binding and receptor activation [176]. The binding pocket of LPAR1 was found to exhibit a higher hydrophobicity than that of LPAR2 and LPAR3, leading to preferential binding to certain LPA molecules [177]. LPAR4-LPAR6 are more distantly related to LPAR1-3 and have distinct structural features [178]. LPAR1 and LPAR2, through G_i/o , G_q , and $G_{12/13}$, lead to activation of Rho signaling and cytoskeletal rearrangements. LPAR3 through G_i/o and G_q , LPAR4, through G_q and $G_{12/13}$, as well as G_s , and LPAR5, on the other hand, only through G_q and $G_{12/13}$ activate different signaling cascades. The final validated LPA receptor, LPAR6, which is a lower-affinity receptor [179], has been shown to activate the signaling pathway through G_s and $G_{12/13}$. This diversity in G-protein coupling leads to varied downstream signaling outcomes [180]. After LPA binding, these receptors bind to G_i/o , $G_q/11$, and $G_{12/13}$, and each triggers separate downstream signaling pathways [181, 182]. The G_i/o pathway suppresses adenylyl cyclase and decreases cAMP levels, while $G_q/11$ stimulates phospholipase C, leading to the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) [182]. However, the $G_{12/13}$ -RhoA-ROCK pathway is particularly crucial for nerve regeneration processes [8, 9, 183]. The $G_{12/13}$ pathway activates guanine nucleotide exchange factors (GEFs) that activate RhoA by catalyzing GDP-to-GTP exchange. Activated RhoA directly interacts with and activates both ROCK1 and ROCK2. ROCK activates its key downstream targets, including myosin light chain

(MLC), LIM kinase, and cofilin, and results in enhanced actomyosin contractility and actin filament stabilization [180, 181]. This ROCK-mediated cytoskeletal rearrangement is important for controlling growth cone motility and axonal tension, which are fundamental for both nerve pathfinding and regeneration [180, 184]. In addition, ROCK regulates focal adhesion formation through phosphorylation of focal adhesion kinase (FAK) and paxillin, processes important for axonal substrate interaction during nerve regeneration [181, 184]. This LPA-LPAR-RhoA-ROCK signaling pathway represents a key mechanism by which LPA accelerates corneal nerve repair after injury [8, 9].

1.4.2 LPA and its receptors in the central and peripheral nervous system

LPA plays a critical role in nerve regeneration in both the central and peripheral nervous systems (CNS and PNS). However, controversial findings have also been found in the literature concerning the effect of LPA on nerve regeneration. While there are studies that observed neuroprotective and regenerative effects of LPA, there are also studies that found inhibitory or neurotoxic properties of LPA. These effects depend on LPA concentration, timing, and cellular context. In the case of peripheral nerve damage, the role of LPA signaling has been suggested to contribute to the stimulation of axon growth [185, 186], regulation of inflammation [187], and improvement of neuronal survival [188], making it a major contributor in the process of regeneration. On the other hand, it has been found that LPA stimulated neurite retraction in PC12 peripheral nerve cells [189] and primary cultured chick DRG neurons [190]. LPA plays an important role in the stimulation of SCs proliferation in an *in vivo* rat model of nerve injury, which could contribute to the regeneration of peripheral nerves [191]. LPA in another study was reported to promote cortical neurite retraction through LPAR1 or LPAR2 via the Rho pathway [192]. Kingsbury *et al.*, however, demonstrated that LPA promoted cortical growth and neural progenitor survival, and the effect is mediated by LPAR1 and LPAR2 [193]. The knockout of *Lpar1* in mice (*Lpar1*^{-/-}) resulted in the elimination of LPA effects, confirming the

importance of LPA and its receptor [194]. However, another study has shown that *Lpar1* knockout mice presented learning and memory deficits [195]. The study investigated how spinal cord injury (SCI) is mediated primarily via the activation of LPAR1. Moreover, the injection of LPA into the spinal cord induced the activation of microglial cells and the demyelination of nerves. Meanwhile, the LPAR1 antagonist reduced demyelination and improved motor function recovery in SCI models [196]. López-Serrano *et al.* have determined that LPAR2 activation is detrimental to the demyelination process following SCI. However, the LPAR2 knockout mouse model presented improved motor function and myelin preservation after SCI [197]. LPAR2 activation through a specific agonist (GRI977143) stimulated the differentiation of the neural stem/progenitor cells (NS/PCs) into neurons through the Erk1/2 signaling pathway of the cortex. LPAR2 activation also enhanced neurite elongation and branch formation in immature neurons [198]. In contrast to that, in our previous study it was shown that LPAR2 plays an inhibitory role in nerve fiber outgrowth in mouse DRG neurons. Additionally, *Lpar2* knockdown enhanced neurite outgrowth and upregulated key neurotrophic markers [9]. In stroke and cerebral ischemia of human patients and mouse models, elevated levels of LPA in the plasma were determined [199, 200]. Another study by Puigdomenech-Poch *et al.* evaluated the high level of *Lpar2* mRNA in the spinal cord of human and mouse models of Amyotrophic Lateral Sclerosis (ALS). They suggest that LPAR2 signaling has a dual role in ALS. Initially, it promotes negative effects by accelerating disease progression through muscle atrophy due to inflammation. At later stages of the disease, it appears to enhance motor neuron viability and thereby extend overall survival in ALS models [201]. Therefore, LPA and its receptors, LPARs, showed highly context-dependent role and often contradictory effects on nerve fibers, from retraction of neurites to stimulation of nerve regeneration/protection, depending on various conditions, including the type of receptor, dosage, time, and cellular environment.

1.5 LPA and its Receptors on the Ocular Surface

Previous studies carried out by Mertsch *et al.* had identified the critical role of ROCK signaling in corneal nerve regeneration through both *in vitro* and *in vivo* models. The study found that the inhibition of Rho kinase significantly increased corneal nerve regeneration following injury, establishing the key role of the Rho-ROCK pathway in corneal nerve repair [8]. Given that LPA is a key upstream activator of the Rho/ROCK signaling pathway and considering the established role of ROCK in corneal nerve regeneration, we hypothesized that LPA and its receptors may represent a novel therapeutic target for promoting corneal nerve repair in NK.

Yang *et al.* examined the significance of LPA signaling in rat retinal ganglion cells (RGCs) during hypoxic conditions. They found that LPAR1 signaling can trigger the degeneration of RGCs during hypoxic conditions, suggesting that LPA can act as a detrimental factor in retinal ischemia. Furthermore, they showed that treatment with an LPAR1 antagonist (THG1603) and ROCK inhibitors provided neuroprotective effects, significantly reducing RGC loss and preserving retinal function under hypoxic stress [202]. Another study determined that LPAR1 and LPAR2 are upregulated in the ganglion cell layer (GCL) and inner nuclear layer (INL) of the retina following ischemic injury in an *in vivo* rat model. Thoreson *et al.* determined that LPA highly stimulated the proliferation of the human retinal pigment epithelial (RPE) cells. They reported that LPA is a key mediator of cell growth in proliferative vitreoretinopathy (PVR). This proves that the main function of LPA is the stimulation of cellular proliferation, rather than cellular migration [203]. Birgbauer *et al.* found that growth cone collapse and retraction of the mouse retinal neurites were promoted by LPA. Their findings showed that LPAR1, LPAR2, and LPAR3 are not responsible for the LPA-induced growth cone collapse, while the knockout mice lacking LPAR1-LPAR3 still exhibited growth cone collapse in response to LPA [204].

Another study found elevated levels of different LPA species and ATX in the vitreous fluid of patients with retinal vein occlusion (RVO) versus control healthy subjects. These elevated levels were positively correlated with proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1), as well as with VEGF

[205]. LPA secreted from corneal epithelial cells exists in the outer layer of the tear film, which, as a polar phospholipid, plays a critical role first in interfacing between the aqueous and nonpolar layers of the tear film as well as enhancing the production of pro-inflammatory cytokines and chemokines, contributing to the pathophysiology of ocular surface diseases such as dry eye disease (DED) [206]. Watsky *et al.* demonstrated that LPA signaling promotes rabbit corneal wound healing by enhancing keratocyte migration and proliferation processes. They showed that cultured corneal keratocytes showed increased expression of *Lpar1* and *Lpar2* during *in vitro* dedifferentiation processes, suggesting that LPA receptor signaling plays a role in keratocyte activation and phenotypic changes that contribute to stromal wound healing responses [207]. Honjo *et al.* demonstrated a remarkable elevation in the levels of ATX, LPA, and LPC in the AH of glaucoma patients versus healthy subjects [208]. It has also been found that Ki16425, an antagonist of LPAR1-3, inhibited the LPA-induced proliferation of the corneal epithelial cells, which suggests the autocrine function of LPA. They also showed that LPA may play a dual role in the ocular surface, acting as both a component of the tear film and a pro-inflammatory factor [209]. Another research by Szepanowski *et al.* in DRG cultures has shown that LPA through LPAR1 promotes demyelination of the SCs. However, following treatment with the LPAR1 antagonist (AM095), the effect of LPA on demyelination and dedifferentiation was highly reduced [210]. In the optic nerves, ATX levels are highly elevated in the optic nerve-derived astrocytes of glaucomatous patients [211]. Conversely, the LPA and LPC levels in the optic nerve of glaucomatous patients were significantly lower than control individuals [212]. Tokumura *et al.* investigated the levels of all LPA species in the AH of an injured cornea in rabbits. They reported that among all LPA species, only LPA 18:2 was found to have higher levels in the AH of injured corneas compared to control AH, while the total amount of LPA in the injured corneas showed no difference compared to normal AH [213]. This indicates the complex dynamics of lysophospholipid metabolism in response to corneal damage.

1.6 Foundational Evidence: LPA-RhoA-ROCK Pathway in Corneal Nerve Regeneration

This thesis builds upon the foundational works carried out by Mertsch *et al.*, who investigated the importance of Rho/ROCK signaling in corneal nerve regeneration using *in vitro* and *in vivo* mouse models. Understanding the mechanisms that regulate corneal nerve regeneration is important for developing effective new treatments for conditions such as NK. In their *in vitro* model, following Rho kinase inhibitor treatment, neurite outgrowth was enhanced in cultured mouse DRG neurons, demonstrating the cellular mechanisms by which ROCK inhibition facilitates nerve regeneration. Their *in vivo* mouse model of corneal nerve injury showed that the topical application of the ROCK inhibitor as an eye drop induced nerve regeneration, compared to untreated controls. This research established the direct evidence of the inhibitory role of ROCK in ocular surface nerve regeneration [8]. Given that LPA is a major upstream factor of the RhoA-ROCK signaling pathway via LPAR1-LPAR6 [214], these foundational findings by Mertsch *et al.* provided us with the scientific rationale for investigating the potential role of LPA in corneal nerve regeneration. Based on this established foundation, the current study investigates whether modulating LPA signaling could promote corneal nerve regeneration.

1.7 Aim of the Hypothesis

Damage to the corneal nerves can significantly impact corneal integrity, leading to NK. Despite therapeutic advances, most treatments focus on restoring the tear film rather than addressing the underlying mechanisms of nerve damage [19, 215]. Preliminary data from our lab by Mertsch *et al.* showed that inhibition of the downstream effector ROCK promotes nerve regeneration following corneal injury *in vitro* and *in vivo*. Despite LPA acting upstream of ROCK, it was critical to determine whether LPA has a role in corneal nerve dysfunction or in

promoting nerve regeneration, and which specific receptor subtypes are involved [8]. LPA has six different receptors that activate several signaling pathways, for example, cell survival, proliferation, neurite retraction, and growth cone collapse. Elevated LPA levels have been observed in epithelial cells of the injured cornea. It has been shown that ATX and LPA levels are significantly enhanced in the AH of different glaucoma subtypes [212]. Based on all these, there were three questions/objectives for us:

1- What is the effect of LPA on corneal nerve regeneration?

The primary goal of this thesis was to determine the role of LPA in corneal nerve regeneration. To investigate the role of LPA, primary mouse DRG neurons were treated with LPA and LPA+ROCKi, and further, the fiber regeneration and signaling pathway changes were investigated. The hypothesis is that whether LPA suppresses corneal nerve regeneration through activation of the Rho/ROCK pathway, and that this inhibitory effect can be reversed by co-treatment with ROCK inhibitor (ROCKi).

2- Among all six LPA receptors, which receptor is responsible for mediating the effects of LPA on DRG neurons?

To identify the responsible LPA receptor subtype for the observed effect of LPA, siRNA-based knockdown experiments were conducted for each LPAR. The hypothesis is that knocking down the responsible receptor for the inhibitory effects of LPA will result in enhanced nerve outgrowth compared to the negative control.

3- Does co-culture of DRG neurons with hLESCs modulate the effects of LPA on nerve regeneration?

To achieve these objectives, the impact of LPA on nerve regeneration was assessed using a co-culture model of DRG neurons with hLESCs (as an accurate model of the real cornea). This experimental approach enables the determination of how neuron-epithelial interactions influence nerve regeneration in the presence of LPA and following *Lpar2* knockdown.

2

2. Materials and Methods

2.1 Materials

This thesis was conducted using the following materials, reagents, and devices.

2.1.1 Animal and Human Cell Sources

Table 3: Mouse line as a source of primary DRG neurons.

Mouse line	Origin
Male C57BL/6J	Tierehaus - Carl von Ossietzky University Oldenburg

Table 4: Mouse cell line as a feeder layer.

Mouse cell line	Origin
3T3 murine fibroblast cells	Sigma-Aldrich, Steinheim, Germany

Table 5: Human corneal donor material numbers as a source of hLESCs.

Human donor intern No.	Origin
SP0021, 27, 31, 33, 81, 86, 92, 94, 108, 112, 115, 123, 124, 125, 127, 128, 134, 139, 145, 272, 283, 297	Deutsche Gesellschaft für Gewebetransplantation, DGFG via PIUS Hospital – Eye Clinic

2.1.2 Instruments, Devices, and Microscopes

Table 6: Instruments, devices, and microscopes with the respective models and manufacturers.

Instrument, Device, and Microscope	Model	Manufacturer
Analytical balances	Kern ABT 100-5NM	Kern & Sohn GmbH, Balingen, Germany
Askina® Brauncel® Cellulose Swabs	4x5 cellulose swabs	B. Braun Melsungen AG, Melsungen, Germany
Benchtop centrifuge	Mega Star 1.6 / 1.6R	VWR International, Darmstadt, Germany
Biometra Thermal Cycler	TAdvanced Series	Analytik Jena GmbH, Jena, Germany
Fine forceps	Dumont #5	Fine Science Tools (F.S.T), Heidelberg, Germany
Incubator HERAcell	vios 250i	Thermo Fisher Scientific Inc., Waltham, USA
IncuCyte S3® Live-Cell Imaging	v2019B Rev2	Sartorius AG, Göttingen, Germany
Inverted Microscope ECLIPSE	Ts2R-FL	Nikon Instruments Inc., Tokyo, Japan
Inverted Microscope	Axio Vert A1	Carl Zeiss (ZEISS), Oberkochen, Germany
Microcentrifuge (refrigerated)	Fresco 21	Thermo Fisher Scientific Inc., Waltham, USA
NanoDrop™	NanoDrop One ^c	Thermo Fisher Scientific Inc., Waltham, USA
Neubauer counting chamber	0.01 mm depth	Marienfeld Superior™ GmbH, Lauda, Germany
QuantStudio™ 3 Real-Time PCR System, 96-well	QuantStudio 3	Thermo Fisher Scientific Inc., Waltham, USA
Scale ABT	220-5DNM	Kern & Sohn GmbH, Balingen, Germany
Spring scissors	564	HERMLE GmbH, Gosheim, Germany
Surgical Microscope	OPMI® Pico	Carl Zeiss (ZEISS), Oberkochen, Germany
Vortex-Genie® 2 Mixer	Z258423-1EA	Scientific Industries, Bohemia, USA
Wacom Drawing tablet (Pen display)	Cintiq Pro 16	Wacom Co., Ltd., Osaka, Japan
Water Bath	1008 (20 L)	GFL Technologies, Burgwedel, Germany

2.1.3. Consumables and Chemicals

Table 7: Consumables and chemicals with the respective manufacturers and catalog numbers.

Reagent	Manufacturer, Cat No.
Adenine	Sigma-Aldrich, Steinheim, Germany, A2786
Calcium chloride (CaCl ₂)	Sigma-Aldrich, Steinheim, Germany, C1016
Chloroform	Sigma-Aldrich, Steinheim, Germany, C2432
Cholera toxin	Sigma-Aldrich, Steinheim, Germany, C8052
Collagenase Type I	Worthington Biochemical Corp., Lakewood, USA, LS004196
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Steinheim, Germany, D8417
Diethyl dicarbonate (DEPC)	Sigma-Aldrich, Steinheim, Germany, D5758
Dispase II	Roche, Mannheim, Germany, 04942078001
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany, D4540
Dulbecco's Modified Eagle Medium / high glucose	Sigma-Aldrich, Steinheim, Germany, D0819
Dulbecco's Modified Eagles Medium / F12	Sigma-Aldrich, Steinheim, Germany, D8062
Dulbecco's Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Steinheim, Germany, D8537
Ethanol > 99.5%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany, 5054.1
Fetal Bovine Serum (FBS) Superior	Sigma-Aldrich, Steinheim, Germany, F7524
Fatty acid-free Bovine Serum Albumin (BSA)	Sigma-Aldrich, Steinheim, Germany, A8806
5-Fluoro-2'-Deoxyuridine (5-FU)	Sigma-Aldrich, Steinheim, Germany, F0503
HiPerfect® Transfection Reagent	QIAGEN, Hilden, Germany, 301704
Hydrocortisone	Sigma-Aldrich, Steinheim, Germany, H0888
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich, Steinheim, Germany, H4034
Insulin	Sigma-Aldrich, Steinheim, Germany, I9278
Isoflurane 1mg/mL	CP-Pharma, Germany, Burgdorf, Germany, 1214
Isopropanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany, CN09.1
Laminin	Sigma-Aldrich, Steinheim, Germany, 11243217001
Magnesium chloride (MgCl ₂)	Sigma-Aldrich, Steinheim, Germany, M8266
Oleoyl-Lysophosphatidic acid (LPA 18:1)	Sigma-Aldrich, Steinheim, Germany, L7260
Paraformaldehyde (PFA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany, 0335.1
Penicillin-Streptomycin (Pen/Strep)	Sigma-Aldrich, Steinheim, Germany, P4333
Poly-D-Lysine (PDL)	Sigma-Aldrich, Steinheim, Germany, P1024

Potassium chloride (KCl)	Sigma-Aldrich, Steinheim, Germany, P3911
ROCK inhibitor (Y27632; ROCKi)	Selleck Chemicals, Houston, USA, S1049
Sodium bicarbonate (NaHCO ₃)	Life technologies, Carlsbad, USA, 25080-060
Sodium chloride (NaCl)	Sigma-Aldrich, Steinheim, Germany, S9888
SYBR™ Green Master Mix	Applied Biosystems, Foster City, USA, 4309155
TaqMan® Master Mix	Applied Biosystems, Foster City, USA, 4352042
Transferrin	Sigma-Aldrich, Steinheim, Germany, T8158
Trypan blue	Carl Roth GmbH + Co. KG, Karlsruhe, Germany, 1680.1
Trypsin-EDTA solution 1x	Sigma-Aldrich, Steinheim, Germany, T3924
Trypsin-EDTA solution 10x	Sigma-Aldrich, Steinheim, Germany, T4174

Table 8: Antibodies and siRNAs with respective manufacturers and catalog numbers.

Antibodies and siRNAs	Manufacturer, Cat No.
Alexa Fluor® 594 AffiniPure Donkey Anti-Mouse Antibody	Jackson ImmunoResearch, West Grove, USA, 715-585-150
Mouse anti- β III Tubulin Antibody	BioLegend, San Diego, USA, 657402
MISSION® esiRNA Mouse <i>Lpar1</i>	Sigma-Aldrich, Steinheim, Germany, EMU054251
MISSION® esiRNA Mouse <i>Lpar2</i>	Sigma-Aldrich, Steinheim, Germany, EMU084191
MISSION® esiRNA Mouse <i>Lpar4</i>	Sigma-Aldrich, Steinheim, Germany, EMU037241
siRNA Universal scramble (Negative Control)	Sigma-Aldrich, Steinheim, Germany, SIC002

Table 9: Mouse primer sequences for SybrGreen-based qRT-PCR (from Eurofins).

Primers	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
<i>Enpp2</i>	CATTTATTGGTGGAAACGCAGA	CTACAAAAACAGTCTGCATGC
<i>Rock1</i>	TGGTAAAGGTAATCGGCAGAGG	ACTATTGGCAAACGCCATGAT
<i>Rock2</i>	TTGGTTCGTCATAAGGCATCAC	TGTTGGCAAAGGCCATAATATCT
<i>RhoA</i>	AGCTTGTGGTAAGACATGCTTG	GTGTCCCATAAAGCCAACCTCTAC
<i>Ras</i>	AGACACGAAACAGGCTCAGG	GCATCGTCAACACCCTGTCT
<i>Pi3k</i>	ACCTTAAATGGTGAGCACGGA	ACACCCCAGCCAATCAAGTC
<i>Plc</i>	CTGAGCGGAGAAGAAAATGG	ACACAGCGACATCCAGACAG
<i>Rac</i>	TGACAGATTGCGTCCCCTCT	ATCGGCTTGTCTTTGCCCC
<i>Mapk</i>	GCTGAAGCGCCATTCAAGTT	ACTTACACCATCTCTCCCTTGC
<i>Calca</i>	GAGGGCTCTAGCTTGGACAG	AAGGTGTGAACTTGTTGAGGT

<i>Tac1</i>	TTTCTCGTTTCCACTCAACTGTT	GTCTTCGGGCGATTCTCTGC
<i>Gdnf</i>	CGCTGACCAGTGACTCCAAT	AAACGCACCCCCGATTTTGTG
<i>Cntf</i>	TTTCACCCCGACTGAAGGTG	TTCTGTTCCAGAAGCGCCAT
<i>Ngf</i>	GTAGACCCCAGACTGTTTAAGAAACG	GTACCATGGGCCTGGAAGTCTAG
<i>Gap43</i>	GGAGAAGGATGATGCTCCCG	TTTGGCTTCGTCTACAGCGT
<i>Cdc42</i>	GAGTGCTCTGCCCTCACAC	GGCTCTTCTTCGGTTCTGG
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table 10: Mouse primer sequence for TaqMan-based qRT-PCR (from Thermo Fisher Scientific).

Primers	Assay ID
<i>Lpar1</i>	Mm00439145_m1
<i>Lpar2</i>	Mm00469562_m1
<i>Lpar3</i>	Mm01312593_m1
<i>Lpar4</i>	Mm01228533_m1
<i>Lpar5</i>	Mm01190818_m1
<i>Lpar6</i>	Mm00613058_s1
<i>Gapdh</i>	Mm99999915_g1

Table 11: Human primer sequences for SyBrGreen-based qRT-PCR (from Eurofins).

Primers	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
<i>LPAR1</i>	GGCTATGTTGCCAGAGGACTAT	TCCAGGAGTCCAGCAGATGATAA
<i>LPAR2</i>	GCTCTGTCGAGCCTGCTTGTCTTC	ACAGTCTTGACCAGGCTGAGCGTG
<i>LPAR3</i>	GGTGAACGTGAGCGGATGT	AGCTTTGTTCTGTCCAGTCA
<i>LPAR4</i>	CCACCTGCTTTGAAGGCTTCTC	AGGCTTGCGAAGAGTTCTCAGC
<i>LPAR5</i>	CGCCATCTTCCAGATGAAC	TAGCGGTCCACGTTGATG
<i>LPAR6</i>	TCAGCATGGTGTGTTGTGCTTGG	CTCCAAATGGCCAATTCCGTGTTG
<i>ENPP2</i>	ACAACGAGGAGAGCTGCAAT	AGAAGTCCAGGCTGGTGAGA
<i>ROCK1</i>	AACATGCTGCTGGATAAATCTGG	TGTATCACATCGTACCATGCCT
<i>ROCK2</i>	TGCGGTCACAACTCCAAGCCTT	CGTACAGGCAATGAAAGCCATCC
<i>RHOA</i>	AGCCTGTGGAAAGACATGCTT	TCAAACACTGTGGGCACATAC
<i>CALCA</i>	GCCTGTGACACTGCCACCTG	AAAGGCTTTGGAACCCACAT
<i>TAC1</i>	CGACCAGATCAAGGAGGAACTG	CAGCATCCCGTTTGCCCAT
<i>GNDF</i>	TGGGTCTGGGCTATGAAACC	ATGCCTGCCCTACTTTGTCA

<i>CNTF</i>	TGATTAGGCCCGCCAAACTT	AACCTGGTATAAGCCGTGCC
<i>NGF</i>	CATCATCCCATCCCATCTTC	GTCTGTGGCGGTGGTCTTAT
<i>BCL2</i>	CTGAGTACCTGAACCGGCA	GAGAAATCAAACAGAGGCCG
<i>BAX</i>	GGGTTGTCGCCCTTTTCTAC	GGAGGAAGTCCAATGTCCAG
<i>CAS3</i>	AGGACTCTAGACGGCATCCA	CAGTGAGACTTGGTGCAGTGA
<i>KI67</i>	TGACCCTGATGAGAAAGCTCAA	CCCTGAGCAACACTGTCTTTT
<i>P63α</i>	GGAAAACAATGCCCAGACTC	ATGATGAACAGCCCAACCTC
<i>PAX6</i>	CTGAGGAATCAGAGAAGACAGGC	ATGGAGCCAGATGTGAAGGAGG
<i>VIM</i>	AGGCAAAGCAGGAGTCCACTGA	ATCTGGCGTTCCAGGGACTCAT
<i>CK12</i>	TATTCTCTCGGGCAATGATGGA	TTGCTGTAATCGCTCTGTGAAG
<i>CK15</i>	GGAGGTGGAAGCCGAAGTAT	GAGAGGAGACCACCATCGCC
<i>GAPDH</i>	ACCCACTCCTCCACCTTTGAC	CATACCAGGAAATGAGCTTGACAA

2.1.4 Kits and Software

Table 12: Kits with the respective manufacturers and catalog numbers.

Kit	Manufacturer, Cat No.
GenElute Mammalian Total RNA Miniprep Kit	Sigma-Aldrich, Steinheim, Germany, RTN350
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Foster City, USA, 4368814

Table 13: Software with the respective versions.

Software	Version
Fiji (ImageJ)	2.14.0
IncuCyte S3® Live-Cell Imaging	2019B (v20192.3.7219.27497)
Zeiss Efficient Navigation (ZEN)	Version 2.6.76.00000 (blue edition)

2.1.5 Culture Media, Buffers, and Solution Components

Table 14: Medium, buffer, and solution components with their concentrations.

Medium, Buffer, and Solution	Component
Antibody (1 st and 2 nd) incubation solution	2% (v/v) FBS in 1x PBS
Blocking solution for immunocytochemistry	5% FBS (v/v) and 0.1% (w/v) Triton X-100 in 1x PBS
Collagenase I solution	1 g (w/v) Collagenase I dissolved in 12.5 mL 1x PBS (10.000 U/mL)
Culture medium for DRG neurons	FBS-free DMEM high glucose 1% (v/v) Pen/Strep (10000 U/mL Pen and 10 mg/mL Strep)
Culture medium for 3T3 fibroblast cells	DMEM high glucose 10% FBS (v/v) 1% (v/v) Pen/Strep (10000 U/mL Pen and 10 mg/mL Strep)
Culture medium for hLESCs (Epithelial cell medium (ECM))	DMEM:F12 10% FBS (v/v) 1% (v/v) Pen/Strep (10000 U/mL Pen and 10 mg/mL Strep) 0.4 µg/mL hydrocortisone (dissolved in 100% ethanol) 0.1 nM cholera toxin (dissolved in deionized water) 0.075% (v/v) NaHCO ₃ (dissolved in deionized water) 18.5 mM Adenine (dissolved in 1 M HCl) 2 nM T3 (dissolved in 0.02 N NaOH) 5 µg/mL transferrin (dissolved in 1x PBS) 5 µg/mL insulin • 10 ng/mL EGF (dissolved in 1x PBS) (Add fresh)
Co-culture medium	50% (v/v) ECM (EGF-free medium) 50% (v/v) DRG neuron medium (FBS-free medium)
DEPC water (RNase-free water)	1 mL DEPC in 1 L deionized water
Dispase II solution	1 g dispase II dissolved in 1x PBS (10 U/mL, stock) • Final concentration 1.2 U/mL
Fixation solution for immunocytochemistry	4% (v/v) PFA (dissolved in 1x PBS)
5-Fluoro-2'-deoxyuridine (5-FU) solution	100 mg 5-FU dissolved in 4065µl sterile water (100 mM, stock) • Final concentration 5 µM
Laminin coating solution	1 mg Laminin dissolved in 2 mL 1x PBS (0.5 mg/mL, stock) • Final concentration 15 ng/mL

LPA preparation buffer	50 mM HEPES (dissolved in sterile water) 138 mM NaCl (dissolved in sterile water) 2.7 mM KCl (dissolved in sterile water) 1 mM CaCl ₂ (dissolved in sterile water) 1 mM MgCl ₂ (dissolved in sterile water) 1% (w/v) fatty acid-free BSA (dissolved in sterile water) • Fill up to 10 mL with sterile water (prepared buffer) • 1 mg of LPA in 881.1 µL prepared buffer (2.6 mM, stock) • Final concentration 10 µM
Mytomycin-C (MMC)	2 mg MMC dissolved in 40 µL DMSO (50 µg/µL, stock) • Final concentration 5 µg/mL
Poly-D-Lysin coating solution	4 mg PDL dissolved in 1 mL 1x PBS (4 mg/mL, stock) • Final concentration 150 ng/mL
ROCK inhibitor (Y27632; ROCKi) solution	50 mg ROCKi dissolved in 15,61 mL sterile water (10 mM, stock) • Final concentration 100 µM
siRNA (for <i>Lpar1</i> , 2, and 4) dilution	20 µg siRNA dissolved in 100 µL TE buffer (200 ng/µL, stock) Molecular weight of 21-mers siRNA: 14490 g/mol • Final concentration in FBS-free DMEM 0.7245 ng/µL or 50 nM
siRNA (scramble) dilution	1 nmol scramble dissolved in 50 µL TE buffer (20 µM, stock) • Final concentration in FBS-free DMEM 50 nM
Trypan Blue solution	0.2% (w/v) in PBS

2.2 Methods

All experiments were conducted at least in independent triplicate ($n = 3$), including the isolation of the primary DRG neurons, with each replicate comprising a mixture of 3 animals ($N = 3$) unless otherwise stated.

2.2.1 Animal and Ethical approval

C57BL/6J male mice (8-12 weeks old) were used for all experiments. All animal housing was conducted under the guidelines of ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under the approval of the University of Oldenburg (approval code: §4-DRG-Mertsch) and the Governmental Institutional Animal Care and Use Committee. All experiments adhered to the regulations of the German federal Animal Welfare Act (TierSchG) and the Laboratory Animal Welfare Ordinance (TierSchVerV).

2.2.2 Neuron preparation of dorsal root ganglia (DRG) and trigeminal ganglia (TG)

For primary neuronal culture, DRGs and TGs were isolated from the mouse backbone and head, respectively, as previously described [8]. Briefly, following euthanasia by cervical dislocation, the mouse was sterilized with 70% alcohol. For DRG isolation, the spinal canal was exposed by removing muscles and cutting bone at a 45-degree angle with scissors. DRG tissues were carefully extracted by grasping nerve endings and preserved in ice-cold DMEM containing 1% Pen/strep. For TG isolation, the skull was opened, and then the brain was removed. The paired TG tissues were carefully dissected from the ventrolateral aspect of the brainstem. Connective tissue was removed from both DRGs and TGs using spring scissors and fine forceps.

DRG and TG dissociation was performed in 2 mL DMEM containing collagenase type I (10.000 U/mL, 500 µL) (Worthington-BioChem) and trypsin (37.5%, 1.5 mL) (Sigma Aldrich, Steinheim, Germany) for 1 hour at 37°C. Cells were mechanically dissociated by aspiration using a syringe, followed by gentle pipetting, then centrifuged at 1000 rpm for 5 min, washed with pre-warmed 1x PBS (performed twice). The cell pellets were resuspended in 1 mL pre-warmed DMEM containing 10% FBS and 1% Pen/Strep for cell counting. The DRG and TG neurons were seeded separately onto PDL (150 µg/mL) (PDL) and laminin (15 µg/mL) pre-coated well plates. The seeding was conducted for 3h at 37°C in an incubator set at 5% CO₂ in DMEM supplemented with 10% FBS and 1% Pen/Strep. After 3h of incubation, the culture medium was changed with a FBS-free medium containing 5 µM of 5-FU. This medium change was conducted to support neuronal growth and inhibit the proliferation of non-neuronal cells. Each experiment had 3 independent biological and technical replicates (n = 3; N = 9). Samples and animal numbers were defined as “n” for the number of independent technical replicates and “N” for the total number of animals used in each experiment.

2.2.3 Primary human limbal epithelial stem cells (hLESCs) culture

Human donor cornea rims were obtained from the PIUS Hospital (Department of Ophthalmology, University Hospital eye clinic, Carl von Ossietzky University of Oldenburg, Germany) with the approval of the local ethics committee (approval Number 2019-055). To culture hLESCs, 3T3 fibroblast cells feeder layers were first prepared to provide essential factors and cell-to-cell interactions that promote the proliferation of limbal epithelial stem cells [216, 217]. 3T3 murine fibroblast cell line (Sigma-Aldrich, Steinheim, Germany) was used as a feeder layer and seeded at a density of 1.8×10^6 cells per T75 flask in DMEM containing 10% FBS and 1% pen/strep. The cells were treated with MMC (5 µg/mL) for 4h at 37°C in an incubator with 5% CO₂ to arrest cell division. To isolate hLESCs, the cornea rims were first washed using 1x PBS and then incubated in Dispase II solution (1.2 U/mL), overnight at 4°C.

This step facilitated the dissociation of the cells from the underlying tissue. After the incubation, the corneas were placed on a petri dish and cut into 4 pieces using a scalpel. HLESCs were isolated from the limbal area through repetitive mechanical scraping with a scalpel. The corneal pieces were rinsed with pre-warmed 1 mL pre-warmed ECM to harvest the cells. Before cell seeding, the DMEM medium was removed from the T75 flask containing the pre-cultured growth-arrested 3T3 fibroblast feeder layer, and the fibroblasts were washed with 5 mL of pre-warmed 1x PBS. Subsequently, 10 mL of ECM medium was added to the T75 flask, along with the hLESCs suspension (1 mL) obtained from the scraped corneal parts. The ECM medium was refreshed every two days, supplemented with 1 mg/mL EGF to support their growth and proliferation. The feeder layer was renewed once a week by adding MMC-treated 3T3 cells when needed, in order to maintain sufficient cell density.

2.2.4 Viability assay

Cytotoxicity of different LPA concentrations was assessed by live-dead assay using Trypan-Blue staining. LPA 18:1 was purchased from Sigma-Aldrich (Steinheim, Germany). A stock solution of 2.6 mM LPA was prepared by dissolving the LPA in a buffer containing 50 mM HEPES, 138 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 1% fatty acid-free BSA (Table 14). Due to the lipid nature of LPA, all procedures were conducted using dark glass bottles and glass pipettes. The stock solution was stored at -20°C until use. Briefly, 2500 neurons/well were seeded on PDL-/laminin-pre-coated 96-well plates. The following day, different concentrations of LPA (5, 10, and 20 µM) were applied to the neurons. Non-treated neurons were used as a control. Cells were incubated for 0h, 24h, and 48h before assessing viability with trypan blue solution (0.2% in 1x PBS). Trypan blue was administered to the cells for 5 min at room temperature (RT), followed by a washing step with pre-warmed 1x PBS. The wells were photographed at three time point, 0h, 24h, and 48h, using the light microscope (Microscope ECLIPSE Ts2R-FL, Nikon, Tokyo, Japan), and the ratio between living and dead

cells was determined in 10 high-power fields (HPF) in each well, with 3 different wells per condition ($n = 3$, $N = 9$).

2.2.5 Single-cell assay

To evaluate the effects of substances on individual neurons fiber length, a single-cell assay was performed. 1000 neurons/well were seeded on PDL/Laminin-pre-coated 24-well plates. After overnight incubation, neurons were treated with LPA (10 μ M) and LPA+ROCKi (100 μ M) (solved in sterile water) [8] (S1049, Selleckchem, Houston, USA) in FBS-free DMEM. The neurons were photographed (Axio Vert A1, ZEISS, Oberkochen, Germany) at 0h (directly before the treatment), 24h, and 48h. For the control group, FBS-free DMEM without substances was used. The media was refreshed every 24h. The overall fiber length of each single neuron without any surrounding cells was examined using ImageJ software with the NeuronJ plugin. For the measurements, 6 neurons per well and 3 well replicates for each treatment group per experiment were utilized ($n = 3$, $N = 9$).

2.2.6 Wound-healing assay

5000 neurons/well were seeded on PDL/Laminin-pre-coated 96-well plates and incubated for 48h to form a dense fiber network. Afterward, a wound was applied using a 100 μ L pipette tip, followed by a gentle washing step with pre-warmed 1x PBS. The neurons were then treated with LPA and LPA+ROCKi, and the media was refreshed every 24h. The wound area was photographed every 30 min for a total 48h using the IncuCyte S3® Live-Cell Imaging and Analysis System (Sartorius AG, Göttingen, Germany). For evaluation, the edge of the wound was marked at 0h (directly after applying the wound) in the Software ImageJ as a Region of Interest (ROI). This ROI was applied to the 24h and 48h photos to measure the fibers that grew

into the wound area using NeuronJ Plugin. Three wells replicate for each treatment group per experiment were evaluated ($n = 3$, $N = 9$).

2.2.7 RNA interference experiments

DRG neurons were cultivated in FBS-free DMEM for single-cell assays (3000 neurons/well) and wound-healing assay (5000 neurons/well), as described before. After 24h (for single-cell assay) and 48h (for wound-healing assay), neurons were transfected (as 0h time point) using HiPerfect® Transfection Reagent (QIAGEN, Hilden, Germany) and siRNA directed against *Lpar1*, 2, and 4 and non-silencing scramble siRNA in a final concentration of 50 nM (Sigma Aldrich, Steinheim, Germany) in a 96-well plate. Transfections were conducted according to the manufacturer's instructions. Briefly, siRNAs targeting *Lpar1*, 2, and 4 and a non-silencing scramble siRNA as a negative control were prepared by dissolving siRNA in 100 μ L TE buffer (200 ng/ μ L stock concentration). siRNA was diluted to 0.724 ng/ μ L in 25 μ L FBS-free DMEM to achieve a final concentration of 50 nM in a total of 200 μ L medium per well. 1.5 μ L of HiPerfect® Transfection Reagent was mixed with 23.5 μ L FBS-free DMEM (50 nM final concentration per well) (the whole procedure was conducted on the ice). Both solutions (25 μ L each) were combined to a total volume of 50 μ L, mixed by pipetting up and down 5 times or gentle vortexing for 5 seconds, and incubated for 5-10 minutes at room temperature to form a transfection complex. Wounds were applied to the neurons, followed by a gentle washing step with pre-warmed 1x PBS. The PBS was removed, and 150 μ L fresh pre-warmed FBS-free DMEM was added to each well, and the transfection complex (50 μ L) was added dropwise to each well (0h). Transfected neurons were incubated at 37°C with 5% CO₂ for 24h. After 24h, the medium containing the transfection complex was removed and replaced with fresh pre-warmed FBS-free DMEM containing LPA or LPA+ROCKi, and incubated at 37°C with 5% CO₂ for the next time points. The media was refreshed every 24h. Neurons were photographed at 0h, 24h, and 48h. To measure the efficiency of siRNA-mediated knockdown for *Lpar1*, 2, and 4,

the neurons were harvested at 24h and 48h after transfection for RNA isolation, and subsequently, qRT-PCR measurement.

2.2.8 Co-culture model of mouse DRG neurons and hLESCs

For co-culturing, primary mouse DRG neurons were seeded onto the PDL and Laminin pre-coated 24-well plates in FBS- and 5-FU-free DMEM as described before (Due to the presence of hLESCs in the co-culture, 5-FU was not used). On the same day and time as neuronal seeding, hLESCs were seeded at 10×10^3 cells per insert in pre-warmed EGF-free ECM (due to the presence of neurons in the co-culture, EGF was not used) onto ThinCert® Cell Culture Inserts (pore diameter: 0.4 μm , 24-well, Greiner Bio-One GmbH, Frickenhausen, Germany). For seeding hLESCs, 3T3 feeder cells were detached from the hLESCs culture flask by adding 1 mL of pre-warmed 1x trypsin, followed by a 3-minute incubation. Subsequently, the detached fibroblasts were removed by adding 4 mL of DMEM to the flask, followed by aspiration of this cell solution and a washing step using pre-warmed 1x PBS. To detach the remaining hLESCs, 2 mL of 10x trypsin was added to the flask and incubated for 5-6 minutes at 37°C with 5% CO₂. The trypsin was then neutralized using 9 mL of ECM, and the cell suspension was transferred to a 15 mL tube and subsequently centrifuged at 1500 rpm for 5 minutes. After pellet resuspension, the hLESCs were counted using a hemocytometer and seeded in the insert with pre-warmed EGF-free ECM. After 24h of cell seeding for single-cell assays and 48h for wound-healing assays, the inserts containing hLESCs were temporarily transferred to clean and sterile well plates to apply the wound and perform siRNA transfection on the DRG neurons. Wounds were applied to neurons, followed by gentle washing with pre-warmed 1x PBS. The PBS was removed, and fresh pre-warmed FBS-free DMEM containing siRNA transfection complex, as described above, was added to each well (characterized as 0h time point). Following transfection, the inserts were immediately returned to the same plates containing neurons to continue co-culturing and incubated at 37°C with 5% CO₂ for 24h. After 24h, the inserts were

again temporarily removed to sterile plates to remove the medium containing the transfection complex and replace it with fresh pre-warmed FBS-free DMEM containing LPA and LPA+ROCKi. The medium in the inserts containing hLESCs was also refreshed with pre-warmed EGF-free ECM, and the inserts were returned to the neuronal plates to continue co-culturing for the next time points. Both media types (FBS-free DMEM in the well plate for neurons and EGF-free ECM in the insert for hLESCs) were refreshed every 24h. Neurons were photographed at 0h, 24h, and 48h. At each photography time point, the inserts were temporarily removed to sterile plates to allow unobstructed imaging of the neurons, and immediately returned to the co-culture plates following photography to continue incubation until the next time point. Both the single-cell and the wound-healing assay were performed pre- and post-siRNA transfection of the neurons, following the previously described protocols.

2.2.9 Quantitative real-time PCR

Total RNA was isolated from DRG neurons at the 48h timepoint using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Steinheim, Germany) following the manufacturer's instructions. RNA concentrations were evaluated using NanoDrop™ One/One C Microvolume UV/VIS Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Total mRNA (0.2 µg) was transcribed into single-stranded cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions, and the reaction mixture is stated in Table 15. QRT-PCR was performed using the SyberGreen Master Mix (Applied Biosystems, Foster City, USA) and TaqMan™ Fast Universal PCR Master Mix (Applied Biosystems, Foster City, USA). The SybrGreen primer sequences for mouse genes *Enpp2*, *Rock1*, *Rock2*, *RhoA*, *Ras*, *Pi3k*, *Plc*, *Rac*, *Mapk*, *Calca*, *Tac-1*, *Gdnf*, *Cntf*, *Ngf*, *Gap43*, and *Cdc42* are presented in Table 9, and TaqMan mouse primers for *Lpar1-6* are shown in Table 10. Human SybrGreen primer sequences for *LPAR1-6*, *ENPP2*, *ROCK1*, *ROCK2*, *RHOA*, *CALCA*, *TAC-1*, *GNDF*, *CNTF*, *NGF*, *BCL2*, *BAX*, *CAS3*, *KI67*, *P63α*, *PAX6*, Vimentin (*VIM*), *CK12*, and *CK15* are listed in Table 11. The reaction

mixtures for SybrGreen and TaqMan™ MasterMix are presented in Table 16 and Table 17, respectively. The data were normalized to *Gapdh*/*GAPDH* as a housekeeping gene, and the quantification of the relative gene expression was conducted using the equation fold change using the $2^{-\Delta\Delta Ct}$ method [218].

Table 15: Reverse transcriptase-PCR details.

Components	Volume for 1 PCR reaction	Final concentration	Program
10x RT Buffer	2 µL	1x	25 °C → 10 min 37 °C → 120 min 85 °C → 5 min 4 °C → Hold
25x dNTP mix	0.8 µL	1 mM	
10x RT random primer	2 µL	1x	
Reverse transcriptase	1 µL	1 U/µL	
RNA Sample	Variable	2 µg	
DEPC water	Variable	-	
Total	20 µL	-	

Table 16: Cycling conditions for SyBrGreen qRT-PCR reaction.

Components	Volume for 1 PCR reaction	Final concentration	Run	Program
			Standard	Avtivation 50 °C → 20 sec Initial Denaturation 95 °C → 10 min Denaturation 95 °C → 15 sec Annealing 60 °C → 1 min Denaturation (pre-melt) 95 °C → 15 sec Annealing (pre-melt) 60 °C → 1 min Melting step 95 °C → 30 sec Final step (pro-melt) 60 °C → 15 sec
SybrGreen Master mix	5 µL	1x		} 40 cycles
DEPC water	3.6 µL	-		
Forward primer	0.2 µL	10 µM		
Reverse primer	0.2 µL	10 µM		
cDNA Sample	1 µL	1:15		
Total	10 µL	-		

Table 17: Cycling conditions for TaqMan qRT-PCR reaction.

Components	Volume for 1 PCR reaction	Final concentration	Run	Program
TaqMan Master mix	10 µL	1x	Fast	Initial Denaturation 95 °C → 20 sec Denaturation 95 °C → 1 sec Annealing 60 °C → 20 sec } 45 cycles
DEPC water	8 µL	-		
Primer	1 µL	1x		
cDNA Sample	1 µL	1:15		
Total	20 µL	-		

2.2.10 Immunocytochemistry

DRG neurons seeded on the PDL/Laminin-pre-coated coverslips after 48h of the experiment were fixed in 4% PFA for 10 min. Subsequently, blocking was administered using 5% FBS with 0.25% Triton X-100 in 1x PBS for 1h at RT. All subsequent washing steps (1x PBS, 3 times, each 5 min) were carried out at room temperature. The mouse anti- β III tubulin antibody (801202, BioLegend, USA; dilution 1:2000) was used as a primary antibody in 2% FBS/PBS overnight at 4°C. As secondary antibody, Alexa Fluor 594 AffiniPure Donkey Anti-mouse antibody (140149, Jackson ImmunoResearch, UK; dilution 1:400) was used. The Nuclei were counterstained with DAPI, and the slides were mounted with Mowiol (Merck, Darmstadt, Germany).

2.2.11 Statistical Analysis

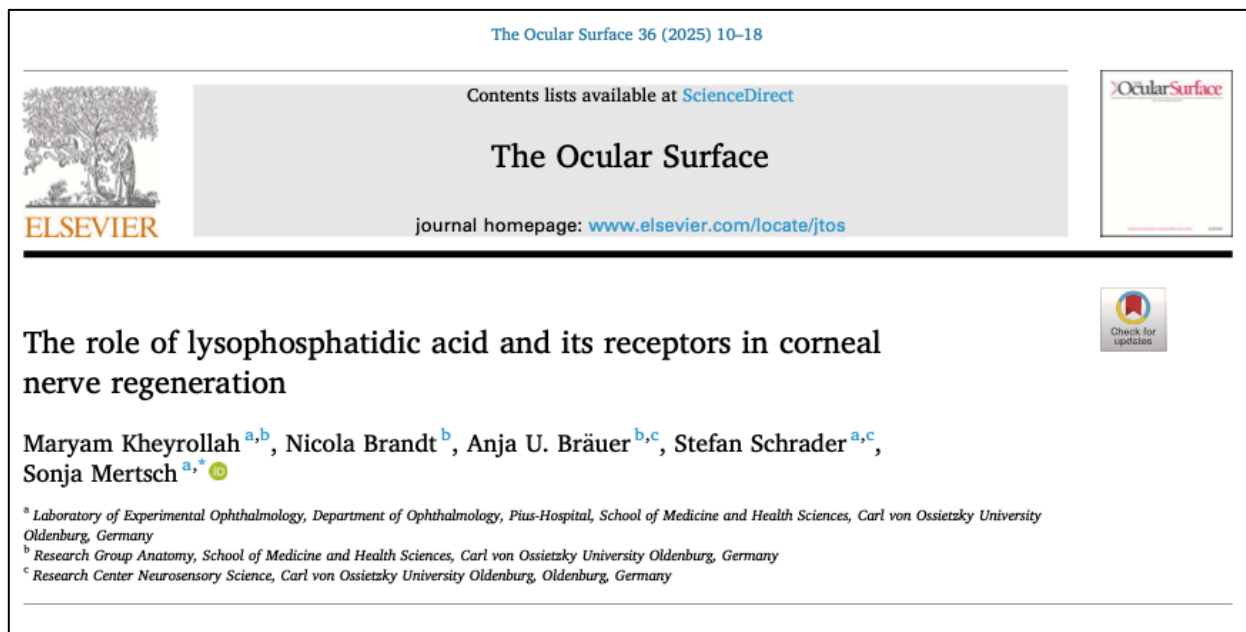
All data were presented as means $\pm$ SEM from three independent biological and technical replicates. All graphs and statistical analyses were performed using Microsoft Excel. Student's t-test was performed to compare differences between the control and treated groups. The threshold for statistical significance was set at $p \leq 0.05$ (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$).

3

3. Results

3.1 Objective 1 and 2: The role of LPA and its receptors on corneal nerve regeneration

The first part of the results presented in this thesis have been published as a peer-reviewed article in “The Ocular Surface”. This publication corresponds to the first and second objectives of the thesis and addresses the investigation of LPA and its receptors regarding corneal nerve regeneration. The subsequent sections of this chapter (section 3.1) provide a comprehensive overview of the experimental work and findings related to these objectives, as detailed in the original publication.



Declaration

Hereby, I confirm that Maryam Kheyrollah contributed to the mentioned study as stated below:

Article:

“The role of lysophosphatidic acid and its receptors in corneal nerve regeneration”

Maryam Kheyrollah, Nicola Brandt, Anja U. Bräuer, Stefan Schrader, Sonja Mertsch

Published in: The Ocular Surface 36 (2025) 10–18

Author contributions:

Maryam Kheyrollah: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. Nicola Brandt: Writing – review & editing, Supervision, Funding acquisition,. Anja U. Bräuer: Writing – review & editing, Supervision, Funding acquisition,. Stefan Schrader: Writing – review & editing, Supervision, Funding acquisition. Sonja Mertsch: Writing – review & editing, Methodology, Validation, Supervision, Project administration, Funding acquisition, Conceptualization, Formal analysis, Data curation.

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_____(Ort, Datum) _____(PD Dr. rer. nat Sonja Mertsch)

3.1.1 Expression levels of *Lpar1-6* mRNA in sensory neurons

The expression levels of *Lpar1-6*, *Rock1*, *Rock2*, and *Enpp2* were determined in TG and DRG neurons by qRT-PCR using the ΔC_t comparison method [218]. qRT-PCR analysis showed mRNA expression of *Lpar1* (DRG: 5.84 ± 0.04 and TG: 5.95 ± 0.08 , $p = 0.37$), *Lpar2* (DRG: 9.72 ± 0.35 and TG: 9.59 ± 0.18 , $p = 0.99$), *Lpar4* (DRG: 10.27 ± 0.03 and TG: 10.19 ± 0.29 , $p = 0.82$), and *Lpar6* (DRG: 4.28 ± 0.19 and TG: 4.01 ± 0.34 , $p = 0.61$) in both DRG and TG neurons. Additionally, *Rock1* (DRG: 6.53 ± 0.05 and TG: 6.77 ± 0.12), *Rock2* (DRG: 4.44 ± 0.03 and TG: 4.49 ± 0.05), and *Enpp2* (DRG: 11.59 ± 0.52 and TG: 11.92 ± 0.53) were detected in both neuronal types. Among all LPA receptors, the highest expression levels were detected for *Lpar1* and *Lpar6*, while *Lpar3* and *Lpar5* were not detected in either TG or DRG neurons. No significant differences were detected between DRG and TG neurons for any individual gene (*Lpar1-6*, *Rock1*, *Rock2*, *Enpp2*). However, within each tissue type, significant inter-gene expression differences were observed with similar patterns in both DRG and TG neurons. In both neuronal types, *Lpar6* showed significantly higher expression compared to *Lpar1*, *Lpar2*, and *Lpar4*. *Lpar1* expression was significantly higher than *Lpar2* and *Lpar4*, while no significant difference was observed between *Lpar2* and *Lpar4*. Additionally, *Rock2* expression was significantly higher than *Rock1* in both tissue types (detailed p-values in Table 18). The consistent expression patterns between DRG and TG neurons indicate their comparable functionality (Figure 5).

Table 18: Statistical p-values of *Lpar1-6*, *Rock1*, *Rock2*, and *Enpp2* expression in DRG and TG neurons.

Comparison	DRG (p-value)	TG (p-value)
<i>Lpar6</i> versus <i>Lpar1</i>	0.003	0.011
<i>Lpar6</i> versus <i>Lpar2</i>	0.0004	0.0003
<i>Lpar6</i> versus <i>Lpar4</i>	1.64E-05	0.0004
<i>Lpar1</i> versus <i>Lpar2</i>	0.0009	0.0001
<i>Lpar1</i> versus <i>Lpar4</i>	2.71E-07	0.0003
<i>Rock2</i> versus <i>Rock1</i>	7.41E-06	0.0001

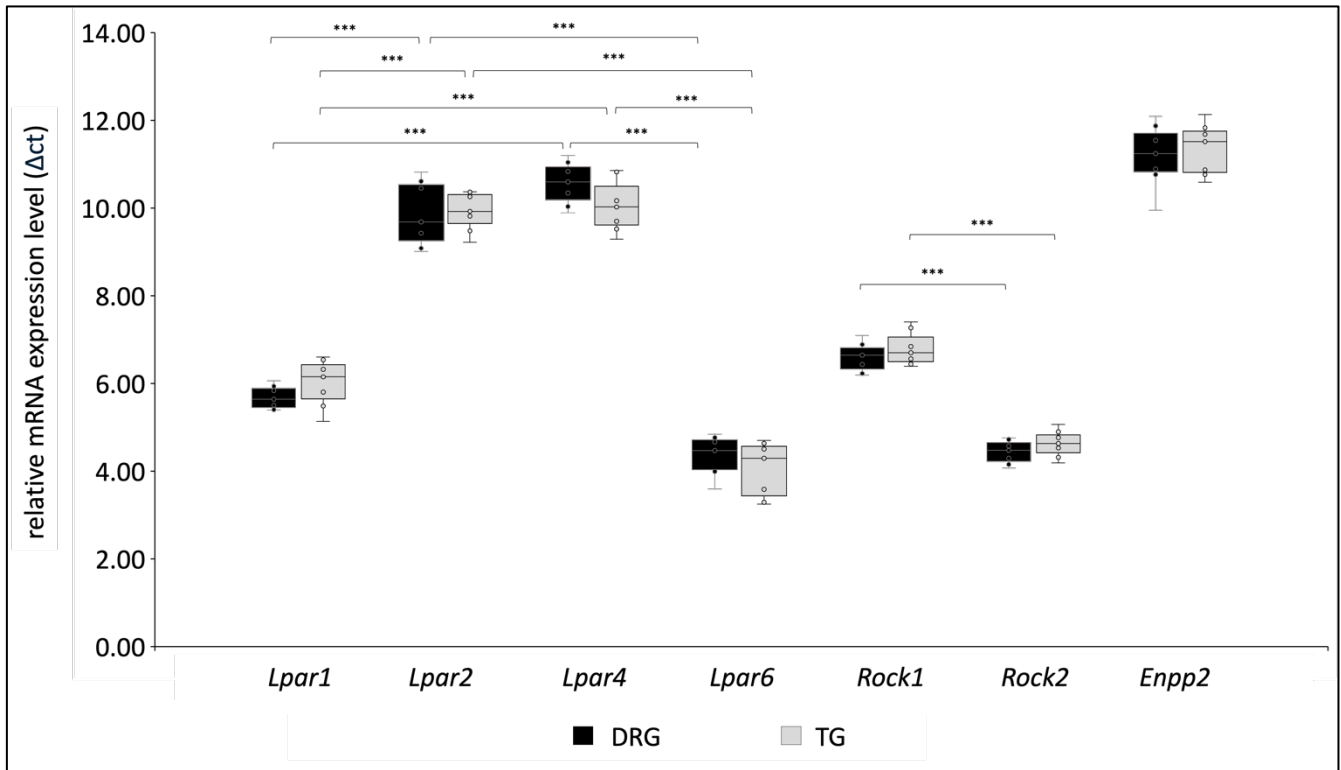


Figure 5: The mRNA expression levels of *Lpar1-6*, *Rock1*, *Rock2*, and *Enpp2* in DRG and TG.

The mRNA expression levels of *Lpar1-6*, *Rock1*, *Rock2*, and *Enpp2* genes in TG and DRG neurons after 48h. Data were normalized to the *Gapdh* gene and presented as relative expression levels compared to the control (Ctrl). The data are presented as mean $\pm$ SEM, and statistical significance is shown by p-values *** $p \leq 0.001$.

3.1.2 Dose-dependent effects of LPA 18:1 on DRG neuron viability

The cytotoxicity measurement of different concentrations of LPA 18:1 (5, 10, and 20 μM) on cultivated DRG neurons for 24h and 48h revealed no significant changes in cell viability for 5 and 10 μM compared to the non-treated control group (Ctrl). However, 20 μM LPA induced significant neuronal death, with a decrease in the live cells ($79.25 \pm 1.26\%$ Ctrl to $70.88 \pm 3.27\%$ LPA 20 μM , $p = 0.042$) and an increase in the dead cells ($21.88 \pm 1.24\%$ Ctrl to $28.88 \pm 1.96\%$ LPA 20 μM , $p = 0.013$) at 48h (Figure 6A-B). Therefore, 10 μM LPA was selected for further experiments.

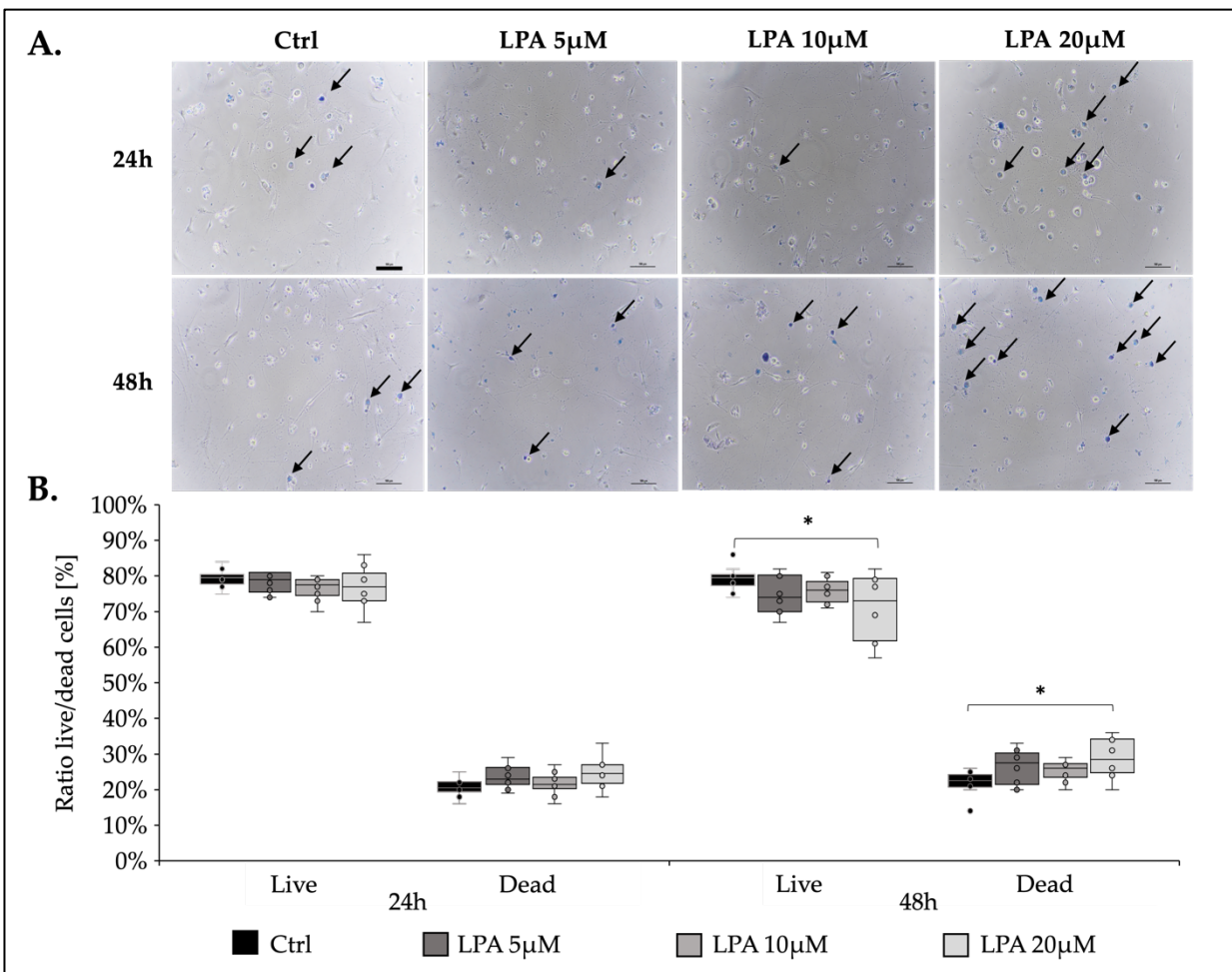


Figure 6: The cytotoxicity effects of different concentrations of LPA on DRG neurons.

The cytotoxicity effects of different concentrations of LPA (5, 10, and 20 μM) on DRG neurons with trypan blue assay. The ratio between live and dead cells is presented as mean $\pm$ SEM. The data are representative of three independent experiments. 10 $\times$ magnification, scale bar, 100 μm .

3.1.3 Functional effect of LPA on sensory fiber growth

As LPA is located upstream in the Rho Kinase pathway, the functional effects of both LPA and LPA+ROCKi on nerve fiber growth were evaluated using the single-cell assay (Figure 7A-B). Measurement of total fiber length per neuron revealed significant suppression effects of LPA at 24h ($0.84 \pm 0.11 \mu\text{m}$ LPA; $2.68 \pm 0.43 \mu\text{m}$ Ctrl, $p \leq 0.00014$; $2.44 \pm 0.27 \mu\text{m}$ LPA+ROCKi, $p \leq 0.0001$ and $4.84 \pm 0.46 \mu\text{m}$ ROCKi, $p \leq 0.0001$) and 48h ($1.03 \pm 0.12 \mu\text{m}$ LPA; $3.22 \pm 0.36 \mu\text{m}$ Ctrl, $p \leq 0.0001$; $2.35 \pm 0.203 \mu\text{m}$ LPA+ROCKi, $p \leq 0.0001$; and $6.78 \pm 0.58 \mu\text{m}$ ROCKi, $p \leq 0.0001$). In line with previous studies [8], it was found that ROCKi significantly elevated the fiber length compared to LPA treatment ($0.84 \pm 0.11 \mu\text{m}$ at 24 and $1.03 \pm 0.12 \mu\text{m}$ at 48h, $p \leq 0.0001$), LPA+ROCKi at ($p \leq 0.0001$, 24h and $p \leq 0.0001$ for 48h), and Ctrl ($p = 0.0008$ at 24h, and $p \leq 0.0001$ at 48h) (Figure 7B).

In the wound-healing assay (Figure 7C-D), where the regrowth of fibers after an additional injury was investigated, LPA again significantly suppressed the fiber growth at 24h ($1761.13 \pm 244.21 \mu\text{m}$, $p = 0.0014$) and 48h ($2131.79 \pm 406.84 \mu\text{m}$, $p = 0.0012$) compared to Ctrl ($3842.5 \pm 451.11 \mu\text{m}$ 24h and $5165.23 \pm 606.334 \mu\text{m}$ 48h). LPA+ROCKi led to a significant increase in fiber length to $4653.32 \pm 852.89 \mu\text{m}$, $p = 0.007$ at 24h and $7142.06 \pm 1196.42 \mu\text{m}$, $p = 0.0017$ at 48h in direct comparison to LPA-treated neurons. Also, ROCKi substantially enhanced fiber regrowth ($7967.86 \pm 1273.03 \mu\text{m}$, at 24h, and $14835.45 \pm 1148.64 \mu\text{m}$, at 48h) compared to Ctrl ($p = 0.010$, at 24h and, $p \leq 0.0001$ at 48h), LPA ($p = 0.0003$ at 24h, and $p \leq 0.0001$ at 48h), as well as to the LPA+ROCKi group at 48h ($p = 0.0004$) (Figure 7D).

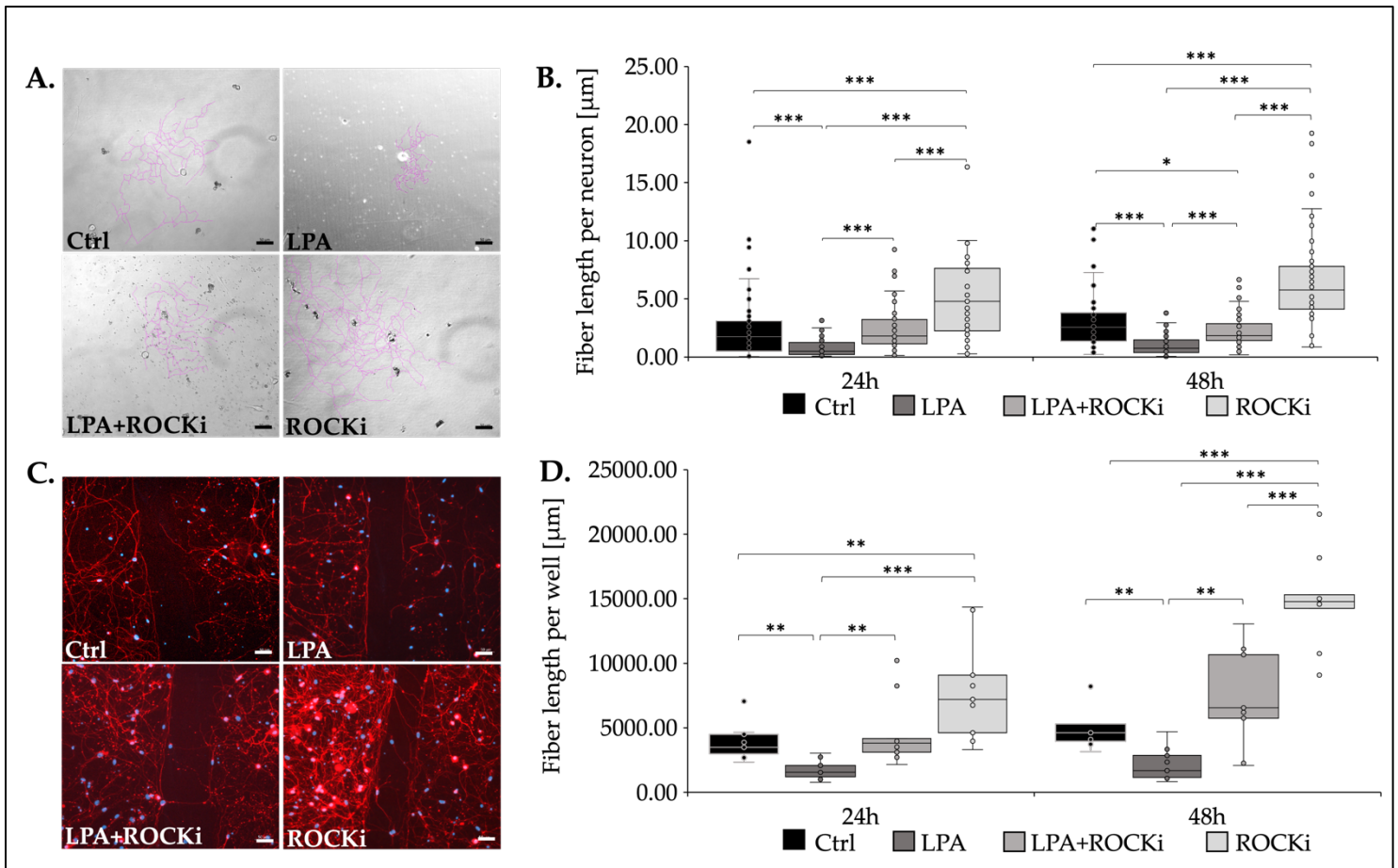


Figure 7: Effect of LPA and ROCKi on neuronal fiber length/outgrowth.

Effect of LPA and ROCKi on neuronal fiber length/outgrowth by single-cell assay and wound-healing assay. A) Photos of single-cell assay measured with ImageJ/NeuronJ showing an untreated control neuron (ctrl), neuron treated with LPA (LPA), with LPA+ROCKi, and ROCKi-treated neuron after 48h. 20 $\times$ magnification, scale bar 50 μ m. B) Neuronal fiber length measurement per neuron in single-cell assay displayed a significant suppression in fiber length in LPA-treated cells at 24h and 48h compared to the Ctrl. LPA+ROCKi and ROCKi revealed a significant enhancement compared to LPA and the Ctrl group at 24h and 48h. C) Immunofluorescence staining of wound-healing assay after 48h for Ctrl, LPA, LPA+ROCKi, and ROCKi-treated neurons. β III-tubulin (red) was used as a neuronal marker, and nuclei were stained with DAPI (blue). 20 $\times$ magnification, scale bar 50 μ m. D) Neuronal fiber outgrowth per well in the wound-healing assay revealed a significant suppression in fiber length at 24h and 48h after wounding in the LPA-treated group compared to the Ctrl. ROCKi showed an enhancement in fiber length at 48h. The data are presented as mean $\pm$ SEM, and statistical significance is indicated by p-values * p $\leq$ 0.05, ** p $\leq$ 0.01, and *** p $\leq$ 0.001. Data were normalized to the corresponding data for each substance at 0h. The images were photographed using the IncuCyte S3® Live-Cell Imaging, with 10 $\times$ magnification.

3.1.4 LPA-ROCK signaling pathways analysis in LPA-treated neurons pre- and post-injury

For a detailed analysis of the observed effect on the neurons, the expression levels of *Lpar1-6*, *Enpp2*, *Rock1*, *Rock2*, *RhoA*, *Ras*, *Cdc42*, *Mapk*, *Pi3k*, *Plc*, *Rac*, *Ngf*, *Calca*, *Tac1*, *Gdnf*, *Cntf*, *Gap-43*, were analyzed by qRT-PCR in LPA-treated non-injured and injured neurons. Non-treated groups for both non-injured and injured samples were used as control and set as 1 (Figure 8). The results were evaluated as n-fold. Among all six LPA receptors, *Lpar2* level was increased by LPA treatment (1.92 ± 0.15) in non-injured cells versus Ctrl ($p = 0.02$) and LPA+ROCKi (1.27 ± 0.08 , $p = 0.03$). After the injury, *Lpar2* was downregulated by LPA (0.59 ± 0.14) compared to the Ctrl ($p = 0.02$). Additionally, in the LPA+ROCKi group, *Rock1* (0.82 ± 0.04 , $p = 0.02$), and *Rock2* (0.66 ± 0.1 , $p = 0.04$) were significantly downregulated after injury versus Ctrl. *RhoA*, which is a downstream factor of LPA receptors, exhibited a substantial upregulation in LPA+ROCKi compared to Ctrl in non-injured (2.68 ± 0.25 , $p = 0.02$) and injured neurons (1.59 ± 0.08 , $p = 0.03$), and after the injury compared to LPA (1.03 ± 0.11 , $p = 0.007$).

In injured neurons, LPA treatment significantly decreased *Ras* (0.81 ± 0.07), *Mapk* (0.5 ± 0.16), and *Plc* (0.55 ± 0.01) levels in comparison to LPA+ROCKi (1.22 ± 0.06 , $p = 0.007$ for *Ras*; 1.33 ± 0.12 , $p = 0.004$ for *Mapk*; and 1.35 ± 0.04 , $p \leq 0.0001$ for *Plc*) and Ctrl ($p = 0.03$ for *Ras*, $p = 0.04$ for *Mapk*, and $p = 0.03$ for *Plc*), whereas post-injury LPA+ROCKi markedly increased *Ras* expression (1.22 ± 0.06 , $p = 0.02$) compared to Ctrl. LPA and LPA+ROCKi led to a significant elevation in *Gdnf* (2.27 ± 0.18 , $p = 0.016$ by LPA; and 3.53 ± 0.23 , $p = 0.007$ by LPA+ROCKi) and LPA significantly enhanced *Ngf* level (1.18 ± 0.18 , $p = 0.04$) versus Ctrl pre-injury. LPA+ROCKi significantly upregulated *Gap-43* (2.37 ± 0.17 , $p = 0.005$) and *Cdc42* (1.42 ± 0.05 , $p = 0.003$) levels versus Ctrl post-injury, while LPA alone significantly reduced *Cdc42* (0.99 ± 0.1 , $p = 0.02$) compared to LPA+ROCKi (1.42 ± 0.05) and enhanced *Gap-43* (3.24 ± 0.29 , $p = 0.013$) compared to Ctrl. LPA led to a substantial reduction in *Tac1* expression (0.45 ± 0.1 , $p = 0.04$ versus Ctrl and LPA+ROCKi (0.92 ± 0.25 , $p = 0.02$) pre-injury (Figure 8).

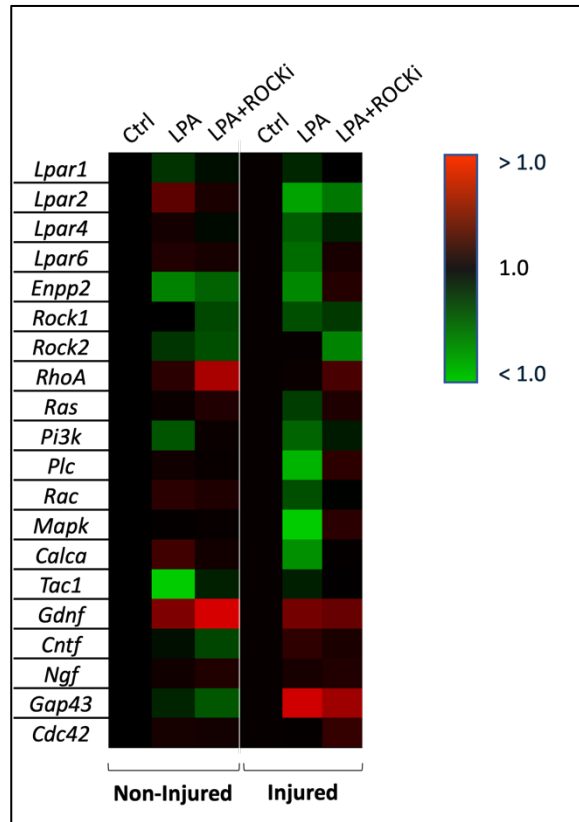


Figure 8: mRNA expression in injured and non-injured DRG neurons.

Heatmap showing differential gene expression between neurons treated with LPA and LPA + ROCKi versus non-treated (Ctrl) neurons at 48h before and after an injury. In the heatmap, red indicates higher expression, green represents lower expression, and black represents no significant alterations. Comparison of signaling pathways before and after injury with LPA, LPA + ROCKi, and ROCKi treatment. LPA treatment led to an upregulation of *Lpar2* levels before the injury, while *Lpar2* was found downregulated after the injury and regrowth. Following the nerve injury, not only the RhoA-ROCK pathway but also *Ras*, *Mapk*, and *Plc* signaling factors were elevated by LPA + ROCKi. Notably, *Gap-43* and *Cdc42* levels were increased by LPA and ROCKi.

3.1.5 Deciphering the receptor responsible for LPA effects on neuronal regeneration

Since LPA showed a suppressive effect on nerve fiber length, a siRNA transfection was performed targeting *Lpar1*, *Lpar2*, and *Lpar4* to elucidate the responsible receptor for the underlying LPA-ROCK signaling pathway affecting nerve regeneration. *Lpar1*, 2, and 4 were knocked down (KD). To establish the optimal time for siRNA transfection and their specificity, we conducted transfection at two different time points, 24 and 48h. The highest efficiency of downregulation was achieved after 48h of transfection with the residual expression of 72% ($p = 0.00013$) for *Lpar1*-KD, 81% for *Lpar2*-KD ($p = 0.00019$), and 63% for *Lpar4*-KD ($p = 0.003$) versus the scramble control (scr). Additionally, this approach allowed us to determine the specificity of the siRNA by assessing its impact on other receptors (Figure 9A-C). Based on this result, 48h transfection time points were selected for the subsequent experiments.

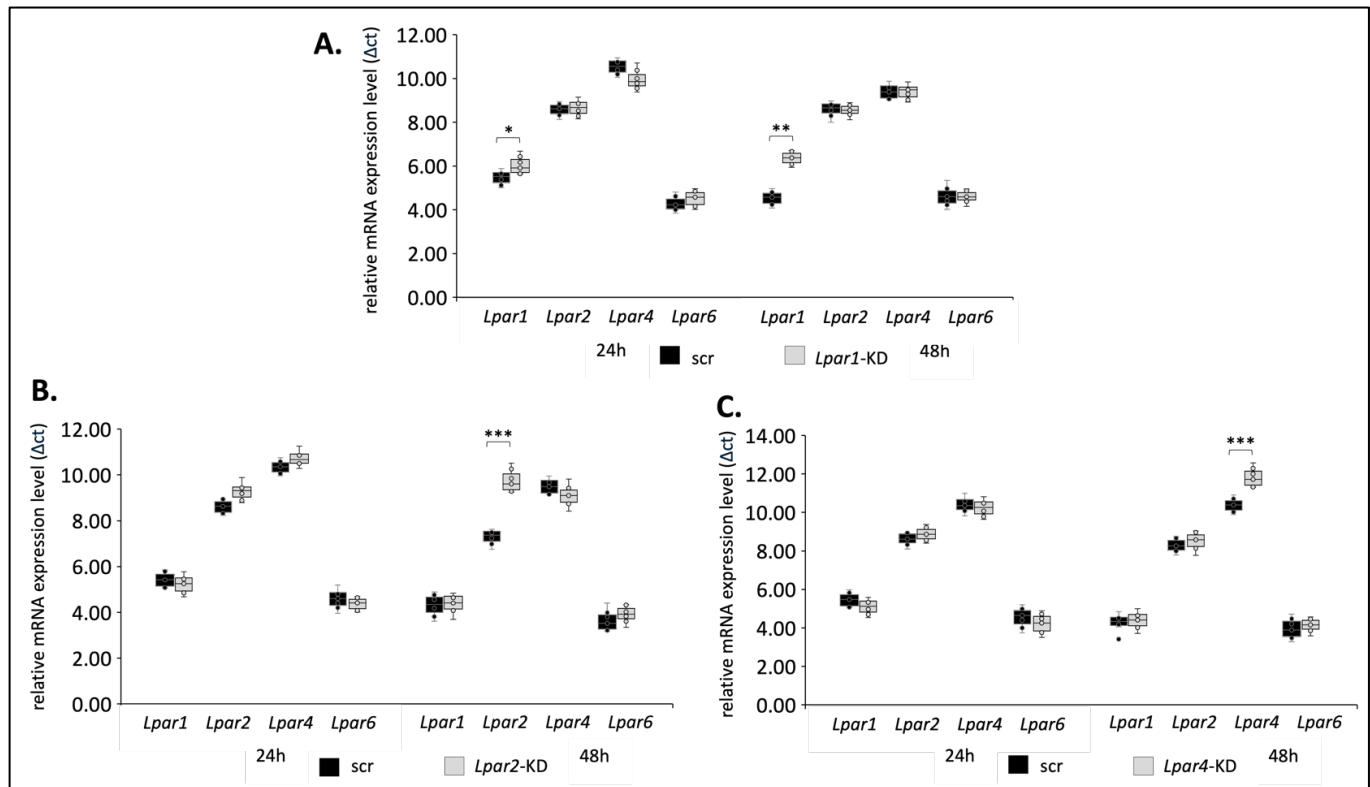


Figure 9: The efficiency and specificity of siRNA transfection.

The knockdown efficacy was assessed on transfected neurons using qRT-PCR relative to the scr at 24 and 48h post-transfection, with the highest efficiency after 48h for A) *Lpar1*, B) *Lpar2*, and C) *Lpar4*. The data are presented as mean $\pm$ SEM, and statistical significance is indicated by p-values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The functional analysis using the single-cell assay revealed that in *Lpar1*-KD cells, no significant difference in fiber growth between the scr and *Lpar1*-KD cells following LPA treatment. Furthermore, LPA+ROCKi led to a significant reduction in fiber length at 48h ($2.91 \pm 0.36 \mu\text{m}$, $p = 0.010$) compared to the scr-LPA+ROCKi ($4.43 \pm 0.45 \mu\text{m}$) (Figure 10), indicating no influence of LPAR1 on the effect of LPA on nerve regeneration. In contrast, *Lpar2*-KD significantly increased the fiber length when adding LPA ($7.04 \pm 0.89 \mu\text{m}$, $p < 0.0001$) compared to scr-LPA neurons ($2.81 \pm 0.21 \mu\text{m}$) after 48h, suggesting that the inhibitory effect of LPA on fiber growth is mediated via the LPAR2. Moreover, scr-LPA+ROCKi neurons after 48h exhibited significant enhancement in fiber length ($4.43 \pm 0.45 \mu\text{m}$, $p < 0.0016$) versus scr-LPA neurons ($2.81 \pm 0.21 \mu\text{m}$). Furthermore, no significant changes were observed between the *Lpar2*-KD-LPA group ($7.04 \pm 0.89 \mu\text{m}$, $p = 0.17$) and *Lpar2*-KD-LPA+ROCKi neurons ($5.67 \pm 0.56 \mu\text{m}$) (Figure 10). No alterations were found for *Lpar4*-KD cells at both time points (Figure 10). The focus of the analysis was on the comparative differences between the scramble and KD groups, therefore, significant alterations between different treatments of scr groups (as well as KD neurons) can be found in Table 19-Table 21.

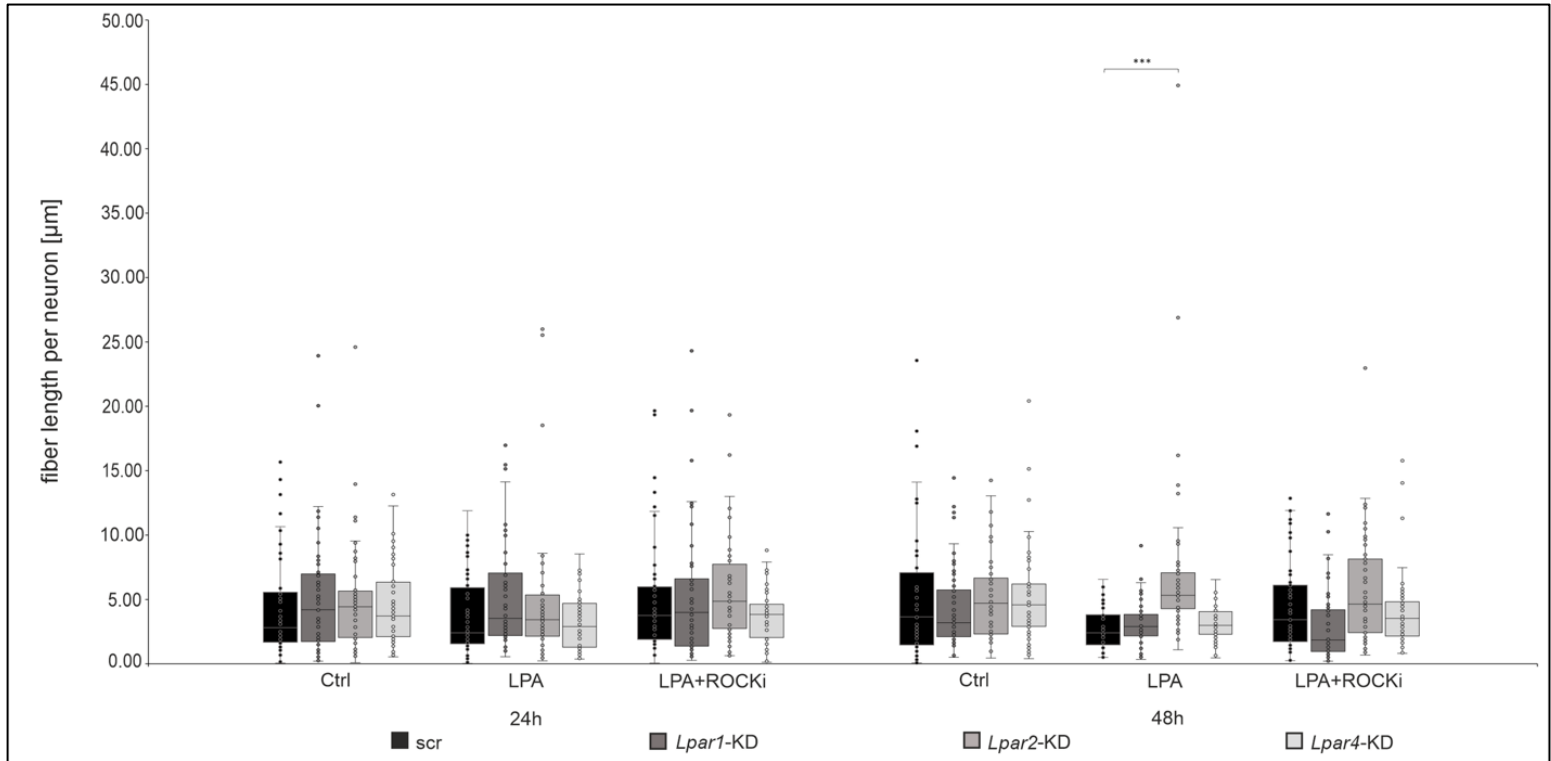


Figure 10: Effect of LPA receptors knockdown on DRG fiber length by single-neuron assay.

Effect of *Lpar1*-, *Lpar2*-, and *Lpar4*-KD on total fiber length per neuron by single-cell assay versus the scramble group. A) Neuronal fiber length measurement per neuron after *Lpar1*-KD displayed no significant effect in fiber length of neurons at 24h, whereas *Lpar1*-KD following LPA+ROCKi treatment after 48h showed a significant reduction compared to scr control. B) Total fiber length per neuron after *Lpar2*-KD revealed no changes after 24h, while a significant enhancement in fiber length of LPA-treated neurons following *Lpar2*-KD was observed compared to the scr-LPA cells after 48h. C) *Lpar4*-KD exhibited no significant effect on fiber length at 24h and 48h after transfection. The data are presented as mean $\pm$ SEM, and statistical significance is indicated by p-values ** $p \leq 0.01$, *** $p \leq 0.001$. Significance was determined in comparison to the results at 0h. Data regarding the significances between different treatments in the scr groups, as well as in KD neurons, can be found in Table 19-Table 21.

Table 19: Statistical p-values in single-cell assay with *Lpar1*-KD neurons.

Group	p-value (48h)
scr (Ctrl) vs scr (LPA)	0.002
scr (Ctrl) vs <i>Lpar1</i> -KD (LPA)	0.007
scr (Ctrl) vs <i>Lpar1</i> -KD (LPA+ROCKi)	0.005
scr (LPA) vs <i>Lpar1</i> -KD (Ctrl)	0.002
<i>Lpar1</i> -KD (Ctrl) vs <i>Lpar1</i> -KD (LPA)	0.016
<i>Lpar1</i> -KD (Ctrl) vs <i>Lpar1</i> -KD (LPA+ROCKi)	0.013
scr (LPA) vs scr (LPA+ROCKi)	0.002
<i>Lpar1</i> -KD (LPA) vs scr (LPA+ROCKi)	0.012

Table 20: Statistical p-values in single-cell assay with *Lpar2*-KD neurons.

Group	p-value (24h)
scr (LPA) vs <i>Lpar2</i> -KD (LPA+ROCKi)	0.009

Group	p-value (48h)
scr (Ctrl) vs scr (LPA)	0.0017
<i>Lpar2</i> -KD (Ctrl) vs scr (LPA)	2.15E-05
scr (LPA) vs <i>Lpar2</i> -KD (LPA+ROCKi)	1.31E-05
scr (LPA) vs scr (LPA+ROCKi)	0.0016
<i>Lpar2</i> -KD (Ctrl) vs <i>Lpar2</i> -KD (LPA)	0.038

Table 21: Statistical p-values in single-cell assay with *Lpar4*-KD neurons

Group	p-value (24h)
<i>Lpar4</i> -KD (LPA) vs scr (LPA+ROCKi)	0.014
<i>Lpar4</i> -KD (Ctrl) vs <i>Lpar4</i> -KD (LPA)	0.014

Group	p-value (48h)
scr (Ctrl) vs scr (LPA)	0.002
scr (Ctrl) vs <i>Lpar4</i> -KD (LPA)	0.004
<i>Lpar4</i> -KD (Ctrl) vs scr (LPA)	3.25E-05
scr (LPA) vs scr (LPA+ROCKi)	0.0016
scr (LPA) vs <i>Lpar4</i> -KD (LPA+ROCKi)	0.006
<i>Lpar4</i> -KD (LPA) vs <i>Lpar4</i> -KD (LPA+ROCKi)	0.02
<i>Lpar4</i> -KD (Ctrl) vs <i>Lpar4</i> -KD (LPA)	0.00012
<i>Lpar4</i> -KD (LPA) vs scr (LPA+ROCKi)	0.005

Given the observed results of the single-cell assay, particularly the significant effects of LPA and LPA+ROCKi 48h after *Lpar2* and *Lpar4* knockdown, respectively, on nerve fiber length, the wound-healing assay focused on LPAR2 and LPAR4 to investigate not only regrowth but regeneration in neurons (Figure 11A). In line with the single-cell results, the knockdown of *Lpar2* led to a significant increase in fiber outgrowth ($8747.75 \pm 1376.21 \mu\text{m}$, $p = 0.0009$ at 24h and $8554.46 \pm 559.22 \mu\text{m}$, $p = 0.0005$ at 48h) compared to the scr-LPA ($2742.13 \pm 300.39 \mu\text{m}$ at 24h and $4699.15 \pm 631.29 \mu\text{m}$, at 48h). No significant alterations were found for *Lpar4*-KD cells at both time points (Figure 11B). The focus of the analysis was on the comparative differences between the scramble and knockdown groups, therefore, significant changes between different treatments of the scr groups (as well as KD neurons) can be found in Table 22-Table 23.

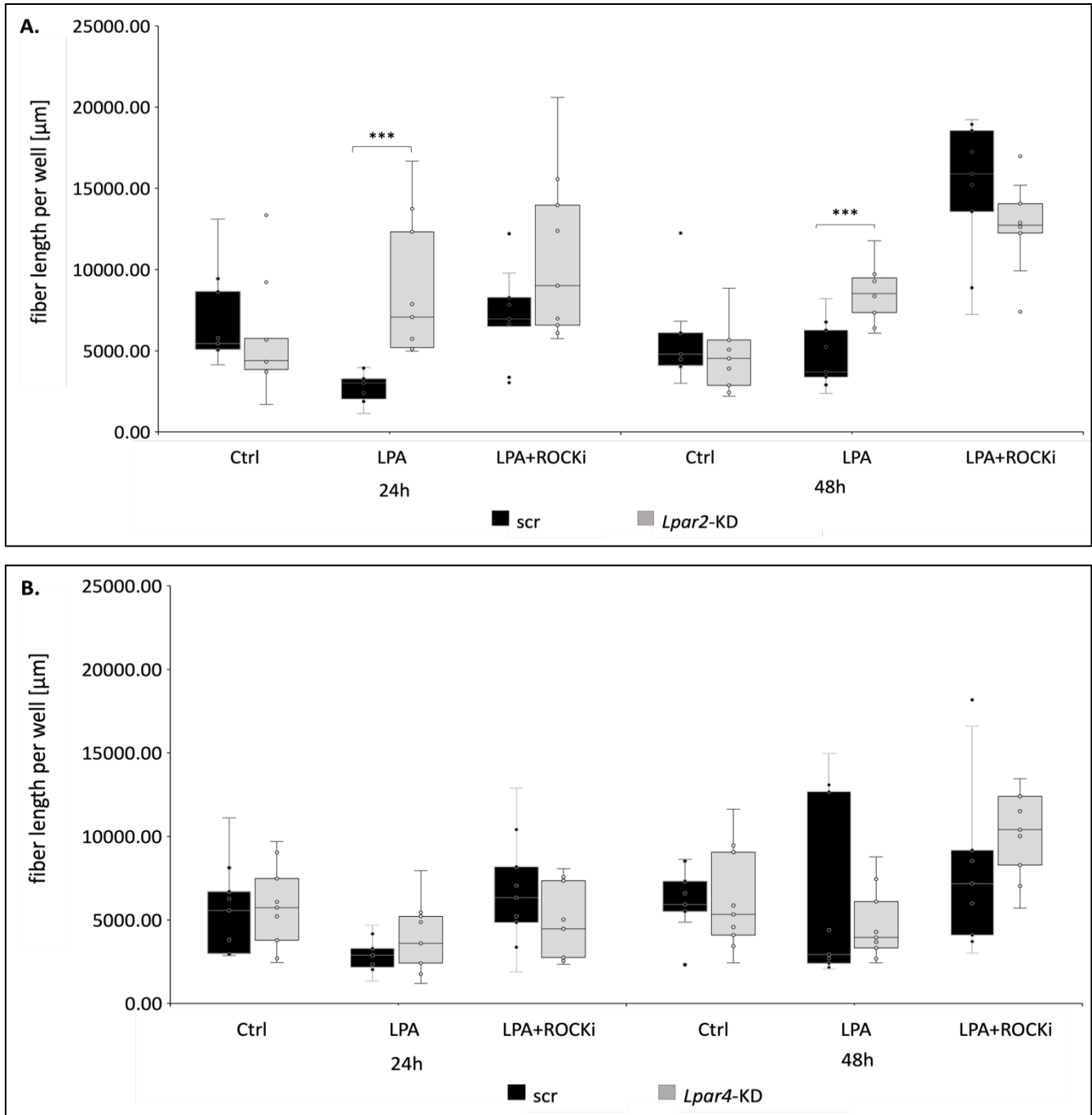


Figure 11: Effect of LPA receptors knockdown on DRG fiber outgrowth by wound-healing assay.

The effect of *Lpar2*-KD and *Lpar4*-KD on nerve regeneration by wound-healing assay versus the scramble group. Re-growing of neuronal fiber per well in transfected neurons with siRNA against *Lpar2* by wound-healing assay revealed that *Lpar2*-KD significantly increased total nerve re-growing per well in LPA-treated neurons compared to the scr-LPA group 24h and 48h after transfection. No significant changes were found for *Lpar4*-KD cells at both time points. The data are presented as mean $\pm$ SEM, and statistical significance is indicated by p-values *** $p \leq 0.001$. Data for 24 and 48h were normalized to the corresponding data for each substance at 0h. Data regarding the significances between different treatments in the scr groups, as well as in KD neurons, can be found in Table 22-Table 23.

Table 22: Statistical p-values in wound-healing assay with *Lpar2*-KD neurons.

Group	p-value (24h)
scr (LPA) vs scr (LPA+ROCKi)	0.0004
scr (Ctrl) vs scr (LPA)	0.0009
<i>Lpar2</i> -KD(Ctrl) vs scr (LPA)	0.02
<i>Lpar2</i> -KD (Ctrl) vs <i>Lpar2</i> -KD (LPA+ROCKi)	0.02
scr (LPA) vs <i>Lpar2</i> -KD (LPA+ROCKi)	0.0003

Group	p-value (48h)
<i>Lpar2</i> -KD (LPA+ROCKi) vs scr (LPA)	3.24E-06
<i>Lpar2</i> -KD (Ctrl) vs <i>Lpar2</i> -KD (LPA)	0.0005
<i>Lpar2</i> -KD (Ctrl) vs <i>Lpar2</i> -KD (LPA+ROCKi)	3.23E-06
<i>Lpar2</i> -KD (LPA) vs <i>Lpar2</i> -KD (LPA+ROCKi)	0.0018
scr (Ctrl) vs scr (LPA+ROCKi)	5.17E-05
scr (LPA) vs scr (LPA+ROCKi)	8.36E-06
scr (Ctrl) vs <i>Lpar2</i> -KD (LPA)	0.015
scr (Ctrl) vs <i>Lpar2</i> -KD (LPA+ROCKi)	5.5E-05
<i>Lpar2</i> -KD (Ctrl) vs scr (LPA+ROCKi)	8.02E-06
<i>Lpar2</i> -KD (LPA) vs scr (LPA+ROCKi)	0.0008

Table 23: Statistical p-values in wound-healing assay with *Lpar4*-KD neurons.

Group	p-value (24h)
<i>Lpar4</i> -KD (Ctrl) vs scr (LPA)	0.006
scr (Ctrl) vs scr (LPA)	0.015
scr (LPA) vs <i>Lpar4</i> -KD (LPA+ROCKi)	0.044
scr (LPA) vs scr (LPA+ROCKi)	0.006

Group	p-value (48h)
scr (Ctrl) vs <i>Lpar4</i> -KD (LPA+ROCKi)	0.002
<i>Lpar4</i> -KD (Ctrl) vs <i>Lpar4</i> -KD (LPA+ROCKi)	0.010
<i>Lpar4</i> -KD (LPA) vs <i>Lpar4</i> -KD (LPA+ROCKi)	0.0002

3.1.6 Effect of *Lpar2*-KD on LPA pathway in non-injured and injured neurons

To elucidate the effect of *Lpar2*-KD on nerve regeneration, mRNA analysis was conducted for non-injured and injured transfected neurons (Figure 12). This experiment involved four experimental groups: scramble (scr, set as 1), non-treated *Lpar2*-KD neurons (*Lpar2*-KD), LPA-treated scramble neurons (scr-LPA), and LPA-treated *Lpar2*-KD neurons (*Lpar2*-KD-LPA), and the results were evaluated as n-fold. The *Lpar2* expression level was significantly downregulated in non-injured (0.65 ± 0.09) and injured *Lpar2*-KD neurons (0.52 ± 0.23) compared to scr ($p = 0.04$) and scr-LPA groups (0.96 ± 0.07 , $p = 0.02$ for non-injured and 1.6 ± 0.14 , $p = 0.008$ following the injury), verifying the successful knockdown. *Enpp2*, the ATX gene, was downregulated in the *Lpar2*-KD-LPA group post-injury (0.3 ± 0.06) versus *Lpar2*-KD (1.11 ± 0.05 , $p = 0.0001$) and scr ($p = 0.0001$), indicating its relevance in regeneration. *Ras* (1.68 ± 0.07 , $p = 0.0013$) and *Pi3k* (1.21 ± 0.02 , $p = 0.006$) were significantly upregulated in *Lpar2*-KD neurons before the injury versus scr group. Signaling pathway factors before the injury, *Ras* (1.89 ± 0.08 , $p = 0.03$), *Pi3k* (1.18 ± 0.06 , $p = 0.03$), and *Mapk* (1.36 ± 0.14 , $p = 0.03$) expressions also showed an elevation in the *Lpar2*-KD-LPA group versus scr-LPA (1.08 ± 0.19 for *Ras*; 0.7 ± 0.19 for *Pi3k*; and 0.6 ± 0.29 for *Mapk*). However, post-injury, *Ras* (1.31 ± 0.03 , $p = 0.016$), *Rac* (1.53 ± 0.19 , $p = 0.05$) and *Mapk* (1.25 ± 0.06 , $p = 0.011$) were also elevated in the *Lpar2*-KD neurons compared to scr-LPA (0.6 ± 0.25 for *Ras*; 0.93 ± 0.12 for *Rac*; and 0.87 ± 0.06 for *Mapk*) group and compared to scr for *Ras* ($p = 0.018$) and *Pi3k* (1.62 ± 0.03 , $p = 0.0003$). In the *Lpar2*-KD-LPA group post-injury, only *Pi3k* showed an upregulation (1.32 ± 0.06 , $p = 0.013$) compared to scr neurons (Figure 12). The regeneration-associated factors *Gdnf* (25.64 ± 0.04 , $p = 0.00011$), *Ngf* (1.74 ± 0.19 , $p = 0.03$), and *Gap-43* (1.49 ± 0.14 , $p = 0.04$), were found upregulated in *Lpar2*-KD non-injured neurons compared to scr group, and *Calca* (1.13 ± 0.06 , $p = 0.04$) and *Tac1* (2.38 ± 0.11 , $p = 0.005$) levels showed an increase in the *Lpar2*-KD-LPA treatment group in comparison with scr-LPA (0.78 ± 0.12 for *Calca*, and 1.24 ± 0.1 for *Tac1*). Additionally, post-injury, a significant upregulation in *Calca*, *Gdnf*, *Cntf*, *Ngf*, *Gap-43*, and *Cdc42* was observed in both *Lpar2*-KD (0.68 ± 0.14 *Calca*; 1.42 ± 0.14 *Ngf*; 3.28 ± 0.22 *Gap-43*; and 1.66 ± 0.05 *Cdc42*) versus scr-LPA (0.06 ± 0.14 , $p = 0.0002$

Calca; 0.99 ± 0.27 , $p = 0.017$ *Gap-43*; and 0.98 ± 0.11 , $p = 0.008$ *Cdc42*) and scr ($p = 0.04$ *Ngf*; $p = 0.02$ *Gap-43*; $p = 0.02$ *Cdc42*) and *Lpar2*-KD-LPA neurons (0.83 ± 0.18 *Calca*; 1.48 ± 0.03 *Gdnf*; 2.74 ± 0.27 *Cntf*; 1.93 ± 0.24 *Ngf*; and 2.11 ± 0.13 *Gap-43*) versus scr-LPA ($p = 0.0002$ *Calca*; $p = 0.02$ *Gdnf*; $p = 0.04$ *Cntf*; $p = 0.04$ *Gap-43*) and scr ($p = 0.012$ *Gdnf*; $p = 0.03$ *Cntf*; $p = 0.04$ *Ngf*). Significant downregulation of *Tac1* was detected by *Lpar2*-KD-LPA (0.35 ± 0.16 , $p = 0.008$) versus scr group (Figure 12). Overall, when directly comparing non-injured with injured neurons, distinct expression patterns were observed versus the scramble control groups. Before the injury, *Lpar2*-KD led to increased levels of *Ras*, *Pi3k*, *Gdnf*, *Ngf*, and *Gap-43*; whereas after the injury, elevated levels of *Ras*, *Pi3k*, *Ngf*, *Gap-43*, and *Cdc42* were noted. In terms of *Lpar2*-KD-LPA neurons compared to scramble control in both non-injured and injured neurons, an upregulation of *Ras*, *Pi3k*, *Mapk*, *Calca*, and *Tac1* were observed in non-injured neurons, while after an injury, there was an upregulation of *Pi3k*, *Calca*, *Gdnf*, *Cntf*, *Ngf*, and *Gap-43* while *Enpp2* and *Tac1* were downregulated.

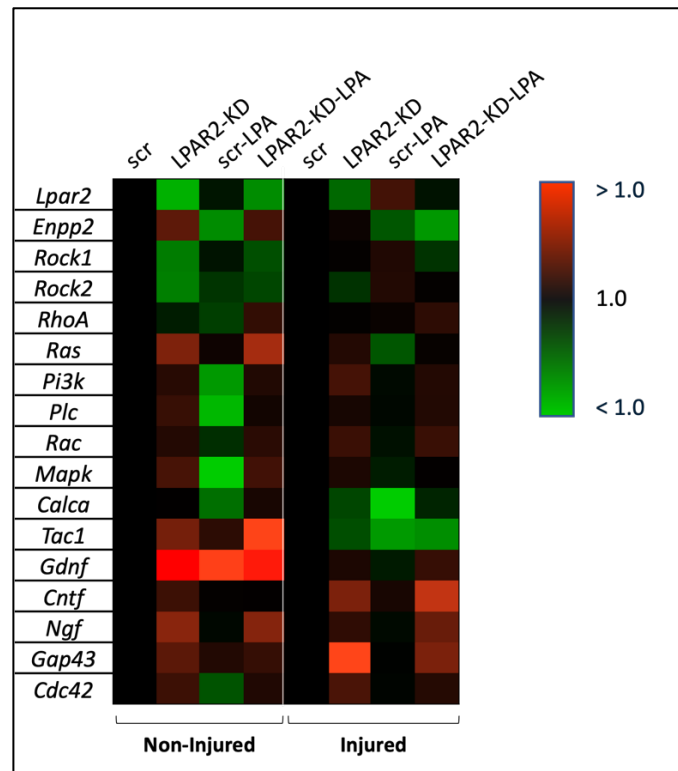


Figure 12: mRNA expression in injured and non-injured transfected-DRG neurons.

Heatmap showing different gene expression in *Lpar2* knockdown neurons with and without LPA treatment versus scramble control (scr) neurons at 48h before and after injury. In the heatmap, red indicates higher expression, green states lower expression, and black represents no significant alterations. A downregulation of *Lpar2* was found in *Lpar2*-KD neurons. In *Lpar2*-KD-LPA neurons, an injury-induced reduction of *Enpp2* was observed. An upregulation of *Ras*, *Pi3k*, and *Mapk* in *Lpar2*-KD and *Lpar2*-KD-LPA cells, pre/post nerve injury was also found. *Lpar2*-KD following LPA treatment led to an enhancement in *Calca*, *Tac1*, and *Gdnf* levels, while following the nerve injury, *Calca*, *Gdnf*, *Cntf*, *Ngf*, *Gap-43*, and *Cdc42* were upregulated.

3.2 Objective 3: Nerve regeneration investigation on LPA-treated neurons in co-culture with hLESCs

The third objective of this thesis was to evaluate whether co-culture of DRG neurons with hLESCs modulates the effects of LPA signaling and ROCK inhibition on nerve regeneration, including experiments with LPA receptor knockdown. This model was designed to better recapitulate the corneal microenvironment by including neuro-epithelial interactions, which are essential for maintaining corneal homeostasis. In the previous section, LPAR2 was identified as the key receptor mediating the suppressive effect of LPA on neurite outgrowth in DRG neuron cultures. Based on this finding, the current section examines whether the presence of hLESCs modulates the LPAR2-dependent response. The aim was to assess whether hLESCs-secreted factors could rescue neurons from the negative effects of LPA.

3.2.1 Functional effect of LPA on DRG neurons co-cultured with hLESCs

To assess the impact of LPA and LPA+ROCKi in the co-culture system, a single-cell assay was first performed on DRG neurons co-cultured with hLESCs to evaluate neurite fiber length/outgrowth. In the single-cell assay at 24h, neurite fiber length in the LPA-treated co-cultured neuron ($1.57 \pm 0.17 \mu\text{m}$) showed no significant decrease compared to the Ctrl ($2.00 \pm 0.18 \mu\text{m}$, $p = 0.086$); however, LPA treatment in DRG alone resulted in significantly reduced neurite length ($0.84 \pm 0.11 \mu\text{m}$), indicating a protective effect of hLESCs for neurons in the co-culture model. In contrast, ROCKi led to a significant elevation in neurite length in co-culture ($3.82 \pm 0.41 \mu\text{m}$, $p \leq 0.00012$) versus Ctrl. LPA+ROCKi resulted in a significant increase in fiber length in co-culture ($2.88 \pm 0.29 \mu\text{m}$) compared to LPA-treated neurons ($p \leq 0.00022$) and the Ctrl group ($p = 0.0128$) (Figure 13).

At 48h, LPA in co-culture significantly reduced neurite length ($1.52 \pm 0.15 \mu\text{m}$) compared to Ctrl ($2.33 \pm 0.19 \mu\text{m}$, $p \leq 0.0014$); however, this reduction was less severe than in DRG neurons

treated with LPA ($1.03 \pm 0.12 \mu\text{m}$), again suggesting a rescuing effect of hLESCs. ROCKi led to a significant elevation in neurite length in co-culture ($4.79 \pm 0.55 \mu\text{m}$, $p \leq 0.0001$) versus Ctrl. Additionally, LPA+ROCKi in co-culture ($2.77 \pm 0.30 \mu\text{m}$, $p \leq 0.00043$) significantly mitigated the suppressive effect of LPA compared to LPA, although neurite length remained significantly lower than in the ROCKi group ($p = 0.0019$) (Figure 13). Both ROCKi and LPA+ROCKi treatments in co-culture showed similar effects to ROCKi and LPA+ROCKi-treated DRG neurons in mono-culture, at both 24h and 48h time points.

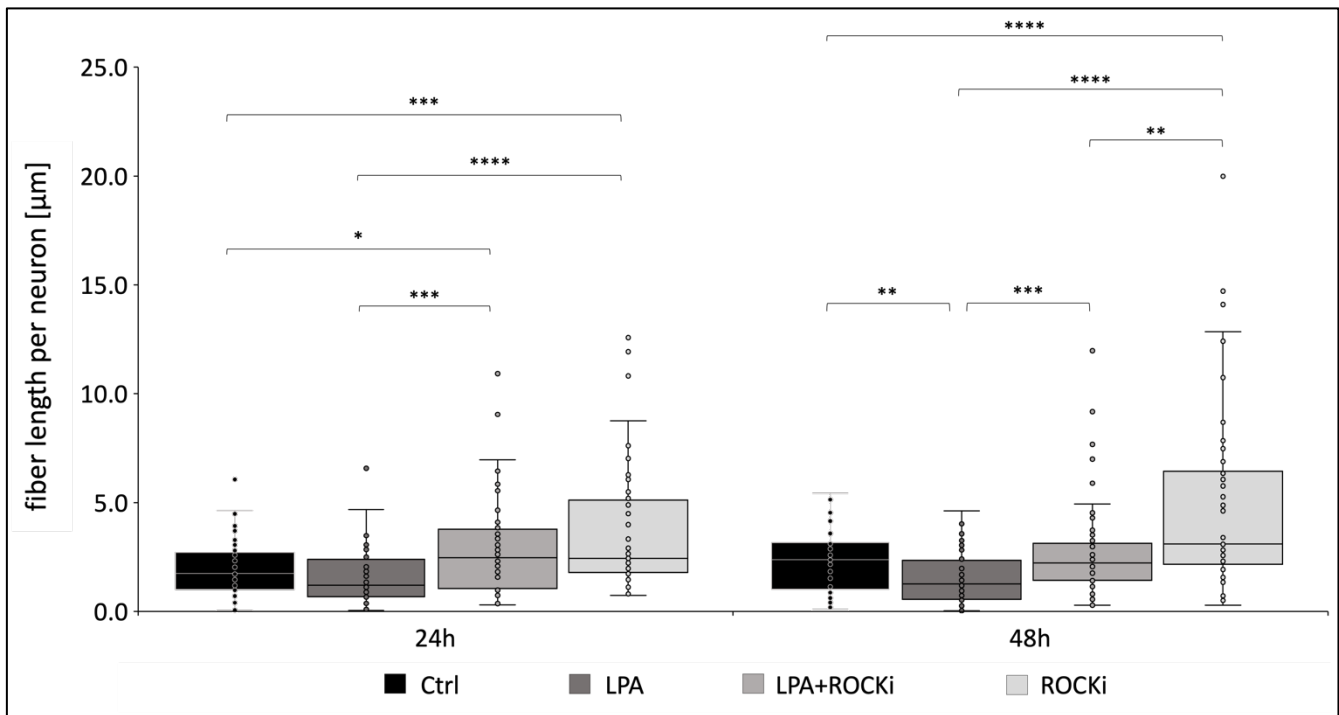


Figure 13: Effect of LPA on fiber length of co-cultured neurons with hLESCs by single-cell assay.

Neuronal fiber length measurement per neuron in coculture with hLESCs in a single-cell assay displayed no suppressive effect in fiber length following LPA treatment at 24h versus Ctrl, while a significant reduction was exhibited at 48h. ROCKi highly enhanced neurite length at both time points, and LPA+ROCKi effectively rescued fiber length suppressed by LPA. The data are presented as mean $\pm$ SEM and were normalized to the corresponding data for each substance at 0h. Statistical significance is indicated by p-values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

In the wound-healing assay at 24h, neurite regrowth in LPA-treated neurons ($3204.90 \pm 413.21 \mu\text{m}$) exhibited no significant reduction compared to Ctrl ($4424.33 \pm 540.06 \mu\text{m}$, $p = 0.110$), while the LPA+ROCKi group showed significant elevation ($5167.40 \pm 621.68 \mu\text{m}$) versus the LPA ($p = 0.0247$). After 48h, LPA treatment again resulted in a non-significant reduction of fiber regrowth ($3469.48 \pm 237.53 \mu\text{m}$) compared to Ctrl ($5071.32 \pm 677.37 \mu\text{m}$, $p = 0.0516$), whereas LPA + ROCKi significantly enhanced regrowth ($9295.54 \pm 801.47 \mu\text{m}$), exceeding both Ctrl ($p = 0.0016$) and LPA ($p \leq 0.0001$) groups (Figure 14).

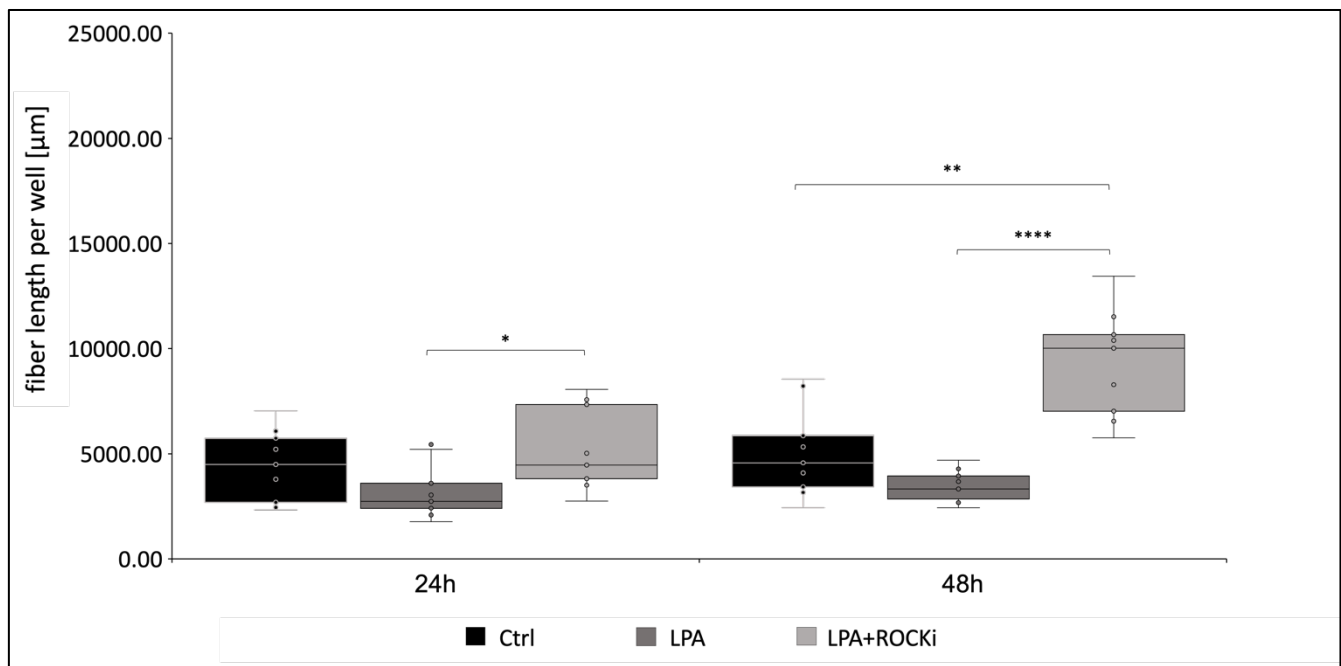


Figure 14: Effect of LPA on fiber length of co-cultured neurons with hLESCs by wound-healing assay.

Neuronal fiber outgrowth measurement per well in coculture with hLESCs by wound-healing assay exhibited no significant alteration for LPA-treated neurons compared to Ctrl at 24h and 48h. LPA+ROCKi significantly elevated fiber regrowth compared to LPA at both time points, and compared to control at 48h. The data are presented as mean $\pm$ SEM and were normalized to the corresponding data for each substance at 0h. Statistical significance is indicated by p-values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

3.2.2 mRNA analysis in LPA-treated co-cultured neurons post-injury

Gene expression analysis was performed 48h post-injury to evaluate transcriptional alterations in regenerating neurons co-cultured with human LSCs. Non-treated injured samples were used as control and set as 1. The results were evaluated as n-fold (Figure 15). The mRNA analysis in this section is based on samples derived from the wound-healing assay. In the co-cultured DRG neurons, expression of *Lpar2* was significantly downregulated by LPA treatment (0.63 ± 0.03 ; $p = 0.0005$) compared to Ctrl neurons, whereas no significant alterations were detected for *Lpar1*, *Lpar4*, or *Lpar6*. *Enpp2* expression remained unchanged across conditions. Significant downregulation of *Rock1*, *Rock2*, and *RhoA* in the LPA+ROCKi group was detected (0.74 ± 0.04 ; $p = 0.0071$; 0.63 ± 0.04 ; $p = 0.0014$, and 0.73 ± 0.04 ; $p = 0.0281$, respectively) versus Ctrl group. No changes were observed in *Enpp2* expression. *Ras*, *Pi3k*, *plc*, and *Mapk* were significantly upregulated in the LPA+ROCKi group compared to Ctrl group (1.35 ± 0.06 , $p = 0.0277$; 1.77 ± 0.06 , $p = 0.0012$; 1.57 ± 0.06 , $p = 0.0012$; and 1.44 ± 0.06 , $p = 0.0019$, respectively). Additionally, *Pi3k*, *plc*, and *Mapk* levels were significantly elevated in LPA+ROCKi treated neurons compared to the LPA group ($p = 0.0114$, $p = 0.0120$, and $p = 0.0045$, respectively). LPA significantly reduced *Mapk* (0.87 ± 0.01 , $p = 0.0007$) versus the Ctrl group. No significant changes were detected in *Rac* expression (Figure 15).

Regarding neuropeptides, *Cac1ca* was significantly upregulated in LPA+ROCKi treated neurons (1.44 ± 0.06) compared to LPA (0.87 ± 0.01 ; $p = 0.0387$) and Ctrl ($p \leq 0.0001$). No alterations were detected for *Tac1* expression. *Gdnf* was significantly increased in both LPA (1.38 ± 0.08 ; $p = 0.0195$) and LPA+ ROCKi treatment groups (1.43 ± 0.11 ; $p = 0.0326$) versus Ctrl group. *Cntf* levels were significantly elevated by LPA+ROCKi treated neurons (1.27 ± 0.07 ; $p = 0.0366$) in comparison to Ctrl neurons. *Ngf* expression increased strongly with LPA (1.38 ± 0.19 ; $p \leq 0.0001$) and further in LPA+ROCKi groups (1.73 ± 0.07 ; $p = 0.0009$ versus Ctrl, $p = 0.0139$ versus LPA group) (Figure 15).

The regeneration marker *Gap43* showed a significant upregulation in both LPA (1.79 ± 0.01 ; $p = 0.0006$) and LPA+ROCKi groups (2.01 ± 0.25 ; $p = 0.0300$) compared to Ctrl neurons. *Cdc42* was similarly upregulated under both conditions, LPA (1.34 ± 0.09 in, $p = 0.0392$) and LPA+ROCK groups (1.76 ± 0.18 , $p = 0.0249$) versus the Ctrl group (Figure 15).

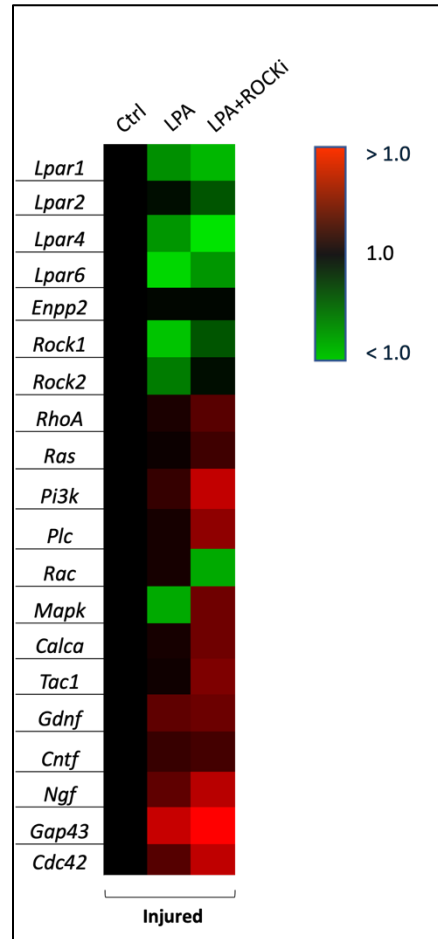


Figure 15: mRNA expression in injured DRG neurons co-cultured with hLESCs.

Heatmap showing differential gene expression in neurons co-cultured with human hLESCs treated with LPA and LPA+ROCKi versus untreated control at 48h after injury. Red indicates upregulation, green indicates downregulation, and black represents no significant change. *Lpar2* was downregulated in the LPA group, while *Rock1*, *Rock2*, and *RhoA* were reduced in the LPA+ROCKi group. *Ras*, *Pi3k*, *plc*, and *Mapk* were upregulated in LPA+ROCKi-treated neurons, with *Pi3k*, *plc*, and *Mapk* further elevated compared to LPA alone. LPA alone modestly increased *Mapk*. An upregulation of *Calca*, *Gdnf*, *Cntf*, *Ngf*, *Gap43*, and *Cdc42* was detected in LPA and/or LPA+ROCKi-treated groups. *Tac1*, *Lpar1*, *Lpar4*, *Lpar6*, *Enpp2*, and *Rac* showed no significant alterations.

3.2.3 Gene expression in hLESCs co-cultured with LPA-treated neurons post-injury

After investigating mRNA expression in injured neurons co-cultured with hLESCs (Figure 15), we next examined the mRNA expression profile of the hLESCs. This aimed to evaluate possible signaling pathway changes in hLESCs when exposed to injured neurons and treated with LPA or LPA+ROCKi (Figure 16). QRT-PCR analysis revealed significant transcriptional changes in hLESCs after 48h of co-culture with injured neurons treated with LPA and LPA+ROCKi, and untreated injured neurons were used as Ctrl) and set as 1. *LPAR2* and *LPAR4* expression were significantly downregulated in both LPA (0.54 ± 0.03 and 0.43 ± 0.01 , respectively) and LPA+ROCKi groups (0.30 ± 0.02 and 0.35 ± 0.09 , respectively) compared to the Ctrl (*LPAR2*: $p = 0.0002$ and $p \leq 0.0001$; *LPAR4*: $p \leq 0.0001$ and $p = 0.0034$, respectively). Additionally, *LPAR2* expression significantly decreased in the LPA group versus the LPA+ROCKi group ($p = 0.0064$). However, *LPAR3* was significantly upregulated in the LPA group (1.34 ± 0.06 ; $p = 0.0107$) compared to Ctrl. No significant changes were detected in hLESCs for *LPAR5*, *LPAR6*, *ENPP2*, *ROCK1*, *ROCK2*, and *RHOA* expression in the LPA and LPA+ROCKi groups (Figure 16). The neuropeptides *CALCA* and *TAC1* were significantly downregulated in the hLESCs of the LPA group versus Ctrl (0.83 ± 0.01 ; $p = 0.0005$, and 0.34 ± 0.08 ; $p = 0.0367$, respectively), while no significant changes were observed in the LPA+ROCKi group. No significant alterations were observed for *GDNF*, *CNTF*, and *NGF*. Apoptotic marker, *CAS3* expression was significantly downregulated in both LPA (0.53 ± 0.07 ; $p = 0.0043$) and LPA+ROCKi groups (0.46 ± 0.06 ; $p = 0.0016$) versus Ctrl. No significant changes were detected for *BCL2* or *BAX* (Figure 16). Stemness- and differentiation-related markers were notably affected by LPA and LPA+ROCKi. *VIM* expression, as a marker of mesenchymal/stem-like cells, was significantly elevated in the LPA group (1.41 ± 0.03 ; $p = 0.0003$) and further increased in the LPA+ROCKi group (1.77 ± 0.03 ; $p \leq 0.0001$) compared to the Ctrl. Additionally, *VIM* levels were significantly upregulated in the LPA+ROCKi group compared to LPA treatment ($p = 0.0027$). *CK12* expression, the corneal epithelial marker, was significantly reduced in the LPA+ROCKi group (0.49 ± 0.06) compared

to both Ctrl ($p = 0.0042$) and LPA groups (0.80 ± 0.03 ; $p = 0.0423$). *CK15*, a marker of basal/stem-like epithelial cells, also showed a significant elevation in the LPA+ROCKi group (1.41 ± 0.11) compared to Ctrl ($p = 0.0390$). No significant differences were observed in *KI67*, *P63 α* , and *PAX6* expression, markers of proliferation, epithelial progenitors, and corneal epithelial marker, respectively (Figure 16).

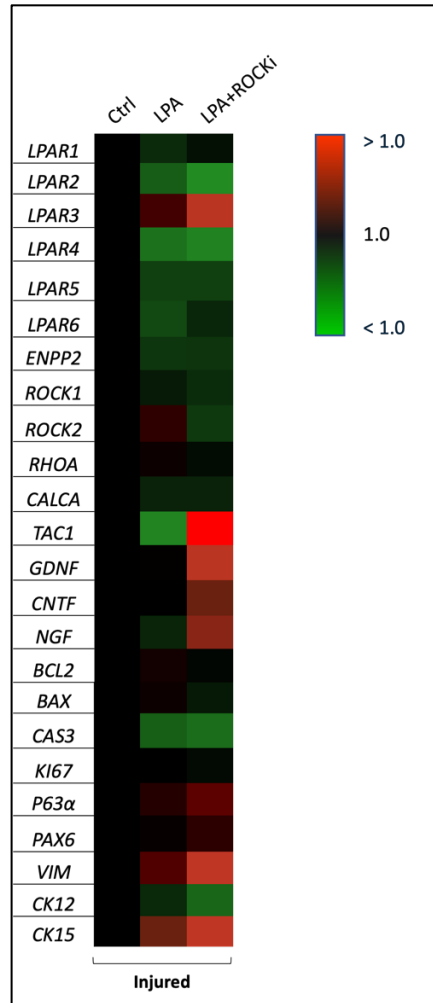


Figure 16: mRNA expression of hLESCs in co-culture with injured DRG neurons.

Heatmap showing differential gene expression in hLESCs co-cultured for 48h with injured DRG neurons treated with LPA and LPA+ROCKi compared to untreated samples (Ctrl). In the heatmap, red indicates higher expression, green exhibits lower expression, and black represents no significant alterations. LPA treatment led to a downregulation of *LPAR2* and *LPAR4*, however, *LPAR3* showed upregulation in the LPA group versus Ctrl. The Neuropeptides gene expression level, *CALCA*, and *Tac1*, were suppressed in the LPA-treated group of hLESCs in the co-culture with injured neurons. Apoptotic marker *CAS3* was significantly reduced in both treatment groups. Notably, *VIM* was significantly upregulated by LPA and further increased by LPA+ROCKi. In contrast, *CK12* was downregulated by LPA+ROCKi compared to both Ctrl and LPA. *CK15* was significantly upregulated in LPA+ROCKi. No significant alterations were observed for *LPAR5*, *LPAR6*, *ENPP2*, *ROCK1*, *ROCK2*, *RHOA*, *P63 α* , *PAX6*, *KI67*, *GDNF*, *CNTF*, *NGF*, *BCL2*, and *BAX*.

3.2.4 Functional effect of LPA on transfected DRG neurons co-cultured with hLESCs

In the previous sections, we evaluated nerve regeneration by single-cell and wound-healing assays on mono-cultured DRG neurons treated with LPA and LPA+ROCKi, before *Lpar2* knockdown (Figure 7), and after *Lpar2* knockdown (Figure 10 and Figure 11) and subsequently on DRG neurons co-cultured with hLESCs before *Lpar2* knockdown (Figure 13 and Figure 14). Here, in this section, we determine whether neurons co-cultured with hLESCs alters the enhanced nerve regeneration observed upon *Lpar2* knockdown. To this end, *Lpar2* knocked down DRG neurons were co-cultured with hLESCs, and their regenerative capacity was assessed using both single-cell assays and wound-healing assays at 24 and 48h (Figure 17 and Figure 18). Non-injured and untreated DRG neurons transfected with scramble siRNA (scr) in co-cultured with hLESCs served as the control group.

In the single-cell assay at 24h, neurite fiber length following LPA+ROCKi treatment was significantly reduced in the *Lpar2*-KD group ($3.63 \pm 0.48 \mu\text{m}$) compared to the scr-LPA+ROCKi group ($6.04 \pm 0.84 \mu\text{m}$; $p = 0.014$). No significant differences were observed between *Lpar2*-KD and scr groups ($2.87 \pm 0.37 \mu\text{m}$ vs. $3.24 \pm 0.41 \mu\text{m}$; $p = 0.5033$), or after LPA treatment ($2.73 \pm 0.29 \mu\text{m}$ vs. $3.38 \pm 0.37 \mu\text{m}$; $p = 0.1766$). At 48h, no significant differences in neurite fiber length were observed between *Lpar2*-KD ($4.83 \pm 0.69 \mu\text{m}$) and scr control group ($4.99 \pm 0.68 \mu\text{m}$; $p = 0.869$). Although LPA treatment slightly increased fiber length in *Lpar2*-KD neurons ($5.22 \pm 0.53 \mu\text{m}$) compared to scr-LPA group ($3.95 \pm 0.36 \mu\text{m}$, $p = 0.05$). The fiber length in LPA+ROCKi treatment in the *Lpar2*-KD group ($6.25 \pm 0.69 \mu\text{m}$) compared to the scr-LPA+ROCKi ($8.07 \pm 1.40 \mu\text{m}$) did not reach statistical significance ($p = 0.2504$) (Figure 17). The focus of the analysis was on the comparative differences between the scramble and *Lpar2*-KD groups, therefore, significant alterations between different treatments of the scr groups (as well as KD neurons) can be found in Table 24.

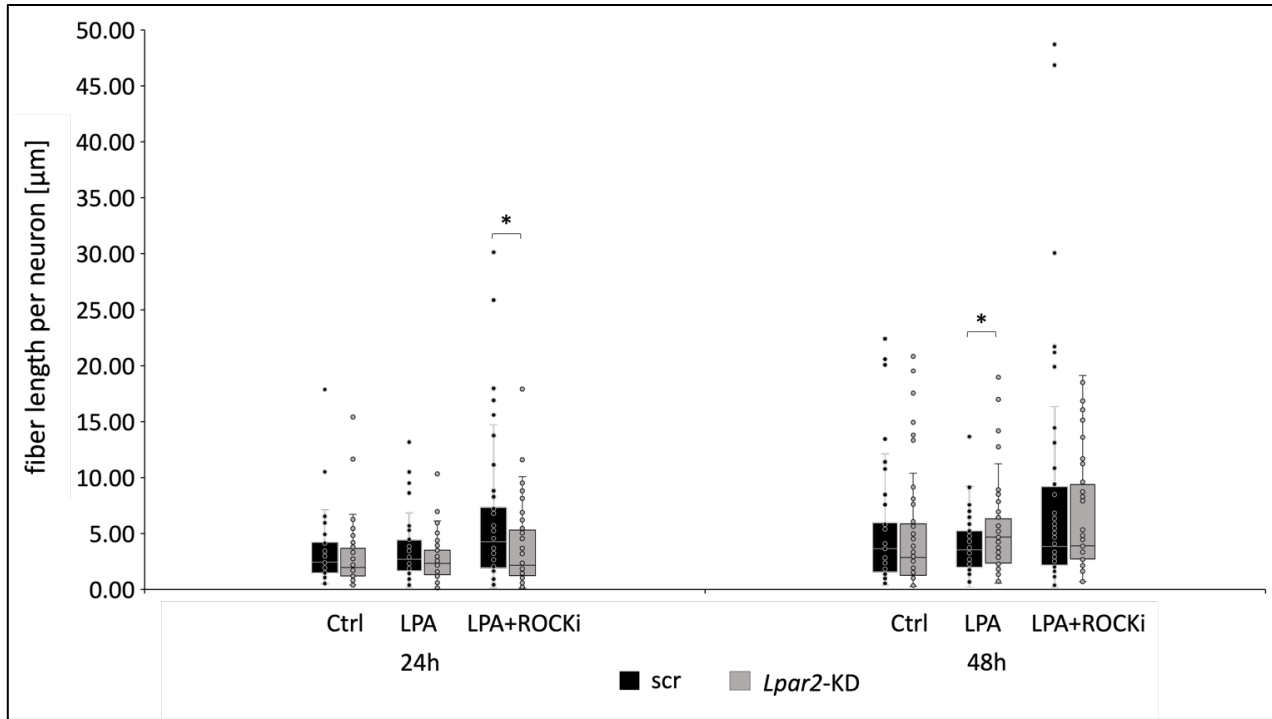


Figure 17: LPA effect on fiber length of co-cultured transfected neurons with hLESCs by single-cell assay.

Neuronal fiber length measurement per transfected neuron in co-culture with hLESCs in single-cell assay displayed that at 24h, *Lpar2*-KD neurons treated with LPA+ROCKi exhibited a significant reduction in fiber length compared to the scr Ctrl under the same condition. No significant differences were observed between *Lpar2*-KD and scr groups in Ctrl group or following LPA treatment alone. At 48h, fiber outgrowth remained comparable between *Lpar2*-KD and scr neurons under Ctrl conditions and after LPA+ROCKi treatment. LPA-treated *Lpar2*-KD neurons showed a trend toward increased fiber length compared to scr controls, but this difference did not reach statistical significance. The data are presented as mean $\pm$ SEM and were normalized to the corresponding data for each substance at 0h. Statistical significance is indicated by p-values * $p \leq 0.05$.

Table 24: Statistical p-values in single-cell assay with *Lpar2*-KD neurons in co-culture with hLESCs.

Group	p-value (24h)
scr (Ctrl) vs scr (LPA+ROCKi)	0.003
scr (LPA) vs scr (LPA+ROCKi)	0.004
<i>Lpar2</i> -KD (Ctrl) vs scr (LPA+ROCKi)	0.0008
<i>Lpar2</i> -KD (LPA) vs scr (LPA+ROCKi)	0.0003

Group	p-value (48h)
scr (LPA+ROCKi) vs scr <i>Lpar2</i> -KD (Ctrl)	0.04
scr (LPA) vs scr (LPA+ROCKi)	0.005
scr (LPA) vs <i>Lpar2</i> -KD (LPA+ROCKi)	0.003

In the wound-healing assay at 24h, no significant differences in fiber regrowth were observed between the *Lpar2*-KD and scr groups under any condition: Ctrl ($5198.19 \pm 696.54 \mu\text{m}$ for *Lpar2*-KD vs. $5323.78 \pm 520.66 \mu\text{m}$ for scr; $p = 0.26$), LPA ($4186.00 \pm 605.00 \mu\text{m}$ *Lpar2*-KD vs. $3008.69 \pm 267.10 \mu\text{m}$ for scr; $p = 0.11$), and LPA+ROCKi ($5004.25 \pm 681.52 \mu\text{m}$ *Lpar2*-KD vs. $5470.98 \pm 577.18 \mu\text{m}$ for scr; $p = 0.63$). At 48h, a significant increase in fiber regrowth was detected in the *Lpar2*-KD-LPA group ($5431.71 \pm 678.54 \mu\text{m}$) compared to the scr-LPA group ($3253.03 \pm 370.08 \mu\text{m}$; $p = 0.017$). No other group comparisons reached statistical significance at this time point: Ctrl ($5619.24 \pm 680.62 \mu\text{m}$ for *Lpar2*-KD vs. $6462.78 \pm 656.88 \mu\text{m}$ for scr; $p = 0.41$) and LPA+ROCKi ($18272.16 \pm 8517.37 \mu\text{m}$ for *Lpar2*-KD vs. $10339.66 \pm 946.26 \mu\text{m}$ for scr; $p = 0.40$) (Figure 18). More significant alterations between different treatments of the scr groups (as well as *Lpar2*-KD neurons) can be found in Table 25.

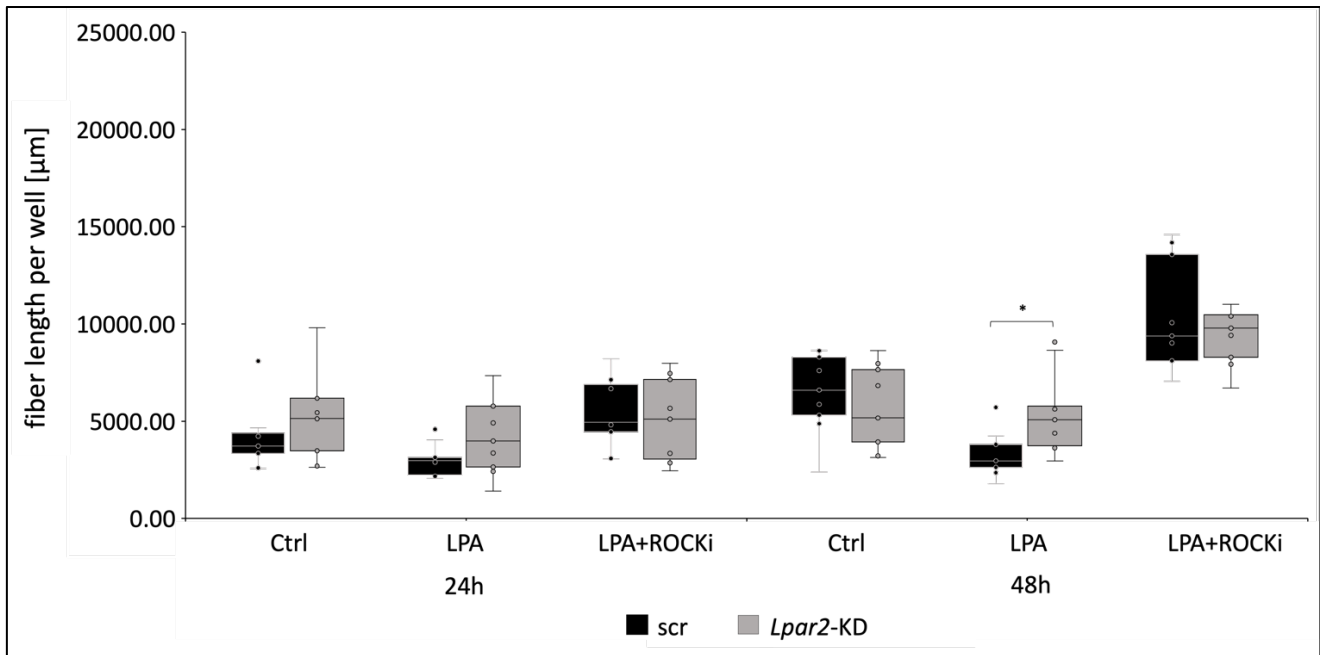


Figure 18: LPA effect on fiber length of co-cultured transfected neurons with hLESCs by wound-healing assay. Neuronal fiber outgrowth measurement per well in transfected neurons cocultured with hLESCs by wound-healing assay exhibited no significant differences in fiber regrowth between *Lpar2*-KD and scr neurons across all treatment conditions at 24h. At 48h, *Lpar2*-KD neurons treated with LPA exhibited a significant enhancement in fiber regrowth versus the scr-LPA group. However, no other treatment conditions showed significant differences between *Lpar2*-KD and scr groups at this time point. The data are presented as mean $\pm$ SEM and were normalized to the corresponding data for each substance at 0h. Statistical significance is indicated by p-values $*p \leq 0.05$.

Table 25: Statistical p-values in wound-healing assay with *Lpar2*-KD neurons in co-culture with hLESCs.

Group	p-value (24h)
scr (LPA) vs <i>Lpar2</i> -KD (Ctrl)	0.013
scr (LPA) vs scr (LPA+ROCKi)	0.002
scr (LPA) vs <i>Lpar2</i> -KD (LPA+ROCKi)	0.02
Group	p-value (48h)
scr (Ctrl) vs scr (LPA)	0.001
scr (Ctrl) vs scr (LPA+ROCKi)	0.005
scr (LPA) vs scr (LPA+ROCKi)	6.38E-06
scr (LPA) vs <i>Lpar2</i> -KD (Ctrl)	0.01
scr (LPA+ROCKi) vs <i>Lpar2</i> -KD (Ctrl)	0.0015
scr (LPA+ROCKi) vs <i>Lpar2</i> -KD (LPA)	0.001

3.2.5 Gene expression in transfected neurons co-cultured with hLESCs pre- and post-injury

To elucidate the molecular mechanisms contributing to neurite outgrowth and regeneration, qRT-PCR analysis was conducted on DRG neurons transfected with siRNA against *Lpar2* or scrambled control, either untreated or treated with LPA, in both non-injured and injured co-culture conditions with hLESCs (Figure 19). In non-injured neurons, *Lpar2* expression showed a significant upregulation in the scr-LPA group (6.17 ± 1.00 , $p = 0.013$) versus the scr. Additionally, *Lpar2* expression was significantly reduced in the *Lpar2*-KD group (1.15 ± 0.08) compared to the scr-LPA group ($p = 0.02$), confirming the knockdown efficiency. Among signaling molecules, *Enpp2* was significantly downregulated in all experimental groups compared to the scr group, including *Lpar2*-KD (0.12 ± 0.007 , $p \leq 0.0001$), scr-LPA (0.44 ± 0.04 , $p = 0.0002$), and *Lpar2*-KD-LPA (0.70 ± 0.08 , $p = 0.037$) treatment groups. Furthermore, *Enpp2* levels in the *Lpar2*-KD group were significantly lower than both the scr-LPA ($p = 0.0021$) and *Lpar2*-KD-LPA ($p = 0.0041$) groups. *Rock1* expression exhibited a significant downregulation in the *Lpar2*-KD group (0.79 ± 0.05 , $p = 0.035$) versus the scr group. *Rock2* was significantly upregulated in scr-LPA group (1.66 ± 0.12 , $p = 0.009$) compared to the scr, and this elevation persisted in *Lpar2*-KD-LPA (1.53 ± 0.16 , $p = 0.053$) versus the scr group, exhibiting a clear LPA-associated trend. *RhoA* was highly downregulated in both *Lpar2*-KD (0.75 ± 0.03 , $p = 0.003$) and *Lpar2*-KD-LPA (0.73 ± 0.02 , $p = 0.0003$) groups versus the scr. *Ras* was significantly upregulated in the *Lpar2*-KD-LPA group (1.89 ± 0.16 , $p = 0.005$) compared to the scr group. *Plc* expression was significantly downregulated in the scr-LPA group (0.61 ± 0.07 , $p = 0.02$) compared to the scr. In contrast, *Rac* was significantly upregulated in the *Lpar2*-KD group (2.22 ± 0.05 , $p \leq 0.0001$) versus the scr group (Figure 19).

Regarding neurotrophic and regeneration-related genes, *Calca* was significantly increased in both scr-LPA (41.52 ± 9.57 , $p = 0.02$) and *Lpar2*-KD-LPA (41.23 ± 11.44 , $p = 0.04$) groups compared to scr, and also significantly elevated in *Lpar2*-KD compared to scr-LPA group ($p = 0.03$). *Gdnf* was upregulated in *Lpar2*-KD (19.33 ± 1.58 , $p = 0.0007$), scr-LPA (17.02 ± 3.14 ,

$p = 0.014$), and *Lpar2*-KD-LPA (15.01 ± 1.53 , $p = 0.0017$) groups relative to scr. *Ngf* expression was significantly increased in *Lpar2*-KD (21.45 ± 3.99 , $p \leq 0.0001$), scr-LPA (17.02 ± 3.14 , $p = 0.014$), and *Lpar2*-KD-LPA (18.21 ± 0.08 , $p \leq 0.0001$) groups versus scr. Moreover, *Ngf* levels were significantly elevated in *Lpar2*-KD-LPA group compared to *Lpar2*-KD ($p \leq 0.0001$), indicating a synergistic effect of LPA treatment under knockdown conditions. *Cdc42* expression was significantly upregulated in *Lpar2*-KD (2.28 ± 0.07 , $p = 0.0001$), scr-LPA (2.09 ± 0.32 , $p = 0.047$), and *Lpar2*-KD-LPA (2.66 ± 0.22 , $p = 0.0028$) groups compared to scr, indicating its consistent activation through both LPA treatment and *Lpar2* knockdown. No significant alterations were detected in *Pi3k*, *Mapk*, *Tac1*, *Cntf*, and *Gap43* levels in non-injured transfected neurons in co-culture with hLESCs (Figure 19).

Upon injury, several transcriptional changes emerged in transfected DRG neurons co-cultured with hLESCs. *Lpar2* expression was significantly downregulated in the *Lpar2*-KD group (0.34 ± 0.02 , $p = 0.0012$) and the *Lpar2*-KD-LPA group (0.61 ± 0.09 , $p = 0.0013$) versus scr, while it was significantly upregulated in the scr-LPA group (1.65 ± 0.16 , $p = 0.035$) relative to scr. Compared to scr-LPA, *Lpar2* levels were also significantly lower in *Lpar2*-KD ($p = 0.0027$) and *Lpar2*-KD-LPA ($p = 0.0079$) groups, confirming effective knockdown. Moreover, *Lpar2* expression in *Lpar2*-KD-LPA remained significantly reduced compared to the *Lpar2*-KD group alone ($p = 0.030$), suggesting an additive suppressive effect under LPA treatment in the knockdown background. *Enpp2* expression was significantly downregulated in the *Lpar2*-KD (0.61 ± 0.10 , $p = 0.0051$), scr-LPA (0.48 ± 0.05 , $p = 0.0005$), and *Lpar2*-KD-LPA (0.56 ± 0.06 , $p = 0.0087$) groups compared to scr. *Rock1* expression exhibited an increasing trend in the *Lpar2*-KD group (1.28 ± 0.15 , $p = 0.057$) compared to scr. A statistically significant difference was observed between scr-LPA (1.73 ± 0.19) and *Lpar2*-KD ($p = 0.027$) groups, suggesting that LPA treatment may enhance *Rock1* expression, which is suppressed upon *Lpar2* knockdown. *Rock2* expression was significantly upregulated in *Lpar2*-KD (1.51 ± 0.15 , $p = 0.002$) and Scr-LPA (1.96 ± 0.10 , $p = 0.002$) groups compared to scr group. Additionally, Scr-LPA exhibited higher *Rock2* levels compared to the *Lpar2*-KD group ($p = 0.008$), while *Rock2* expression in the *Lpar2*-

KD-LPA group (1.60 ± 0.07) remained elevated versus scr-LPA group ($p = 0.03$). In the injured condition, *Plc* expression was significantly elevated in the scr-LPA group (1.17 ± 0.03) compared to scr group ($p = 0.02$), while no significant differences were observed among other groups. *Rac* was significantly upregulated in the *Lpar2*-KD group (1.73 ± 0.16 , $p = 0.015$) compared to the scr). *Mapk* was significantly elevated in scr-LPA group (1.68 ± 0.18) compared to both scr ($p = 0.006$) and *Lpar2*-KD ($p = 0.04$) groups, suggesting LPA-induced *Mapk* activation that is not maintained under *Lpar2* knockdown conditions. *Calca* expression was significantly upregulated in the *Lpar2*-KD group (2.16 ± 0.33 , $p = 0.02$) versus scr group, while no significant changes were observed in the scr-LPA or *Lpar2*-KD-LPA groups. *Tac1* was significantly elevated in the *Lpar2*-KD-LPA group (1.41 ± 0.09 , $p = 0.002$) versus scr, indicating increased neuropeptide expression under combined knockdown and LPA stimulation. No significant differences were detected in other groups (Figure 19). *Gdnf* expression was significantly upregulated in all experimental groups relative to scr, including *Lpar2*-KD (1.93 ± 0.17 , $p = 0.011$), Scr-LPA (1.78 ± 0.29 , $p = 0.018$), and *Lpar2*-KD-LPA groups (1.64 ± 0.14 , $p = 0.019$). *Cntf* level was significantly upregulated in *Lpar2*-KD (1.60 ± 0.13 , $p = 0.04$) and *Lpar2*-KD-LPA (2.00 ± 0.12 , $p = 0.0003$) groups compared to scr group. *Ngf* expression showed a robust increase across all groups compared to scr group, including *Lpar2*-KD (2.12 ± 0.29 , $p = 0.001$), scr-LPA (2.86 ± 0.39 , $p = 0.001$), and *Lpar2*-KD-LPA (4.25 ± 0.33 , $p \leq 0.0001$) groups. Notably, *Ngf* was further elevated in *Lpar2*-KD-LPA group versus scr-LPA ($p = 0.02$) and *Lpar2*-KD ($p = 0.0008$) groups. *Cdc42* expression was significantly upregulated in all experimental groups versus scr, including *Lpar2*-KD (1.86 ± 0.12 , $p = 0.0004$), scr-LPA (1.76 ± 0.13 , $p = 0.006$), and *Lpar2*-KD-LPA (1.93 ± 0.09 , $p = 0.004$). No statistically significant changes were observed in the expression levels of *RhoA*, *Ras*, *Pi3k*, *Plc*, *Rac*, *Mapk*, and *Gap43* across all experimental groups following injury in the transfected neurons in the co-culture with hLESCs (Figure 19).

To determine how injury influences transcriptional responses in DRG neurons in co-culture with hLESCs, we compared gene expression levels between non-injured (single-cell) and injured (wound-healing assay) neurons under the same treatment conditions. In non-injured

neurons, *Lpar2*-KD led to a strong suppression of *Enpp2*, *RhoA*, and *Rock1*, while upregulating *Rac*, *Ngf*, *Gdnf*, *Cdc42*, and *Calca*. Following injury, the same *Lpar2*-KD condition maintained suppression of *Lpar2* and *Enpp2*, but showed increased expression of *Rock2*, *Ngf*, *Gdnf*, *Cntf*, *Tac1*, and *Cdc42*. Notably, *Ngf* was significantly more elevated by *Lpar2*-KD-LPA treatment after injury, suggesting a synergistic effect. Some markers, such as *Rac* and *Calca*, were strongly induced in the *Lpar2*-KD group only before injury, while *Cntf* and *Tac1* upregulation emerged only after injury. These results highlight that the regenerative and signaling response of *Lpar2*-KD neurons is context-dependent and dynamically modulated by injury status (Figure 19).

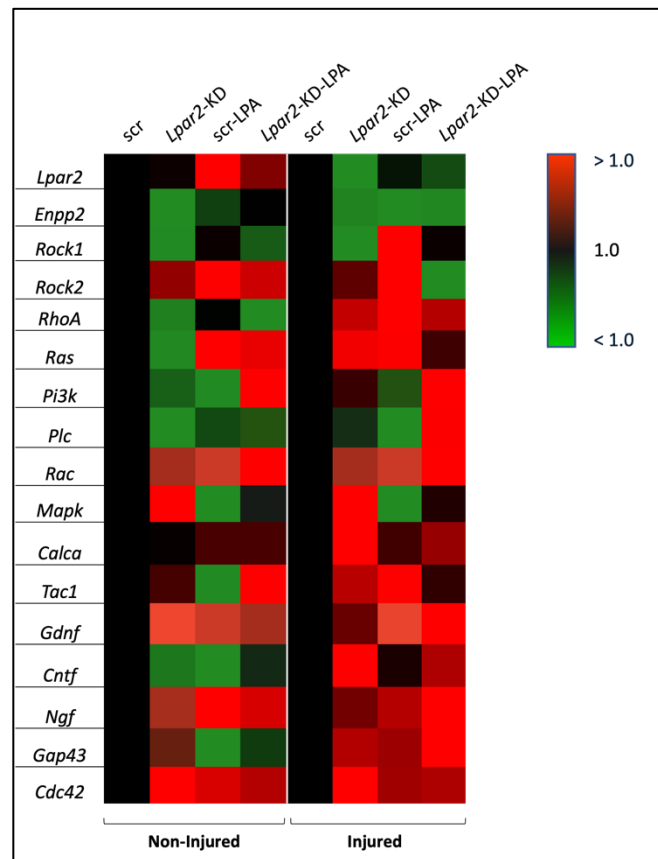


Figure 19: mRNA expression of transfected neurons in co-culture with hLESCs pre- and post-injury.

Heatmap showing differential gene expression in DRG neurons co-cultured with hLESCs under non-injured and injured conditions, following LPA treatment and/or *Lpar2*-KD. Red indicates higher expression, green indicates lower expression, and black exhibits no significant change relative to the scr group. In non-injured neurons, *Lpar2*-KD suppressed *Enpp2*, *RhoA*, and *Rock1*, while inducing *Rac*, *Ngf*, *Gdnf*, *Calca*, and *Cdc42*. After injury, *Lpar2*-KD maintained suppression of *Lpar2* and *Enpp2*, while upregulating *Rock2*, *Ngf*, *Gdnf*, *Cntf*, *Tac1*, and *Cdc42*. Notably, *Ngf* was further increased by LPA treatment in the knockdown background. *Rac* and *Calca* were specifically induced before injury, whereas *Cntf* and *Tac1* were selectively upregulated after injury.

3.2.6 Gene expression in hLESCs co-cultured with transfected neurons pre- and post-injury

The transcriptional response of hLESCs co-cultured with transfected neurons pre- and post-injury was also evaluated using qRT-PCR. The aim was to determine whether LPA has an effect on the hLESCs as well and if the knockdown of *Lpar2* in DRG neurons, alone or in combination with LPA treatment, modulates gene expression in adjacent hLESCs. To assess the transcriptional response of hLESCs co-cultured with transfected DRG neurons pre- and post-injury, qRT-PCR was performed across key genes related to signaling, apoptosis, and epithelial markers (Figure 20).

Before injury, in hLESCs co-cultured with *Lpar2*-KD neurons, *LPAR2* (0.61 ± 0.03 , $p = 0.0005$) and *ENPP2* (0.27 ± 0.16 , $p = 0.0210$) were significantly downregulated versus scr. Additionally, *LPAR2* level was significantly reduced in the *Lpar2*-KD group versus scr-LPA group ($p = 0.0123$). *ENPP2* remained suppressed in the *Lpar2*-KD-LPA group (0.20 ± 0.13 , $p = 0.006$ relative to scr. *ROCK1* and *ROCK2* levels were significantly downregulated in the *Lpar2*-KD group (*ROCK1*: 0.65 ± 0.004 , $p < 0.0001$; *ROCK2*: 0.51 ± 0.11 , $p = 0.02$) versus scr, and remained suppressed in the *Lpar2*-KD-LPA group (*ROCK1*: 0.68 ± 0.06 , $p = 0.016$; *ROCK2*: 0.57 ± 0.12 , $p = 0.048$) compared to scr. *ROCK1* was also significantly lower in the *Lpar2*-KD group versus scr-LPA ($p = 0.0213$). *ROCK2* expression was significantly elevated in the scr-LPA group compared to scr (1.27 ± 0.08 , $p = 0.04$), but remained significantly lower in both *Lpar2*-KD ($p = 0.0106$) and *Lpar2*-KD-LPA ($p = 0.017$) groups versus scr-LPA group (Figure 20). Neuropeptides *CALCA* and *TAC1* showed strong treatment-specific responses. *TAC1* expression was significantly elevated in the *Lpar2*-KD group (19.06 ± 3.17 , $p = 0.009$) relative to scr group, while changes in scr-LPA and *Lpar2*-KD-LPA groups did not reach statistical significance. In contrast, *CALCA* levels increased significantly in the scr-LPA group (20.41 ± 1.73 , $p = 0.0008$) versus scr group, and this upregulation was also significant when compared to the *Lpar2*-KD ($p = 0.0012$) and the *Lpar2*-KD-LPA groups ($p = 0.002$), suggesting that LPA stimulation in the scramble group was the primary driver of *CALCA* induction. *GDNF* was significantly upregulated in all groups,

Lpar2-KD (3.85 ± 0.33 , $p = 0.002$), scr-LPA (3.78 ± 0.50 , $p = 0.0106$), and *Lpar2*-KD-LPA (3.26 ± 0.27 , $p = 0.002$) compared to the scr group. *NGF* expression was significantly elevated in both the *Lpar2*-KD (4.35 ± 0.73 , $p = 0.02$) and the *Lpar2*-KD-LPA group (3.83 ± 0.63 , $p = 0.02$) versus the scr group (Figure 20). Among apoptosis-related markers, *BCL2* was downregulated in all conditions (*Lpar2*-KD: 0.08 ± 0.05 ; scr-LPA: 0.04 ± 0.01 ; *Lpar2*-KD-LPA: 0.07 ± 0.05 , all $p \leq 0.0001$) compared to scr group. *BAX* remained unchanged in *Lpar2*-KD and scr-LPA groups but was significantly downregulated in *Lpar2*-KD-LPA group (0.69 ± 0.002 , $p < 0.0001$ vs. scr). *BAX* level was significantly reduced in the *Lpar2*-KD-LPA group (0.69 ± 0.003) versus scr ($p < 0.0001$), *Lpar2*-KD ($p = 0.02$), and versus scr-LPA ($p = 0.0003$). *CAS3* expression was significantly decreased in all experimental groups (*Lpar2*-KD: 0.63 ± 0.08 , $p = 0.0181$; scr-LPA: 0.21 ± 0.10 , $p = 0.0034$; *Lpar2*-KD-LPA: 0.51 ± 0.001 , $p < 0.0001$) compared to the scr group (Figure 20).

Proliferation marker *KI67* was significantly reduced in the *Lpar2*-KD group (0.41 ± 0.11 , $p = 0.013$) and in the *Lpar2*-KD-LPA group (0.44 ± 0.13 , $p = 0.02$) relative to the scr, and also exhibited a significant reduction in the *Lpar2*-KD versus scr-LPA ($p = 0.04$). *P63 α* expression was significantly elevated in both the *Lpar2*-KD (1.49 ± 0.10 , $p = 0.014$) and *Lpar2*-KD-LPA (1.36 ± 0.07 , $p = 0.011$) groups compared to the scr. These elevations were also significant when compared to scr-LPA ($p = 0.0009$ and $p = 0.0004$, respectively), where *P63 α* expression was markedly downregulated in scr-LPA (0.37 ± 0.03 , $p = 0.0001$) compared to scr. Similar to *P63 α* , *PAX6* expression was enhanced by all groups (*Lpar2*-KD: 1.88 ± 0.02 , $p < 0.0001$; scr-LPA: 1.81 ± 0.18 , $p = 0.0243$; *Lpar2*-KD-LPA: 1.66 ± 0.13 , $p = 0.0164$) all versus scr. The corneal epithelial marker *CK12* was significantly downregulated in all conditions compared to the scr (*Lpar2*-KD: 0.12 ± 0.002 , $p < 0.0001$; scr-LPA: 0.07 ± 0.0006 , $p < 0.0001$; *Lpar2*-KD-LPA: 0.13 ± 0.002 , $p < 0.0001$). Furthermore, expression levels differed significantly between *Lpar2*-KD and scr-LPA ($p < 0.0001$) groups, between *Lpar2*-KD and *Lpar2*-KD-LPA ($p = 0.02$) groups, and between scr-LPA and *Lpar2*-KD-LPA ($p < 0.0001$) groups. In contrast, *CK15* was significantly upregulated in the *Lpar2*-KD group (1.59 ± 0.14 , $p = 0.03$) and scr-LPA group (2.45 ± 0.33 , $p = 0.02$) compared

to the scr. The expression levels of *RHOA*, *CNTF*, and *VIM* did not show statistically significant changes across any of the experimental groups (Figure 20).

Following the injury to the transfected DRG neurons, qRT-PCR analysis was again performed to evaluate the gene expression profile in the co-cultured hLESCs. *LPAR2* was found to be significantly downregulated in the *Lpar2*-KD group (0.52 ± 0.10 , $p = 0.018$) versus the scr group. While, *LPAR2* expression was markedly upregulated in the scr-LPA group (1.42 ± 0.05 , $p = 0.0016$) versus scr, and significantly higher compared to both *Lpar2*-KD ($p = 0.0027$) and *Lpar2*-KD-LPA ($p = 0.01$) groups. *ENPP2* was significantly reduced in the *Lpar2*-KD-LPA group (0.67 ± 0.07 , $p = 0.02$) compared to scr, with no changes observed in the other conditions. *ROCK1* was significantly downregulated in the *Lpar2*-KD group (0.82 ± 0.03 , $p = 0.0053$) relative to the scr and the scr-LPA groups (1.18 ± 0.03 , $p = 0.0019$), while upregulation was observed in the scr-LPA group ($p = 0.0086$) versus scr. A significant downregulation was determined in the *Lpar2*-KD-LPA group ($p = 0.018$) compared to the scr-LPA. Similarly, *ROCK2* was strongly downregulated in the *Lpar2*-KD (0.60 ± 0.01 , $p < 0.0001$) and *Lpar2*-KD-LPA (0.42 ± 0.09 , $p = 0.0051$) versus scr, and both were significantly lower than scr-LPA ($p = 0.014$ and $p = 0.011$, respectively). *RHOA* was significantly elevated in *Lpar2*-KD (1.29 ± 0.01 , $p = 0.0001$) and *Lpar2*-KD-LPA (1.21 ± 0.05 , $p = 0.0319$), while reduced in scr-LPA (0.81 ± 0.05 , $p = 0.0388$) compared to the scr group. Expression levels in *Lpar2*-KD and *Lpar2*-KD-LPA groups were both significantly higher than the scr-LPA group ($p = 0.0017$ and $p = 0.0114$, respectively) (Figure 20). Among neurotrophic and neuropeptide genes, *TAC1* was strongly upregulated in all groups (*Lpar2*-KD: 2.63 ± 0.13 , $p = 0.0005$; scr-LPA: 2.75 ± 0.10 , $p = 0.0001$; *Lpar2*-KD-LPA: 3.02 ± 0.14 , $p = 0.0003$) relative to the scr group. *GDNF* expression was significantly increased in *Lpar2*-KD (2.28 ± 0.17 , $p = 0.0039$), scr-LPA (1.92 ± 0.05 , $p = 0.0001$), and *Lpar2*-KD-LPA (2.21 ± 0.02 , $p < 0.0001$) groups compared to the scr, with scr-LPA showing significantly lower expression than *Lpar2*-KD-LPA group ($p = 0.0101$). Similarly, *CNTF* and *NGF* expression were significantly upregulated in all three groups (*CNTF*: 2.36 ± 0.05 , 1.67 ± 0.05 , 2.11 ± 0.06 ; all $p < 0.001$; *NGF*: 3.42 ± 0.28 , 3.25 ± 0.60 , 3.91 ± 0.29 ; $p = 0.002$, 0.03 , 0.001 , respectively) versus scr. Additionally, *CNTF* expression in the

Lpar2-KD group was significantly higher than in the scr-LPA ($p = 0.001$) and *Lpar2*-KD-LPA ($p = 0.04$) groups, while the *Lpar2*-KD-LPA group also showed significantly elevated levels compared to the scr-LPA ($p = 0.008$) (Figure 20).

Proliferation marker *KI67* level was significantly elevated in the scr-LPA group (1.47 ± 0.07) compared to the scr ($p = 0.007$), *Lpar2*-KD ($p = 0.0146$), and *Lpar2*-KD-LPA ($p = 0.0390$) groups. *P63α* and *PAX6* expression were significantly upregulated in all conditions (*P63α*: *Lpar2*-KD: 1.45 ± 0.08 , $p = 0.01$; scr-LPA: 1.84 ± 0.19 , $p = 0.02$; *Lpar2*-KD-LPA: 2.01 ± 0.16 , $p = 0.007$; *PAX6*: *Lpar2*-KD: 1.50 ± 0.12 , $p = 0.02$; scr-LPA: 0.73 ± 0.02 , $p = 0.0008$; *Lpar2*-KD-LPA: 1.88 ± 0.22 , $p = 0.03$) all compared to the scr group. Additionally, *PAX6* levels were significantly upregulated in both *Lpar2*-KD ($p = 0.007$) and *Lpar2*-KD-LPA ($p = 0.01$) groups compared to the scr-LPA group. *VIM* level was elevated in the *Lpar2*-KD group (1.11 ± 0.02) compared to the scr ($p = 0.01$) and scr-LPA ($p = 0.0138$) groups. *CK12* expression was strongly downregulated in all three groups: *Lpar2*-KD (0.01 ± 0.001 , $p < 0.0001$), scr-LPA (0.01 ± 0.001 , $p < 0.0001$), and *Lpar2*-KD-LPA (0.01 ± 0.005 , $p < 0.0001$) compared to the scr. In contrast, *CK15* expression was significantly upregulated in all conditions: *Lpar2*-KD (2.30 ± 0.28 , $p = 0.02$), scr-LPA (2.71 ± 0.35 , $p = 0.016$), and *Lpar2*-KD-LPA (3.13 ± 0.40 , $p = 0.011$) groups compared to the scr group. No significant changes were detected for *CALCA*, *BCL2*, *BAX*, and *CAS3* in any conditions (Figure 20).

To assess the impact of injury on transcriptional responses in hLESCs co-cultured with *Lpar2*-knockdown DRG neurons, we analyzed gene expression differences between non-injured (single-cell assay) and injured (wound-induced) conditions across treatment groups. In summary, *LPAR2* and ROCK pathway components were consistently downregulated in *Lpar2*-KD conditions across both injury states, while LPA treatment induced distinct effects depending on injury status. Neuropeptides such as *TAC1* were highly upregulated post-injury, whereas *CALCA* showed a more prominent response pre-injury. Neurotrophic factors (*GDNF*, *CNTF*, *NGF*) remained robustly elevated in all conditions, with injury upregulating *CNTF* and *NGF* responses. Apoptotic markers such as *BCL2*, *BAX*, and *CAS3* were suppressed pre-injury

but maintained consistent levels. Proliferation and stemness markers (*KI67*, *P63α*, *PAX6*) responded differently depending on the condition, with *P63α* and *PAX6* consistently elevated in *Lpar2*-KD groups, and *KI67* enhanced only after injury in LPA-treated conditions. Corneal epithelial markers diverged, with *CK12* persistently downregulated and *CK15* upregulated under all conditions. These findings highlight both conserved and injury-dependent gene regulation patterns in the co-cultured hLESCs in response to neuronal signals (Figure 20).

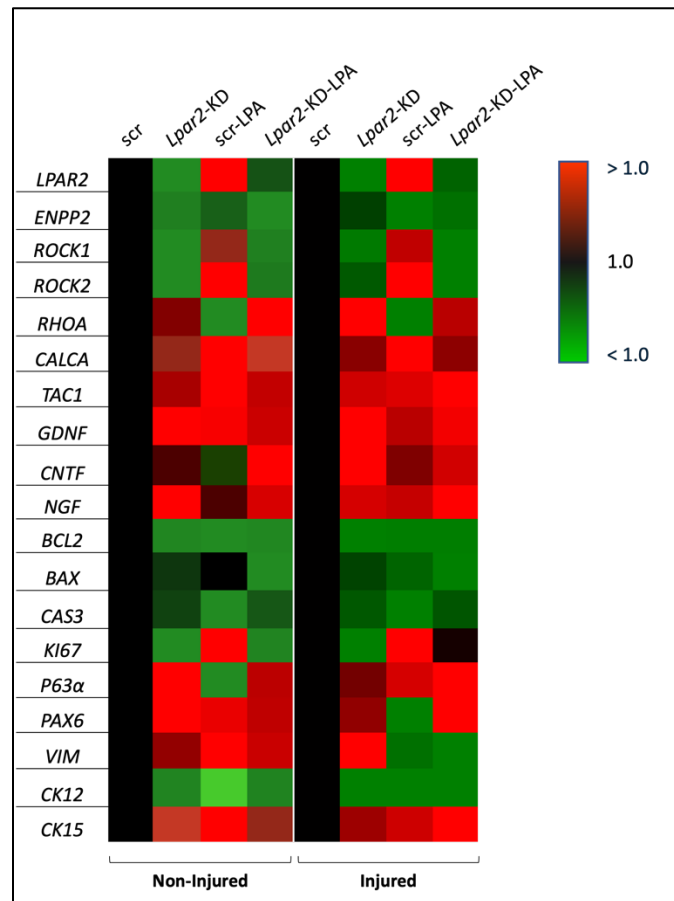


Figure 20: mRNA expression of hLESCs in co-culture with transfected neurons pre- and post-injury.

Heatmap exhibiting differential gene expression in hLESCs co-cultured with transfected DRG neurons under non-injured and injured conditions, with or without LPA treatment. Red indicates upregulation, green indicates downregulation, and black represents no significant change relative to the scr group. Before injury, *Lpar2*-KD reduced *LPAR2*, *ENPP2*, and *ROCK1/2*, while increasing *TAC1*, *NGF*, *GDNF*, and *PAX6*. *P63α* was induced in both *Lpar2*-KD and *Lpar2*-KD-LPA groups but suppressed by scr-LPA. After injury, *Lpar2*-KD and *Lpar2*-KD-LPA groups maintained suppression of *LPAR2* and *ROCK2*, and further elevated *CNTF*, *NGF*, *PAX6*, and *CK15*. *CK12* was consistently downregulated across all conditions, while *KI67* was selectively increased in scr-LPA only after injury.

4

4. Discussion

4.1 Functional Implications of *Lpar2*-KD in Nerve Regeneration

NK is a rare disease with prevalence rates of 1-5 cases per 10.000 individuals in Europe. It progresses through three stages: reduced corneal sensitivity, epithelial breakdown, and stromal involvement with ulceration [19]. Current treatments include preservative-free lubricating eye drops, conjunctival flaps, and amniotic membrane transplantation (AMT), and are mainly symptomatic [56, 65, 219]. Cenegermin is the only non-surgical curative treatment that addresses the underlying pathophysiology of NK. It was found to be generally well-tolerated in clinical trials, with the most commonly reported ocular symptoms (such as eye pain) reflecting the therapeutic mechanism of action, the restoration of corneal innervation and sensitivity [112]. However, it has limitations that restrict its application, including high cost, side effects such as corneal deposits and infections, and its withdrawal from the German market in 2020, despite demonstrated clinical efficacy [111, 112, 220]. Therefore, there is a high clinical need for new curative therapeutic approaches to treat NK. A recent study by Mertsch *et al.* on corneal nerve regeneration showed that the inhibition of ROCK significantly increased the regeneration of damaged corneal nerve fibers *in vitro* and *in vivo* [8]. Since LPA is an upstream factor of ROCK in the signaling pathway [214], it was critical to determine whether LPA plays a role in corneal nerve regeneration, and which specific LPA receptor subtypes are involved.

LPA causes neurite retractions in the CNS, whereas overexpression of plasticity-related gene 1 (PRG-1) alleviates this effect [221]. In the PNS, it has also been found that LPA signaling stimulates corneal wound healing in rabbits by increasing the migration and proliferation rates of keratocytes, and that LPA concentration increases in aqueous humor (AH) and tear fluid

after corneal injury [207]. Moreover, Honjo *et al.* found remarkably higher levels of ATX (autotaxin, the major enzyme for converting LPC to LPA), LPA, and LPC in the AH of glaucoma patients compared to healthy individuals [208]. Therefore, the purpose of the present study was to examine the effect of LPA on the regeneration of corneal nerves and to identify the involved LPA receptor.

Analyzing the effect of exogenous LPA on the regenerative capacity of sensory nerve fibers, revealed that LPA leads to an inhibition of fiber growth as well as of regeneration in the sensory neurons *in vitro*. To further evaluate the downstream signaling pathway responsible for this effect, neurons were treated with LPA alone and LPA combined with the ROCK inhibitor (LPA+ROCKi). Inhibition of Rho Kinase showed enhanced fiber regeneration and growth compared to the control, which aligns with other studies that have reported ROCKi promotes neurite outgrowth, axonal sprouting, and nerve regeneration in corneal nerves as well as in the optic nerve [8, 222, 223]. Our findings exhibited that the combination of LPA with ROCKi resulted in a significant rescue effect of the LPA-induced fiber length suppression [9], indicating that the inhibitory effect of LPA on fiber elongation is mediated via the ROCK pathway.

Yang *et al.* reported that LPA-induced spinal cord injury (SCI) in rats was reduced by *Lpar4* and *Lpar6* knockdown [224]. Inoue *et al.* showed that in a sciatic nerve injury mouse model, LPA accumulated in the wound area, primarily originating from degenerated/damaged neurons [225]. It has also been found that blocking *Lpar1* could attenuate the LPA-induced effect in Wallerian degeneration [210]. Although LPA and its receptors are known to affect synapse formation, dendrite, and axon growth [226], its specific effect in sensory nerve regeneration is unknown. Li *et al.* reported that different types and subtypes of DRG neurons (based on their size) have unique gene expression patterns. They found that a subset of C57BL/6 mouse DRG cells (C5 and C6) express *Lpar3*, while other subtypes (C3 and C4) do not express *Lpar3* [227]. JW Robering has shown that mouse DRG neurons exhibited higher *Lpar1*, *Lpar3*, and *Lpar6*

expression compared to *Lpar2*, *Lpar4*, and *Lpar5* [228]. The present study demonstrated the distinct expression of *Lpar1*, 2, 4, and 6 in murine DRG neurons and no expression of *Lpar3* and 5. Furthermore, comparable expression patterns of *Lpar1-6*, *Rock1*, *Rock2*, and *Enpp2* in murine TG and DRG neurons were found, which indicated that DRG neurons are an appropriate model for analyzing corneal nerve regeneration.

Lpar1- and *Lpar4*-knockdown (*Lpar1*-KD and *Lpar4*-KD) in neurons combined with LPA treatment revealed no functional changes in the fiber length. However, in *Lpar2*-KD neurons, the addition of exogenous LPA led to a significant enhancement in fiber length after 48h compared to the scramble negative control treated with LPA. This finding indicates that the negative effect of LPA on nerve fiber growth is mediated by the LPAR2. A diverse role of LPAR2 in different neuronal contexts has been reported in the literature. López-Serrano *et al.* demonstrated that increased levels of *Lpar2* were detected in SCI [197]. This upregulation is consistent with our findings that LPAR2 mediates the inhibitory effects of LPA on DRG fiber growth. However, Uenaka *et al.* demonstrated that LPAR2 activation promotes neurite elongation and branch formation in neocortical neurons [198]. This contrasting finding highlights the cell-type-specific function of LPAR2, where it promotes growth in cortical neurons but inhibits growth in sensory DRG neurons. The present study further supports this context-dependent role, as *Lpar2*-KD attenuated the suppressive effect of LPA on DRG neuronal fiber length and enhanced fiber outgrowth.

The impact of *Lpar2*-KD on signaling pathways comparing injured and non-injured neurons was additionally evaluated in this study. No alteration was found in *Enpp2* expression before injury in the *Lpar2*-KD group, however, after the injury, *Lpar2*-KD neurons led to an *Enpp2* downregulation (the encoding gene for ATX). In glaucoma patients, the levels of ATX and LPA were found to be elevated in the AH [208]. Nagano *et al.* showed in a glaucoma mouse model that by applying an ATX inhibitor as an ophthalmic drug named Aipreon, the ATX activity (LysoPLD activity) in the AH was reduced, followed by a reduction in the intraocular pressure

[229]. To further investigate changes in nerve regeneration signaling pathways, the expression of key Rho GTPases (*Cdc42* and *RhoA*) was analyzed. It has been reported that CDC42 promotes nerve regeneration [230] and cell survival [231], whereas RhoA induces the formation of actin stress fibers, leading to neurite retraction [230]. In this present study, *Cdc42* levels were upregulated post-injury in both non-transfected neurons treated with LPA+ROCKi (compared to LPA) and in transfected *Lpar2*-KD cells (compared to scr negative control). Notably, *RhoA* was significantly elevated in non-transfected neurons treated with LPA+ROCKi pre- and post-injury compared to Ctrl and LPA groups. These results are in agreement with the study by Mertsch *et al.*, who previously found this RhoA-ROCK feedback mechanism, where RhoA expression is upregulated in injured corneal nerve fibers 28 days after treatment with ROCKi *in vivo* [8]. Although LPA is known to activate RhoA protein via LPA receptor signaling in mouse neuroblastoma cells [184], in the present study, LPA treatment alone, in all conditions (pre- and post-injury, as well as before and after neuronal transfection) did not change *RhoA* mRNA expression levels. These results demonstrate that while LPA activates RhoA at the protein level [184], it does not affect *RhoA* mRNA levels in DRG neurons [9], whereas ROCKi increased *RhoA* mRNA levels in injured DRG neurons [8].

NGF and GDNF have been shown to facilitate nerve regrowth/regeneration through RAS/MAPK/Pi3K/PLC/ERK pathways [232–235], especially in sensory neurons [236, 237]. Our findings showed that *Lpar2*-KD neurons, with and without LPA treatment pre-injury, led to increased levels of *Ras*, *Pi3k*, *Plc*, *Mapk*, *Ngf*, and *Gdnf*. Similarly, *Lpar2*-KD in injured neurons resulted in upregulation of *Ras*, *Pi3k*, *Mapk*, *Gdnf*, and *Ngf* compared to the scr and scr-LPA treatment groups. Matsumoto *et al.* found that PLC inhibition reduced neurite length in DRG neurons, highlighting PLC and MAPK/ERK's role in nerve regeneration by NGF [238]. Notably, *Lpar2*-KD neurons (without LPA treatment) showed upregulation of *Plc*, *Mapk*, and *Ngf* compared to the scr and scr-LPA treatment groups before injury. This finding suggests that LPAR2 may have an inhibitory effect on these regeneration-promoting factors (PLC-MAPK pathway) even in the absence of exogenous LPA, either via basal receptor activity or

endogenous LPA production by the neurons themselves, since mouse DRG neurons are shown by Anliker *et al.* to produce LPA endogenously [239]. Although basal activity (ligand-independent) has not been demonstrated specifically for LPAR2, many G protein-coupled receptors (GPCRs) are known to display basal activity in the absence of their ligand stimulation [240]. The knockdown of *Lpar2* would therefore enhance expression of pro-regenerative genes, such as *Plc*, *Mapk*, and *Ngf*. These findings support the critical role of the PLC-MAPK pathway in nerve regeneration following *Lpar2* knockdown in DRG neurons. Additionally, injured *Lpar2*-KD cells showed elevated *Cntf* levels, likely due to the neuroprotective effect of CNTF found in the literature [241]. CNTF has been reported to activate three important signaling pathways, including JAK-STAT, MAPK/ERK, and PI3K/Akt [242, 243]. The JAK-STAT pathway promotes mouse motor neuron survival [244], and the MAPK/ERK and the PI3K/Akt pathways both support neurite outgrowth and axonal regeneration in mouse embryonic DRG neurons [245]. The upregulation of *Cntf* in injured *Lpar2*-KD neurons versus the scr negative control was accompanied by increased expression of *Pi3k* and *Mapk*, as described above. This coordinated upregulation suggests that knockdown of *Lpar2* in DRG neurons activates a nerve regenerative signaling pathway where CNTF and its downstream PI3K and MAPK pathways work synergistically to promote nerve regeneration. RAC, a member of the Rho GTPase family (alongside RhoA and CDC42), is downstream of PI3K and promotes actin polymerization and neurite outgrowth [246, 247]. Spillane *et al.* showed that RAC induces axon branching and neuronal elongations in embryonic chick DRG neurons [248], consistent with our results, which showed a significant elevation of *Rac* in injured *Lpar2*-KD neurons compared to the scr-LPA group. The concurrent upregulation of *Rac*, *Mapk*, *Pi3k*, *Cdc42*, and *Cntf* in injured *Lpar2*-KD neurons indicates activation of a coordinated pro-regenerative signaling network that promotes nerve regeneration.

GAP-43 is a marker for axonal regeneration and maturation following nerve injury [249]. The present study revealed that *Gap-43* expression was upregulated in *Lpar2*-KD-LPA post-injury. Previous findings have reported that *Gap-43* was upregulated in a mouse model of NK 28 days

after ROCKi treatment [8]. *CALCA* and *TAC1* both contribute to nerve regeneration in rat DRG neurons and restore corneal sensitivity in diabetes-induced mice [250, 251]. In line with that, *Lpar2*-KD-LPA neurons pre- and post-injury and *Lpar2*-KD-LPA before the injury, showed an upregulated *Calca* and *Tac1* expression, while LPA treatment significantly reduced *Tac1* in *Lpar2*-KD neurons post-injury.

After identifying that the negative effect of LPA on fiber growth of DRG neurons is based on Rho Kinase activation and that the main responsible receptor might be LPAR2, a co-culture system was established for further analysis. Here, the effects of LPA, LPA+ROCKi, as well as the knockdown of *Lpar2*, were investigated in a co-culture system of DRG neurons with primary limbal epithelial stem cells (hLESCs) to mimic the native cornea and to gain insights into the direct interaction of corneal nerve fibers and epithelial cells concerning nerve regeneration.

4.2 Regulation of Neurons and hLESCs in Co-Culture Systems

The primary results of this doctoral thesis showed that LPA significantly suppressed neurite regrowth in DRG neurons while ROCKi treatment promoted neurite elongation. In more detail, LPA treatment resulted in elevated levels of *Rock2*, which consequently led to the reduction of nerve fiber length. On the other hand, using ROCKi (in the LPA+ROCKi group) not only alleviated the inhibitory effect of LPA but also significantly enhanced neurite outgrowth/regeneration [9]. Our experimental design involved a co-culture model of DRG neurons with hLESCs. This model mimics the corneal epithelial-nerve microenvironment, where physical and paracrine interactions between neurons and epithelial cells are known to influence each other.

The observation that suppressive effect of LPA on neurite length in the co-culture with hLESCs only became significant at 48h, with no significant reduction at 24h in the single-cell assay, suggests that the hLESCs provide an initial protection against LPA-induced neurite retraction. HLESCs are known to produce and secrete various neurotrophic factors that support neuronal survival, growth, and regeneration. These include but are not limited to NGF, BDNF, GDNF, and basic fibroblast growth factor (bFGF), which generally enhance neurite outgrowth and cytoskeletal stabilization through signaling pathways that inhibit RhoA-ROCK activity, for example the PI3K/Akt and MAPK pathways. It's possible that during the first 24h, the balance between the inhibitory signals of LPA and regeneration-associated gene (RAG) signals of hLESCs temporarily counteracts the inhibitory effect of LPA [252, 253]. A study using a keratinocyte co-culture with DRG neurons showed that additional keratinocyte signaling significantly elevated neurite outgrowth [254].

Gene expression analysis by qRT-PCR showed that injured DRG neurons treated with LPA+ROCKi in co-culture with hLESCs (before *Lpar2* knockdown) exhibited decreased *Rock1* and *Rock2* expression and increased levels of *RhoA*, *Ras*, *Pi3k*, *Mapk*, neurotrophic factors (*Ngf*, *Gdnf*, *Cntf*), *Cgrp*, and regeneration markers (*Gap43*, *Cdc42*) compared to the Ctrl group. Also,

when compared to LPA treatment alone, LPA+ROCKi specifically enhanced the expression of *Pi3k*, *Plc*, *Mapk*, *Cgrp*, and *Ngf*. These findings confirm that Rho kinase inhibition alleviates the inhibitory effect of LPA on nerve regeneration, even in the co-culture with hLESCs. These results are in line with a study by Edström and Ekström, who showed that PI3K is critical for DRG neurite growth [255]. Moreover, these findings are consistent with the pro-regenerative role of CGRP in peripheral sensory nerve regeneration in rats [256] and corneal wound healing in mice, where CGRP promotes nerve fiber regeneration via MAPK activation [257]. In parallel, gene expression analysis of hLESCs co-cultured with injured neurons (before *Lpar2* knockdown) revealed that both LPA and LPA+ROCKi treatments decreased *LPAR2* and *LPAR4* expression levels compared to the non-treated control. Also, the expression of *RHOA*, *ROCK1*, *ROCK2*, and *ENPP2* showed no significant changes in hLESCs co-cultured with injured DRG neurons treated with LPA and LPA+ROCKi. These findings are in line with the study by Yin *et al.*, who reported that while LPA increased ROCK activities (increased phosphorylation of ROCK), the total protein levels of ROCK1 and ROCK2 remained unchanged in human corneal epithelial cells [258], suggesting that LPA primarily regulates ROCK through post-translational activation rather than transcriptional regulation in epithelial cells.

The comparison of DRG neurons in mono-culture and DRG neurons co-cultured with hLESCs offers strong evidence for the potent pro-regenerative capacity of healthy hLESCs. The observed improvements in DRG neurite outgrowth, in the presence of hLESCs, align strongly with the studies on the beneficial interactions between various stem cell types and neurons within regenerative contexts [259, 260]. HLESCs and related corneal stromal stem cells have been known for their critical role in tissue repair and neuroprotection [259, 261, 262]. Bammidi *et al.* showed that in a mouse model of corneal injury, corneal stromal stem cell secretome effectively supported the regeneration of sensory nerves, as indicated by the restored expression of β -III tubulin and substance P, and the re-establishment of the corneal nerve plexus as compared to control treatments [261]. In line with these findings, Di *et al.* determined that during corneal wound healing, the corneal epithelium highly upregulates NGF and GDNF,

which closely correlate with the restoration of the subbasal nerve plexus and enhance neurite outgrowth from mouse TG neurons. These secreted factors directly promote nerve regeneration and stimulate neurite outgrowth [51]. These findings suggest that hLESCs can act as a therapeutic resource for corneal nerve regeneration, not only via their stemness features but also through their neurotrophic factors secretion which can affect the neuronal microenvironment. Moreover, the neurotrophic capacity of hLESCs is highly dependent on their health status [263]. Under pathological situations such as deficiency of limbal stem cell, chronic inflammation, chemical injuries, or trauma induced by contact lenses, hLESCs lose their beneficial functions, and their supportive niche is disrupted, compromising their ability to maintain corneal nerve homeostasis [264–267]. This paracrine mechanism of hLESCs that support nerve cells is similar to that played by Schwann cells (SCs) in peripheral nerve regeneration. SCs become specialized repair cells when the peripheral nerves are injured, thereby increasing the production of neuroprotective factors such as GDNF, BDNF, NGF, and Artemin, which promote neuronal survival and axonal regrowth [268, 269]. This suggests that cell-therapy based using hLESCs might provide neuroprotective benefits for corneal nerve injury when endogenous repair mechanisms are impaired.

When comparing mono-culture and co-culture DRG neurons before *Lpar2* knockdown, LPA showed inhibitory effects on neurite outgrowth at both 24h and 48h (both single-cell and wound-healing assays) in mono-cultured DRG neurons. However, in the co-culture model, no inhibitory effect was observed for LPA at 24h in either single-cell or wound-healing assays. At 48h, the protection of hLESCs persisted only in the wound-healing assay, confirming that hLESCs provide neurotrophic support against LPA-induced inhibitory effect on neuronal regrowth. DRG neurons treated with LPA+ROCKi showed comparable increases in fiber length/regrowth in both mono-culture and co-culture models (before *Lpar2* knockdown).

Following *Lpar2*-KD, the comparison of mono-cultured and co-cultured DRG neurons showed similar responses in single-cell and wound-healing assays. In both mono-culture and co-culture following *Lpar2* knockdown, LPA-treated neurons (*Lpar2*-KD-LPA) significantly increased

fiber length at 48h compared to scrambled neurons treated with LPA (scr-LPA group), demonstrating that LPAR2 might be a key mediator for the inhibitory effect of LPA [9]. However, in mono-culture following *Lpar2* knockdown, LPA+ROCKi group showed no significant changes at both 24h and 48h timepoints, whereas in co-culture, neurite fiber length was significantly decreased only at 24h in the *Lpar2*-KD-LPA+ROCKi group compared to the scr-LPA+ROCKi group. The transient inhibitory effect observed at 24h in the *Lpar2*-KD-LPA+ROCKi group may be explained by compensatory signaling via other LPA receptors (LPARs). Our previous experiments confirmed the specificity of each siRNA, demonstrating that knockdown of *Lpar2* does not affect the expression of other LPARs. Since other LPARs (LPAR1, LPAR4, and LPAR6) remain functional in the DRG neurons following *Lpar2*-KD, and by some studies it has been shown that these receptors activate ROCK downstream via $G\alpha_{12/13}$ -RhoA signaling [270, 271], the remaining LPARs signaling may temporarily counteract the beneficial effects of *Lpar2*-KD and ROCK inhibition at early timepoints (*Lpar2*-KD-LPA+ROCKi group at 24h). At 48h, however, the impact of *Lpar2* knockdown becomes dominant, resulting in enhanced neurite outgrowth despite the presence of other LPA receptors. To further evaluate the role of Rho kinase inhibition in the neuron-hLESCs crosstalk, we examined the effect of LPA+ROCKi on neurotrophic gene expression in co-cultured hLESCs both before and after neuronal *Lpar2* knockdown. Interestingly, while LPA+ROCKi treatment showed no effect on neurotrophic gene expression in hLESCs co-cultured with injured neurons (before *Lpar2* knockdown), following *Lpar2* knockdown, co-cultured hLESCs with injured neurons significantly upregulated the expression of neurotrophic factors (*TAC1*, *NGF*, *CNTF*, and *GDNF*) in the *Lpar2*-KD group compared to the scr group. This upregulation was particularly pronounced for *GDNF* and *CNTF* in the *Lpar2*-KD-LPA versus scr-LPA groups, suggesting that neuronal *Lpar2* knockdown, but not only direct Rho kinase inhibition, enables a constructive neurotrophic crosstalk between injured neurons and hLESCs.

Our data showed that LPAR2 might be the dominant receptor responsible for the inhibitory effects of LPA in DRG neurons [9]. However, other studies have found that LPA induced dose-

dependent growth cone collapse in retinal neurons, yet mice lacking LPAR1-3 continued to exhibit full growth cone collapse in response to LPA [204]. This indicates a possible compensation by other LPA receptors in that system, as mentioned above. However, our experimental findings in both mono-cultured DRG neurons and DRG neurons co-cultured with hLESCs exhibited that LPAR2 plays an essential role, as its knockdown highly abrogated the suppressive effect of LPA, suggesting that LPAR2 might be the main receptor in adult peripheral sensory neurons under these conditions. This discrepancy could be due to cell-type and developmental stage differences in expression patterns of the receptors in the CNS versus the PNS. In the CNS, studies on mouse brain development have shown that hippocampal neurons highly expressed *Lpar1*, *Lpar2*, *Lpar4*, and *Lpar6*, with *Lpar3* and *Lpar5* being undetectable [272]. Also, *Lpar1* showed the highest expression in CNS neurons and was detected in all developmental stages, while *Lpar2*, *Lpar4*, and *Lpar6* were expressed in both immature and mature mouse hippocampal neurons. Our data demonstrate that adult DRG/TG neurons significantly express *Lpar2*, and its knockdown remarkably abrogates the inhibitory effect of LPA [9]. In contrast, the growth cone collapse remained unchanged in the *Lpar1-3* knockout mice when treated with LPA, indicating functional compensation by other LPA receptors, most likely LPAR4-6 [204].

Molecular analysis of *Lpar2* knockdown neurons co-cultured with hLESCs showed the upregulation of *Lpar2* in the scr neurons treated with LPA (both non-injured and injured). This suggests the same fact that the effect of LPA might be mediated via LPAR2, as LPA leads to increased *Lpar2* levels, creating a positive feedback mechanism where neurons may increase their responsiveness to LPA by upregulating its receptor LPAR2. The significant reduction of *Lpar2* expression in the *Lpar2*-KD groups, even in the presence of LPA, proves the efficacy of the siRNA knockdown process in the co-culture system, allowing us to attribute observed molecular changes to the reduction of *Lpar2*. The downregulation of *Enpp2* in all conditions, especially with *Lpar2*-KD and LPA treatment, is unexpected, as ATX is the primary enzyme synthesizing LPA [273], however, this might be a compensatory mechanism to reduce

endogenous LPA production when exogenous LPA is present or when *Lpar2* signaling is knocked down. However, in mono-cultured DRG neurons before injury, the *Lpar2*-KD group showed no effect on *Enpp2* expression but, *Enpp2* was downregulated after the injury. This suggests that the presence of hLESCs in the co-culture model may affect the relationship between LPA-LPAR2 signaling and *Enpp2* expression, which influences LPA metabolism and synthesis pathways. In non-injured transfected neurons in a co-culture system, *RhoA* and *Rock1* downregulation by *Lpar2*-KD implies that the knocked down LPAR2 signaling might reduce the expression of these inhibitory components downstream, creating a regenerative environment for neurite outgrowth in the co-culture model. However, the upregulation of *Rock2* by LPA in both non-injured and injured groups (scr-LPA and *Lpar2*-KD-LPA) shows that LPA consistently activates this major downstream effector, even after *Lpar2* knockdown. This regulation of RhoA-ROCK in the co-culture model showed the involvement of hLESCs-derived neurotrophic factors. The mRNA analysis of hLESCs in the co-culture with injured neurons before *Lpar2* knockdown revealed that hLESCs highly upregulated *NGF* and *GDNF* expression. This is supported by a study using the *in vivo* mouse model, where it has been found that *NGF* and *GDNF* gene expression significantly elevated in regenerating corneal epithelium after nerve injury, peaking during active nerve regrowth and returning to baseline levels when reinnervation is complete [51]. Moreover, LPA treatment maintained this epithelial *NGF* and *GDNF* elevation by suppressing nerve regeneration, keeping the epithelium in a "non-regenerated nerve" status that continuously releases neurotrophic factors for neurons. This explanation can also be supported by a study by Ueno *et al.*, who found that denervated corneas in an *in vivo* mouse model of NK have increased *NGF* mRNA in the epithelium [274]. In contrast, when neuronal *Lpar2* was knocked down in the co-culture model, successful nerve regeneration occurred even in the presence of LPA, and *NGF* and *GDNF* levels were upregulated in hLESCs co-cultured with injured DRG neurons following *Lpar2*-KD.

In contrast, mono-cultured DRG neurons exhibited different RhoA-ROCK regulation patterns: LPA treatment did not change *RhoA*, *Rock1*, and *Rock2* mRNA expression levels in any

condition (pre- and post-injury, before and after *Lpar2* knockdown). This diverse regulation implies that hLESCs in co-culture affect the responsiveness of the neurons to LPAR2 signaling, enabling *Lpar2* knockdown to influence the expression levels of *RhoA* and *Rock1*, whereas in the mono-cultured DRG neurons, these factors are not responsive to LPA. On the other hand, the continuous upregulation of *Rock2* by LPA in co-cultured neurons, despite the knockdown of *Lpar2*, suggests that *Rock2* expression can also be regulated by other LPA receptors that are still active and functional in the signaling. This differences in the regulation of ROCK1 and ROCK2 largely depend on their specific functions and regulatory mechanisms. This is in line with the findings of Mertsch *et al.*, who showed that ROCK isoforms exhibit opposing signaling effects that play a crucial role in the determination of substrate specificity/migration patterns in the case of glioblastoma cells [275], other cellular contexts [276]. A small GTPase, RAC, plays a key role in the formation of filopodial and lamellipodial protrusions, which are important for the initial steps in neurite extension and growth cone progression, often acting antagonistically to RhoA [277]. The continues upregulation of *Rac* levels in co-cultures of DRG neurons with hLESCs after *Lpar2*-KD, both prior to and after injury, compared to scrambled untreated neurons is a key finding. However, in mono-cultured DRG neurons, *Rac* expression was found to be higher in the *Lpar2*-KD untreated group versus the scr-LPA group only after injury, highlighting the effect of hLESCs crosstalk in the co-culture model in sustaining *Rac* activation even in uninjured neurons. This upregulation in *Lpar2*-KD neurons suggests that *Lpar2* knockdown stimulates a growth-promoting cytoskeletal rearrangement, thereby explaining the functional trends reflected in our previous result for the co-culture model [9]. The simultaneous upregulation of *Rac* in DRG neurons and neurotrophic factors (*Ngf*, *Gdnf*, and *Cntf*) in both DRG neurons and hLESCs within *Lpar2*-KD and *Lpar2*-KD-LPA groups in the co-culture model is correlated with the enhanced functional outcomes observed in the single-cell assay and wound-healing assay. The RAS-MAPK-PI3K-PLC pathway regulation is more variable. The RAS-ERK pathway plays a significant role in axon regeneration through cytoskeletal rearrangement and activating regeneration-associated genes in peripheral

neurons. In adult rat superior cervical ganglion (SCG) neurons, activation of RAS-ERK signaling increases axonal elongation [278]. Also active RAS has been shown to highly promote axon regeneration after optic nerve injury, reaching 2.5 mm from the injury region in an *in vivo* mouse model, establishing RAS activation as a key factor of regenerative capacity in neurons [279]. Our findings showed that *Ras* expression was elevated in DRG neurons after *Lpar2* knockdown in both mono-culture and co-culture models. In mono-cultured DRG neurons, *Lpar2*-KD resulted in *Ras* upregulation both pre- and post-injury versus the scrambled neurons. Also, before injury, *Ras* expression showed elevation in the *Lpar2*-KD-LPA group versus the scr-LPA. Similar to mono-cultured DRG neurons, in co-culture with hLESCs, *Lpar2*-KD-LPA neurons exhibited elevated levels of *Ras* in non-injured neurons compared to the scrambled controls. These findings suggest that *Lpar2* knockdown activates *Ras* regardless of culture model or injury status, establishing RAS as a key mediator for nerve regeneration. The potential regenerative outcomes exhibited in mono-culture, where *Ras* upregulation in the *Lpar2*-KD group persisted even in the presence of LPA (*Lpar2*-KD-LPA) versus the scr-LPA group, indicate that *Lpar2* knockdown alleviates the inhibitory effects of LPA, maintaining RAS activation that supports enhanced regenerative capacity. NGF and GDNF facilitate nerve regeneration through activation of RAS-MAPK-PI3K-PLC-ERK signaling pathways [236, 237]. In mono-cultured DRG neurons, the *Lpar2*-KD group exhibited upregulation of *Ras*, *Pi3k*, *Plc*, *Mapk*, *Ngf*, and *Gdnf*, both with and without LPA treatment before injury. However, in co-culture with hLESCs, the signaling profile changed: while *Lpar2*-KD-LPA neurons maintained elevated *Ras* expression in non-injured conditions compared to scrambled controls, the downstream effectors *Pi3k*, *Plc*, and *Mapk* remained unchanged. This suggests that in the presence of hLESCs in the co-culture model, RAS activation may activate other downstream pathways, or maybe the epithelial-derived neurotrophic factors modulate the signaling cascade in a different way than we observed in the mono-culture model. This is consistent with the findings of Catozzi *et al.*, who showed that RAS signaling is highly context-dependent, with pathway selection determined by local protein abundances and the cellular microenvironment

[280], the same finding as we observed between mono-culture and co-culture models in the present study. This multi-layered regulation of the LPA signaling and the context-dependent functions of other signaling highlight the complexity of LPA signaling.

The upregulation of *Cdc42* in co-cultured DRG neurons in all treatment groups (scr-LPA, *Lpar2*-KD, and *Lpar2*-KD-LPA), both pre- and post-injury, show its critical role in neurite initiation. Brown *et al.* reported that in primary chick spinal cord neurons CDC42 stimulates neurite outgrowth and formation of growth cone filopodia and lamellipodia [281]. This suggests that while LPA and *Lpar2*-KD have distinct effects on other signaling pathways, elevated *Cdc42* expression represents a conserved molecular response necessary for nerve fiber regeneration in co-culture model.

The upregulation of neurotrophic factors (*Ngf*, *Gdnf*, *Cntf*) and neuropeptides (*Calca* and *Tac1*) in both non-injured and injured co-cultured neurons following *Lpar2*-KD versus the scrambled controls is a great finding. This effect was particularly evident for *Ngf* levels in LPA-treated groups (*Lpar2*-KD-LPA versus scr-LPA) post-injury. These results demonstrate how injury itself, along with the presence of hLESCs in the system, induces the expression of these important factors. Similarly, in mono-cultured DRG neurons before injury, *Lpar2*-KD neurons elevated *Gdnf* and *Ngf* levels, while *Cgrp* and *Tac1* were upregulated in *Lpar2*-KD-LPA neurons versus both the scr and the scr-LPA groups. Post-injury, both *Lpar2*-KD and *Lpar2*-KD-LPA groups showed significant upregulation of *Gdnf*, *Cntf*, *Ngf*, and *Cgrp* compared to both scrambled controls and scr-LPA groups. However, *Tac1* was downregulated in *Lpar2*-KD-LPA neurons post-injury versus the scr group. To determine whether the upregulation of these factors observed in the co-culture model results from *Lpar2* knockdown, the effect of hLESCs in the system, or their combined effect, we evaluated and compared the expression of these factors under LPA-induced inhibitory conditions (scr-LPA versus scr) in mono-cultured DRG neurons, co-cultured DRG neurons, and hLESCs within the co-culture system, pre- and post-neuronal injury. In co-cultured neurons, *Ngf*, *Gdnf*, and *Cgrp* remained upregulated even under LPA-mediated inhibitory conditions (scr-LPA versus the scr). In contrast, mono-cultured

neurons exhibited significant downregulation of *Tac1* and *Cgrp* post-injury under the same inhibitory state. Additionally, hLESCs in co-culture with non-injured neurons in the same inhibitory LPA group (scr-LPA versus the scr) upregulated *CGRP* and *GDNF*, and when co-cultured with injured neurons, increased *TAC1*, *GDNF*, *NGF*, and *CNTF* levels. These results indicate that epithelial cells perceive the signal from LPA as pro-neurotrophic, not inhibitory. This reveals that while neuronal *Lpar2* knockdown enhances regenerative capacity, the presence of hLESCs has a more potent and predominant effect on nerve regeneration in the co-culture system, since the presence of the epithelial environment completely suppresses the inhibitory effect of LPA regardless of the integrity of the LPA-LPAR2 signaling pathway in neurons. Given the potent neurotrophic support offered by hLESCs under inhibitory conditions mediated by LPA, we investigated whether LPA signaling can directly affect the proliferative potential and stemness characteristics of hLESCs, which could explain their ability to sustain neurotrophic factor production even when neurons are treated with LPA. Critically, LPA treatment (scr-LPA) significantly increased *KI67* expression in co-cultured hLESCs both before and after neuronal injury compared to the *Lpar2*-KD-LPA and scr groups, and the higher proliferative response was found in hLESCs co-cultured with injured neurons (scr-LPA versus scr, *Lpar2*-KD, and *Lpar2*-KD-LPA). This proliferative response is in line with the well-known evidence that LPA is a potent mitogen for epithelial cells by activating the MAPK/ERK signaling pathway, leading to cell cycle progression and stem cell accumulation [162]. These findings are further supported by the study of Xu *et al.*, that LPA facilitates proliferation and epithelial wound healing by the activation of EGFR-ERK-AKT signaling pathways [282]. In addition to proliferation, our co-culture model revealed how neuronal interactions and LPA signaling influence hLESCs stemness and corneal epithelial identity. HLESCs are characterized by stem/progenitor markers (*P63α* and *CK15*), differentiation marker (*CK12*), and the key ocular surface transcription factor *PAX6* [283–287]. Co-cultured hLESCs with non-injured and non-transfected DRG neurons treated with LPA (before *Lpar2* knockdown) showed no significant changes for *P63α*, *CK15*, and *PAX6*, and significant downregulation for *CK12*,

indicating hLESCs remained undifferentiated, which suggests that LPA alone doesn't force co-cultured hLESCs differentiation in the co-culture with non-injured neurons. Indeed, prior studies showed LPA tends to stimulate proliferation/migration rather than immediate differentiation in epithelial cells [282, 288]. However, hLESCs in co-culture with transfected neurons, in the *Lpar2*-KD group, exhibited an upregulation level of *P63α* and *PAX6* (versus scr and scr-LPA for *P63α*, and compared to the scr, scr-LPA, and *Lpar2*-KD-LPA groups for *PAX6*) (both before and after the injury), indicating that *Lpar2* knockdown in neurons promotes the maintenance or enhancement of the stemness phenotype of the hLESCs. Despite the significant downregulation of *P63α* in co-cultured hLESCs in the scr-LPA group versus the scr group pre-neuronal injury, the significant upregulation of *CK15* level in the *Lpar2*-KD group compared to all groups (the scr, scr-LPA, and *Lpar2*-KD-LPA groups) further supports the preservation of stemness of hLESCs both before and after neuronal injury. These findings are in line with those of Ueno *et al.*, who showed that hLESCs were critically dependent on ocular surface innervation. In their denervated model of NK, they showed that sensory nerve deprivation significantly reduced stem cell markers (*P63α*, *ABCG2*, *HES1*) as well as a reduction of stem cell frequency (approximately 75%), highlighting the essential role of DRG neurons-hLESCs interactions in maintaining the limbal niche [274]. The significant downregulation of *CK12* within all conditions, before and after *Lpar2* knockdown, both pre- and post-neuronal injury, supports the idea that the co-culture of hLESCs with DRG neurons, regardless of injury, LPA treatment or *Lpar2* knockdown, promotes a situation where hLESCs are maintained in an undifferentiated state rather than differentiating into mature corneal epithelial cells, maintaining their plasticity and regenerative potential.

Apoptosis-related genes in hLESCs in both non-injured and injured co-culture models, before and after neuronal *Lpar2* knockdown in all treatment groups, exhibited low expression of pro-apoptotic factors (*BAX*, *CASP3*) and anti-apoptotic genes (*BCL-2*). This is consistent with a supportive environment, which states that intact corneal nerves continuously release factors (such as SP, CGRP, and trophic peptides) that help maintain epithelial cell viability and

suppress excessive apoptosis [52–54]. However, the results showed that injured neurons in the co-culture model also (before and after *Lpar2* knockdown) retained sufficient capacity to release neuropeptides and maintain a supportive environment adequate for hLESCs survival. Vimentin (VIM) is an intermediate filament protein commonly associated with mesenchymal cells and is often used as a marker for mesenchymal properties, cell activation, and migration. However, Polisetti *et al.* detected a low level of VIM expression in the basal limbal epithelial cells, which were positive for PAX6 [289]. This highlighted that cultural conditions can induce VIM expression [290, 291]. Aligning with these findings, in our co-cultured hLESCs with non-injured neurons following *Lpar2* knockdown, no changes were observed for VIM levels; however, VIM upregulation in co-cultured hLESCs with injured neurons in the *Lpar2*-KD group versus scr and scr-LPA groups suggests that injured neurons may induce a wound healing or migratory response in hLESCs, as VIM is associated with highly motile corneal epithelial cells and is transiently upregulated during corneal wound repair. Previous studies have demonstrated that VIM upregulation in corneal epithelial cells is associated with enhanced cellular motility and wound repair responses [292, 293]. The upregulation of VIM in our *Lpar2*-KD post-injury co-culture hLESCs may reflect an adaptive cellular response to neuronal injury, potentially indicating an enhanced motility state of hLESCs in response to injured neurons.

The gene expression analysis of co-cultured hLESCs highlights the profound and dynamic interaction in the co-culture model between DRG neurons and hLESCs. Neuronal *Lpar2* knockdown, LPA treatment, and injury status all independently and synergistically regulate the mRNA profile of hLESCs. The consistent downregulation of *LPAR2*, *RHOA*, *ROCK1*, and *ROCK2* in co-cultured hLESCs with injured DRG neurons (in *Lpar2*-KD and *Lpar2*-KD-LPA groups, both compared to the scr and scr-LPA groups) suggests a feedback mechanism where neuronal LPAR2 activity affects the ability of hLESCs to respond to LPA and its effect. The observed all changes in co-cultured hLESCs with neurons (either pre- and post-injury both before and after neuronal *Lpar2* knockdown) and discussed in this section, such as enhanced neurotrophic factors secretion, low expression of apoptosis markers, and maintenance of

stemness/epithelial markers with suppression of differentiation marker, especially after *Lpar2*-KD, highlight that hLESCs are becoming more supportive and regenerative. This highlights that *Lpar2* knockdown in neurons not only has a direct effect on neuronal regeneration itself, but also regulates the supportive microenvironment of co-cultured hLESCs for healing and regeneration. This dual benefit underscores the potential of targeting LPA signaling for nerve regeneration in NK.

Our data showed that there were obvious differences in regenerative responses between mono-cultured DRG neurons and co-cultured with hLESCs, especially when neurons treated with LPA or *Lpar2* were knocked down. In mono-cultured DRG neurons before *Lpar2* knockdown, LPA alone showed a suppressive effect on neurite outgrowth. However, this inhibition was less pronounced in the co-cultured DRG neurons. For example, while LPA significantly reduced the fiber length of mono-cultured DRG neurons, the same treatment in the co-culture model did not reach statistical significance at early time points (24h), suggesting that secreted neurotrophic factors from hLESCs in the early phase of regeneration provide partial neuroprotection against LPA-induced growth suppression. Interestingly, in both mono- and co-cultured DRG neurons, the knockdown of *Lpar2* increased nerve regeneration, but it had a significantly more pronounced effect on co-cultured Neurons. For example, in the single-cell assay of co-cultured neurons, the *Lpar2*-KD-LPA group versus the scr-LPA group showed significantly higher values of neurite fiber length with a more significant p-value than the same comparison groups in the mono-cultured DRG neurons. This enhanced regenerative effect in co-culture is directly supported by the qRT-PCR analysis of hLESCs in co-culture with DRG neurons, which showed that hLESCs co-cultured with injured neurons after *Lpar2* knockdown upregulated neurotrophic factors (*TAC1*, *GDNF*, *NGF*, and *CNTF*), particularly in the *Lpar2*-KD-LPA versus scr-LPA comparison, where *GDNF* and *CNTF* showed the most pronounced elevation. Also, LPA treatment (scr-LPA versus scr) downregulated trophic factors in mono-cultured neurons post-injury, however, the same LPA treatment (scr-LPA versus scr) highly increased these factors in both co-cultured cells: DRG neurons and hLESCs. These findings

demonstrate that epithelial cells recognize LPA as a pro-neurotrophic factor rather than as an inhibitory stimulus to support DRG neurons. The observed supportive mechanism of hLESCs in our co-culture model is marked by the constant release of neurotrophic factors by hLESCs, as well as increased proliferative ability, and maintained stemness features that preserve regenerative capacity under LPA treatment. This mechanism explains the superior regenerative outcomes observed when we knocked down *Lpar2* within the DRG neurons-hLESCs co-culture model, since hLESCs remarkably alleviate the suppressive effect of LPA on neurite outgrowth via complementary neurotrophic support. These findings highlighted the significant advantages of the LPA-LPAR2 signaling modulation (*Lpar2* knockdown) combined with the microenvironmental support (provided by hLESCs in the co-culture model). The significance of such interactions may be crucial in designing therapeutic strategies for peripheral nerve regeneration in ocular surface or corneal models, where epithelial-neuronal cross-talk plays an important role.

4.3 Conclusion

Our data reinforce that LPA exerts a strong inhibitory role in the regeneration of DRG neurons, by triggering the RhoA-ROCK pathway via LPAR2 [9, 294]. Moreover, knockdown of *Lpar2* abolished the inhibitory effect of LPA and promoted nerve fiber regrowth and increased the levels of regeneration expression (*Ngf*, *Gap43*, and *Cdc42*) despite LPA exposure. Additionally, the findings of this study showed that Rho kinase inhibition rescued DRG neurons by the inhibitory effect of LPA and increased neuronal fiber length/regrowth [9], similar to the Mertsch *et al.* observations that Rho kinase inhibition significantly enhanced nerve regrowth *in vitro* and *in vivo* [8]. These results confirmed that the LPAR2-RhoA-ROCK axis as the key mechanistic pathway for nerve regeneration. Importantly, our DRG neurons-hLESCs co-culture model revealed that hLESCs have a modulatory influence on neuronal regeneration via the high levels of neurotrophic factors secretion such as, *NGF* and *GDNF*. This is in agreement with the findings of Di *et al.*'s who reported that neurotrophic factors secreted by corneal epithelium promote nerve regeneration [51]. hLESCs effectively mimicked Schwann cells for corneal nerves, functioning similarly to the corneal epithelial cell-nerve interaction described by Stepp *et al.* [295]. In co-culture, hLESCs maintained their stemness characteristics by sustained expression of stemness markers (*P63 α* and *CK15*) and suppression of the differentiation marker (*CK12*). At the same time, hLESCs co-cultured with DRG neurons after *Lpar2* knockdown (with and without LPA treatment) upregulated neurotrophic factors (*TAC1*, *GDNF*, *NGF*, and *CNTF*), effectively alleviated LPA-induced nerve regrowth suppression, and promoted superior nerve fiber regrowth in co-cultured DRG neurons compared to the mono-cultured DRG neurons. Methodologically, this co-culture model used in our study mimics the neuron-epithelium interactions that exist in the native cornea. This enables us to deeply investigate the bidirectional dynamics in both cells (with and without LPA treatment, before and after *Lpar2* knockdown). Overall, by examining LPAR2 [9] and its downstream RhoA-ROCK signaling pathway as inhibitors of nerve regeneration [8], this thesis offers a mechanistic insight for the development or improvement of therapeutic approaches, such as delivered

LPAR2 antagonists or ROCK inhibitors locally into the cornea tissue, to enhance corneal nerve repair in NK patients. These strategies may offer an improvement over the existing cenergermin eye drop, which, despite promoting corneal healing, is expensive and not universally available [147].

4.4 Future perspective

Based on the observations of this doctoral thesis, future studies should examine the long-term *in vivo* outcomes of LPAR2 knockdown therapy to validate long-lasting nerve regeneration and full functional recovery over time. It will be worth investigating more extensive LPA signaling pathways that may also modulate regeneration. By integrating molecular insights into nerve-epithelial interactions with a physiologically relevant co-culture model, this study provides a foundation for developing regenerative therapies to overcome the limitations of current NK treatments and restore corneal innervation and health. Given the outcome from the co-culture model, which showed healthy hLESCs can enhance neurite outgrowth, it is plausible that hLESCs exert paracrine effects via extracellular vesicles (EVs). Future research may shed more light on these EVs by purification and characterization as regards protein and RNA content, as well as investigating the regeneration capacity of these EVs on DRG neurons with or without LPA or *Lpar2* knockdown. Moreover, single-cell transcriptome analysis of hLESCs co-cultured with DRG neurons may identify particular subpopulations or adaptive gene programs initiated by neuronal damage or LPA signaling. While the findings of this thesis demonstrate enhanced neurite elongation, future studies examining axon guidance molecules and synaptic markers should be investigated to confirm whether this neurite elongation/regrowth is organized, target-directed, and results in functional synaptic connections. Calcium imaging of the regenerated neurons would also confirm the restoration of neuronal excitability and signal transmission. Future studies may investigate the role of dendritic cells (DCs) in corneal nerve regeneration. Since DCs are near subbasal nerves and can regulate both inflammation and the epithelial repair process, they may affect the neurons-hLESCs interaction. The combining of DCs within co-culture models or analysis of DCs-secreted factors (such as IL-10 or TGF- β) could provide an overview of neuro-immune regulation that may promote corneal nerve regeneration. The delivery of ROCK inhibitors, LPAR2 antagonists, or hLESCs-derived EVs with biodegradable eye-compatible hydrogels may provide a cell-free and non-invasive therapeutic approach for NK patients. Recently, CRISPR-based therapeutic options have been

used in clinical trials for human eye diseases, such as CRISPR/Cas9 gene editing (EDIT-101) for Leber congenital amaurosis type 10 and CRISPR/Cas9 antiviral therapy (BD111) for herpes simplex keratitis. Currently, both are successfully in the clinical trials phase 1/2 and have shown promising results. Based on these successful approaches, the silencing of LPAR2 by CRISPR in the corneal neurons may become an effective strategy for enhancing nerve regeneration in NK patients.

Together, these directions provide us with a roadmap for the development of the observed *in vitro* results into more comprehensive, functionally validated, and clinically applicable options to corneal nerve regeneration.

5

5. References

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6. Publications

- **Original Research Article**

1. **“The role of lysophosphatidic acid and its receptors in corneal nerve regeneration”**

Authors: Maryam Kheyrollah, Nicola Brandt, Anja U. Bräuer, Stefan Schrader, Sonja Mertsch

Journal: The Ocular Surface 36 (2025) 10–18

Publisher: Elsevier

Publication Date: 01.04.2025

- **Poster Presentations**

2. **“ATX-LPA Signaling Modulation for Corneal Nerve Regeneration - An *In Vivo* Study in a Neurotrophic Keratopathy Mouse Model”**

Authors: Maryam Kheyrollah, Nicola Brandt, Anja U. Bräuer, Stefan Schrader, Sonja Mertsch

Deutsche Ophthalmologische Gesellschaft (DOG), Oct 2024, Berlin, Germany.

3. **“Role of Lysophosphatidic Acid and Its Receptors on Corneal Nerve Regeneration: Implications for Neurotrophic Keratopathy”**

Authors: Maryam Kheyrollah, Nicola Brandt, Anja U. Bräuer, Stefan Schrader, Sonja Mertsch

Association for Research in Vision and Ophthalmology (ARVO), May 2024, Seattle, USA.

- **Oral Presentation**

4. **“Role of Lysophosphatidic Acid on Corneal Nerve Regeneration”**

Authors: Maryam Kheyrollah, Nicola Brandt, Anja U. Bräuer, Stefan Schrader, Sonja Mertsch

Deutsche Ophthalmologische Gesellschaft (DOG), Oct-Nov 2023, Berlin, Germany.

7. Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und keine als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit weder in ihrer Gesamtheit noch in Teilen einer anderen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat und dass ich die Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg befolgt habe. Weiterhin versichere ich, dass im Zusammenhang mit dem Promotionsvorhaben keine kommerziellen Vermittlungs- oder Beratungsdienste (Promotionsberatung) in Anspruch genommen worden sind.

_____ (Ort, Datum) _____ (Maryam Kheyrollah)

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و یک تشکر ویژه از پدر و مادرم، که همیشه با عشق، صبوری، و حمایت بی‌پایان‌شان در تمام مراحل زندگی و این مسیر دشوار در کنار من بودند. هر موفقیتی که به دست آورده‌ام، نتیجه دعای خیر، محبت بی‌حد و مرز، و ایمان عمیق شما به من است. بدون حضور و پشتیبانی شما، هیچ‌یک از این دستاوردها ممکن نبود. از صمیم قلب قدردان تمام فداکاری‌ها، ایثار و محبت بی‌دریغ شما هستم.