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ROLE OF PIEZO1 MECHANOSENSITIVE CHANNEL IN ELEVATED PRESSURE-INDUCED MICROGLIA REACTIVITY

Dissertação no âmbito do Mestrado em Investigação Biomédica, com especialização em Ciências da Visão, orientada pela Professora Doutora Ana Raquel Sarabando Santiago e coorientada pela Doutora Raquel Sofia Freitas Boia e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Setembro 2023

Faculdade de Medicinada Universidade de Coimbra

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Abbreviations

AD - Alzheimer's disease

BBB – blood-brain barrier

BCA – bicinchoninic acid

BRB – blood-retina barrier

BSA – bovine serum albumin

Ca^{2+} - calcium ions

CEDs – C-terminal extracellular domains

CNS – central nervous system

CTCF – corrected total cell fluorescence.

CTDs – C-terminal domains

DAF-FM – 4-Amino-5-Methylamino-2',7'-Difluorofluorescein diacetate

DAMPs – damage-associated molecular patterns

DAPI – 4',6-diamidino-2-phenylindole

EHP – elevated hydrostatic pressure

ECL – enhanced chemiluminescence

FBS – fetal bovine serum

GABA – gamma-aminobutyric acid

GCL – ganglion cell layer

HCs – horizontal cells

hEHP – hypoxia elevated hydrostatic pressure

HRP – horseradish peroxidase

HIF-1α – hypoxia-inducible factor 1-alfa

INL – inner nuclear layer

iNOS – inducible nitric oxide syntetase

IOP – intraocular pressure

IPL – inner plexiform layer

IHs – inner helices

IS – inner segments

KHR – Krebs-Henseleit ringer solution

NFL – nerve fibre layer

NO – nitric oxide

OHs – outer helices

ONL – outer nuclear layer

OPL – outer plexiform layer

OS – outer segments

PBS – phosphate-buffered saline

PFA – paraformaldehyde

PHs – peripheral helices

PVDF – poly(vinylidene difluoride)

RGCs – retinal ganglion cells

RIPA – radioimmunoprecipitation assay

RPE – retina pigment epithelium

RPMI – roswell park memorial institute

ROS – reactive oxygen species

RT – room temperature

SEM – standard error of the mean

TAZ – transcriptional coactivator with PDZ-binding motif

TBS-T – tris-buffered saline with Tween-20

THUs – transmembrane helical units

YAP – yes-associated protein

Resumo

O glaucoma, uma das principais causas de perda irreversível da visão, é caracterizado pela perda de células ganglionares da retina e danos no nervo ótico, onde os principais fatores de risco são a pressão intraocular elevada e o envelhecimento. Foi demonstrado que a neuroinflamação induzida pela microglia desempenha um papel crucial no início e progressão da doença. Tem sido mostrado que o controlo da neuroinflamação mediada pelas células da microglia é suficiente para proteger as células ganglionares da retina de danos, reforçando o papel crucial da microglia na doença. No nosso grupo, foi mostrado que a exposição da microglia à pressão elevada induz a liberação de fatores citotóxicos que promovem a morte das células da retina, nomeadamente das células ganglionares da retina. No entanto, o sensor na microglia que deteta as alterações da pressão na membrana plasmática ainda não foi identificado.

Piezo1, um canal iónico mecanosensível, deteta alterações na pressão e tensão de cisalhamento cuja ativação tem sido associada à inflamação. Piezo1 é expresso na microglia, mas o seu papel na retina e a sua contribuição para a neuroinflamação no glaucoma ainda não foram explorados. Este estudo teve como objetivo estudar o papel da ativação do Piezo1 na reatividade da microglia induzida por pressão elevada.

Células BV-2 foram expostas a pressão hidrostática elevada (70 mmHg acima da pressão normal) em condições de normoxia (EHP; 20% O_2) e em condições de hipoxia (hEHP; 2% O2) por 4 e 24 horas, na presença ou ausência de 0,5, 1 e 10 μM GsMTx4 (inibidor do Piezo1). As células foram incubadas com 1, 5 e 10 μM Yoda1 (agonista do Piezo1). Os níveis de proteicos do canal Piezo1 foram quantificados por Western blot. A fagocitose foi avaliada com microesferas fluorescentes. A produção de NO foi avaliada pelo ensaio da reação de Griess e com a sonda DAF-FM. Os níveis proteicos de iNOS, TAZ, YAP e p-YAP (S397) foram quantificados por Western blot.

Este trabalho mostrou que Piezo1 é expresso em células BV-2 e que, com a exposição ao hEHP, houve um ligeiro aumento na expressão do canal. Curiosamente, a ativação do Piezo1 mostrou um ligeiro aumento na eficiência da fagocitose, enquanto a exposição ao hEHP não mostrou alterações na eficiência da fagocitose.

O efeito da ativação de Piezo1 no stress nitrosativo não mostrou alterações na concentração média de nitrito. No entanto, a avaliação direta da produção de NO, revelou que a exposição a hEHP aumentou significativamente a produção de NO.

A ativação de Piezo1 não afetou os níveis proteicos totais de TAZ, YAP e p-YAP (S397). Em relação à sua localização subcelular, a ativação do Piezo1 não afetou os níveis proteicos, quer a nível nuclear quer a nível do citoplasma, de TAZ nem YAP, no entanto, exibiu uma tendência a diminuir os níveis citoplasmáticos de p-YAP (S397).

Este trabalho mostrou que Piezo1 é expresso em células BV-2 e pode desempenhar um papel na reatividade desta linha celular de microglia, uma vez que mostrou uma tendência a aumentar a eficiência da fagocitose e a produção de NO com incubação de Yoda1, bem como sob EHP e hEHP, respetivamente. O efeito de ativação de Piezo1 não mostrou efeitos significativos na localização subcelular de YAP, TAZ e p-YAP (S397), no entanto, é necessária uma investigação mais aprofundada para compreender melhor os mecanismos de indução de reatividade na microglia induzidos pela ativação do Piezo1.

Palavras-chave: Microglia; glaucoma; pressão hidrostática elevada; canal Piezo1; neuroinflamação; via de sinalização *Hippo*.

Abstract

Glaucoma, a leading cause of irreversible vision loss, is characterized by retinal ganglion cell loss and optic nerve damage, where the main risk factors are elevated intraocular pressure and ageing. It has been shown that microglia-induced neuroinflammation plays a crucial role in the disease's onset and progression. The control of microglia-mediated neuroinflammation is sufficient to protect retinal ganglion cells from damage, reinforcing the crucial role of microglia in disease. In our group, it has been demonstrated that exposing microglia to elevated pressure induces the release of cytotoxic factors that promote retinal death. However, the sensor of elevated pressure in microglia has not been identified yet.

Piezo1, a mechanosensitive ion channel, detects alterations in pressure and shear stress and its activation has been linked to inflammation. Piezo1 is expressed in microglia but its role in the retina and its contribution to neuroinflammation in glaucoma has not been explored. This study aimed to investigate the role of Piezo1 activation in elevated pressure-induced microglia reactivity.

BV-2 cells were exposed to elevated hydrostatic pressure (70 mmHg above normal pressure) in normoxic conditions (EHP; 20% O2) and in hypoxic conditions (hEHP; 2% O2) for 4 and 24 hours, in the presence or absence of 0.5, 1 and 10 µM GsMTx4 (Piezo1 inhibitor). Cells were incubated with 1, 5 and 10 µM Yoda1 (Piezo1 agonist). Piezo1 protein levels were quantified by Western blot. Phagocytosis was assessed with fluorescent microbeads. NO production was assessed by Griess reaction assay and with the NO-sensitive probe DAF-FM. Protein levels of iNOS, TAZ, YAP and p-YAP (S397) were quantified by Western blot.

This work showed that Piezo1 is expressed in BV-2 cells and the exposure to hEHP, leads to a slight increase in the channel expression. Intriguingly, Piezo1 activation showed a slight increase in phagocytosis efficiency, while exposure to hEHP showed no alterations in phagocytosis efficiency.

The effect of Piezo1 activation in nitrosative stress showed no alterations in the medium nitrite concentration. However, when directly examining the production of NO, revealed that the exposure to hEHP significantly increased NO production.

Piezo1 activation did not affect the total protein levels of TAZ, YAP, and p-YAP (S397). Regarding their subcellular location, Piezo1 activation did not affect TAZ nor YAP nuclear and cytosolic protein levels, however, it exhibited a tendency to decrease cytoplasmic levels of p-YAP (S397).

Altogether this work showed that Piezo1 is expressed in BV-2 cells and might play a role in the reactivity of these microglia cell line once it showed a tendency to enhance phagocytosis efficiency and increase NO production with Yoda1 incubation as well as under EHP and hEHP, respectively. Piezo1 activation effect showed no significant effects in the YAP, TAZ and p-YAP (S397) subcellular location, however, it is required further investigation to better understand Piezo1 activation – induced microglia reactivity mechanisms.

Keywords: Microglia; glaucoma; elevated hydrostatic pressure; Piezo1 channel; neuroinflammation; Hippo signalling pathway

Chapter 1

Introduction

1.1 The eye: anatomy and physiology

The eye's primary function involves the conversion of incident light into electrical signals, which are subsequently transmitted through the optic nerve to the brain (Purves et al., 2015; Willoughby et al., 2010). It can be divided into two distinct parts, the anterior and the posterior segments. In the anterior segment, the outermost layer is the cornea, a transparent tissue that acts as a protective cover and refracts excessive light. The iris controls the size of the pupil, regulating the amount of light that enters the eye. The lens is a flexible structure that adjusts its shape enabling the focus of objects in different planes. The ciliary body, a ring-like structure surrounding the lens that produces the aqueous humour, a clear and watery fluid that provides support for the eyeball and nutrients to the cornea and lens (Downie et al., 2021; Willoughby et al., 2010).

The posterior segment comprises the vitreous humour that fills the space between the lens and the retina, supporting the eye's shape and providing structural integrity (DelMonte & Kim, 2011; Gao et al., 2008). The retina is a layer of light-sensitive neuronal cells lined in a well-organized structure that is responsible for the conversion of light stimuli into electrical signals (Demb & Singer, 2015; la Cour & Ehinger, 2005). The choroid is a vascular layer that provides oxygen and nutrients to the outer retinal layers (Willoughby et al., 2010). The outermost layer is the sclera, whose main role is to maintain the shape and integrity of the eye and helps to keep the eye's intraocular pressure stable (Boote et al., 2020; Watson & Young, 2004) (Figure 1).

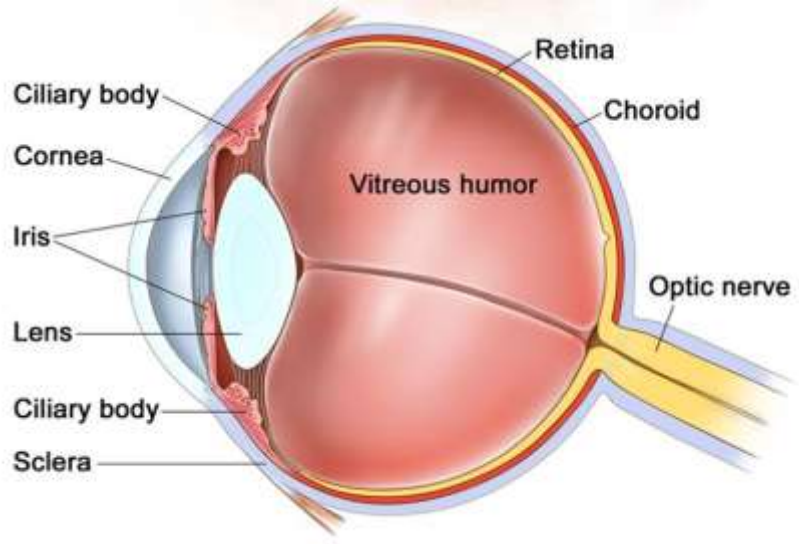

Figure 1 | Anatomy of the human ocular globe. Illustration of the eye structure, including the cornea, iris, ciliary body, lens, vitreous humour, retina, sclera, choroid and the optic nerve. Adapted from NationalCancerInstituteUS 2021.

1.2 The retina: structure and function

The retina is a tissue located in the posterior part of the eye and just like the brain and spinal cord, it is part of the central nervous system (CNS), where its main function is to convert light stimuli in the form of photons into neuroelectric impulses so that the brain can process the images captured by the eyes (Hoon et al., 2014; Ptito et al., 2021).

This sensory tissue presents a well-organized structure composed of layers of cell bodies, the nuclear layers, intercalated with the plexiform layers, where the retinal cells establish synapses. The outermost layer is the retinal pigment epithelium (RPE), a monolayer of cuboid and pigmented cells whose main goal is to provide support and pigment production for the photoreceptors (rods and cones), as well as the removal of cell debris. Outer and inner segments (OS/IS) of photoreceptors connect with the upper part of the RPE. The cell bodies of the photoreceptors are located in the outer nuclear layer (ONL) and establish synapses with bipolar cells in the outer plexiform layer (OPL). The inner nuclear layer (INL) contains the cell bodies of the bipolar, horizontal, and amacrine cells, that form synapses with retinal ganglion cells (RGCs) in the inner plexiform layer (IPL). The nuclei of RGCs and displaced amacrine cells are located in the ganglion cell layer (GCL). Next is the nerve fibre layer (NFL) composed by the RGCs' axons that form the optic nerve that will transmit the signal to the visual cortex in the brain, where the information will be processed (Madeira, Boia, et al., 2015b; Masland, 2001; Santiago et al., 2020).

Alongside neurons, the retina also contains glial cells, endothelial cells, and pericytes. Retinal glial cells form a supportive and protective network, establishing complex interactions with neurons and blood vessels. There are three main types of glial cells in the retina: Müller cells, astrocytes, and microglia (Bringmann et al., 2009; Reichenbach & Bringmann, 2020).

Müller cells, the most abundant glia in the retina, extend throughout its entire thickness, with their cell bodies located at the INL. Astrocytes are predominantly confined to the innermost retinal layers, and their cell bodies are mostly restricted to the NFL. Microglia cells are the resident immune cells of the retina and are typically found in the inner parts of the retina (Fischer et al., 2010; Vecino et al., 2016).

The retina is a highly active and energy-demanding tissue that requires a substantial blood supply for its functions. The primary blood supply is provided by the choroid, which is characterized by fenestrated vessels, allowing efficient exchange of oxygen and nutrients (Joyal et al., 2018; Purnyn, 2013). The blood vessels are formed by endothelial and surrounded by pericytes that provide structural support and regulate blood flow. Another vascular circulation

supply is provided by the central retinal artery, located at the centre of the optic nerve (Overbeeke, J.J. & Sekhar, N., 2003; Singh Hayreh, 2001).

This system is associated with a blood-retinal barrier (BRB) formed by a non-fenestrated network of vessels that maintain tight junctions of the retinal endothelial cells, representing the inner BRB. The outer BRB is maintained by tight junctional complexes between RPE cells and the barrier formed by Bruch's membrane. BRB function comprises the control of the movement of substances between the blood and the retina, ensuring proper retinal function and protection (Campbell & Humphries, 2013; Cunha-Vaz et al., 2011; Runkle & Antonetti, 2011) (Figure 2).

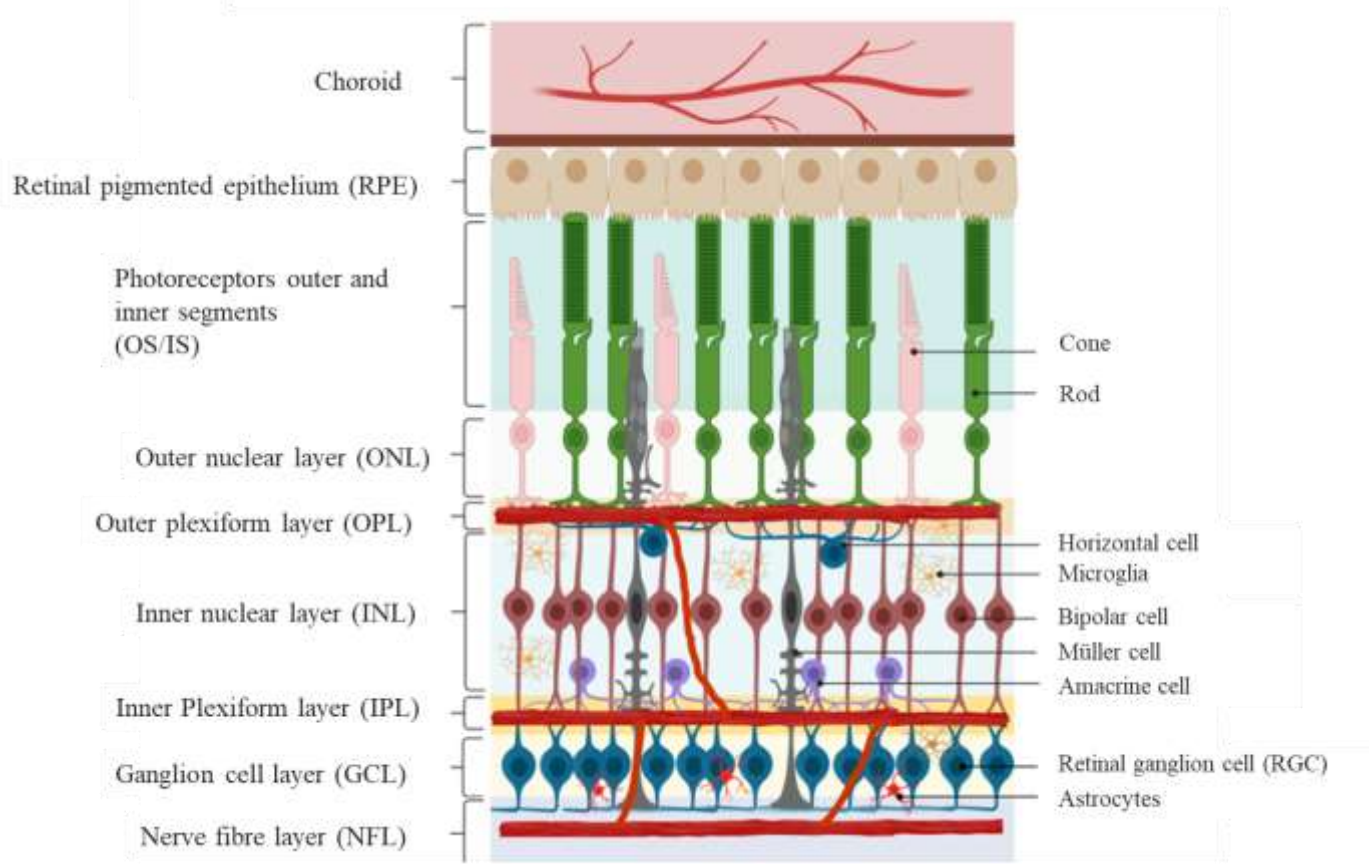

Figure 2 | Schematic representation of the retinal structure. The neuroretina cells include rod/cone photoreceptors, bipolar cells, horizontal cells, amacrine cells and retinal ganglion cells; as well as three types of glial cells (Müller cells, astrocytes, and microglia). Retinal layers (from the most internal to the outer layers): nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptors outer and inner segments (OS/IS), retinal pigment epithelium (RPE), choroid (Ch). Image adapted from Zhou & Chen (2023).

1.2.1 Retinal neuronal cells

There are five types of neurons in the retina: photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells, and RGCs. Visual information follows two pathways for processing and transmission: the vertical pathway (direct pathway) and the horizontal pathway (lateral pathway) (Ptito et al., 2021).

The vertical pathway is a direct and unidirectional transmission of visual information, involving a sequential flow of retinal neuronal cells from photoreceptors to bipolar cells, and finally to RGCs. The RGCs' axons extend to form the optic nerve, which in turn carries this information to the brain for further processing and interpretation (Byzov et al., 1977; Rosso et al., 2021). The horizontal pathway involves horizontal and amacrine cells and is responsible for lateral interactions, such as lateral inhibition, enhancing visual contrast and sharpness. It improves spatial resolution and sensitivity to light and dark contrasts, refining visual information before transmitting it to the brain (Masland, 2004).

1.2.1.1. Photoreceptors

Photoreceptors are responsible for phototransduction, a process that converts light photons into a biological signal, culminating in the release of glutamate at the photoreceptor synapse (Mahroo & Duignan, 2022). There are two classes of photoreceptors: rods and cones. Rods are characterized by higher light sensitivity and slower kinetics, mainly mediating scotopic vision, while cones function under bright light, conveying brightness, colour information and visual acuity (Zang & Neuhauss, 2021).

Rods and cones outer segment (OS) comprise tightly packed membrane discs containing visual pigments such as rhodopsin and other proteins involved in phototransduction, while the inner segment (IS) contains all the cell organelles (Santo & Conte, 2022). In order to prevent metabolic stress caused by continuous exposure to light, the most distal portion of the OS undergoes daily shedding by RPE, with the incorporation of newly formed discs (Arendt, 2003).

1.2.1.2. Horizontal cells

As part of the horizontal pathway, horizontal cells are GABAergic interneurons that receive information from photoreceptors and modulate the signal output, facilitating long- and short-range interactions (Schubert et al., 2010). Horizontal cells also offer lateral feedback inhibition that maintains the visual system's sensitivity to brightness and supports contrast enhancement (Chapot et al., 2017).

Horizontal cells present dendrites with many clustered terminals that connect individually with photoreceptors. Depending on the species, horizontal cells can be subdivided

according to its morphology and function (Miller & Peichl, 1993). The most highlighted difference relies on the presence or absence of the axon: A-type cells are axon-less contacting exclusively with cones through its dendritic trees; B-type cells present axon terminal systems that contact exclusively with rods, whereas its dendritic trees contact exclusively with cones (Boije et al., 2016; Mangel, 1991).

1.2.1.3. Bipolar cells

Bipolar cells act as an intermediary for visual information transmission from photoreceptors and horizontal cells to amacrine cells and finally RGCs (Euler et al., 2014). Bipolar cells can be categorized as ON or OFF bipolar cells, depending on their response to glutamate (Snellman et al., 2008).

ON bipolar cells depolarize in response to increased light levels, transmitting information about the presence of light or brightness. In contrast, OFF bipolar cells hyperpolarize when light decreases, conveying information about darkness or the absence of light. (Burkhardt, 2011; Puller et al., 2017).

Bipolar cells can also be distinguished according to the type of photoreceptor they interact with: rod bipolar cells receive input from multiple rod photoreceptors, allowing them to function well in low-light conditions; cone bipolar cells interact with a smaller number of cone photoreceptors and are primarily responsible for processing visual information in brighter light conditions and in colour vision (Hildebrand & Fielder, 2011).

1.2.1.4. Amacrine cells

Amacrine cells are retinal interneurons located in the INL and are involved in lateral interactions within the retina. They lack axons and modulate visual signals by releasing gamma-aminobutyric acid (GABA) and glycine, both inhibitory neurotransmitters (Johansson et al., 2010; Masland, 2012).

These features enable amacrine cells to inhibit the activity of other neurons, enhancing visual contrast by adjusting sensitivity to light and adapting the retina to different lighting conditions. Displaced amacrine cells in the GCL are also engaged in detecting light contrast and movement (Euler et al., 2014; Lechner et al., 2017).

1.2.1.5. Retinal ganglion cells

RGCs cell bodies are inserted in the GCL and their axons converge at the optic disc, forming the optic nerve. RGCs exhibit diversity with various subtypes, each responding to different aspects of visual stimuli such as colour, motion, contrast, or orientation. A notable characteristic of RGCs is the partial crossing-over of their axons at the optic chiasm,

contributing to binocular vision in forward-facing eyes (Chidlow & Osborne, 2003; Schiller, 2010).

Melanopsin-containing RGCs are photosensitive, expressing melanopsin, and are involved in circadian rhythm regulation, pupillary light reflex, and sleep (Lax et al., 2019). As a neuronal cell type, RGCs have limited regenerative capacity after the establishment of synaptic connections shortly after birth, making them susceptible to injuries and contributing to ocular pathologies like glaucoma (Boia et al., 2020; Santiago et al., 2014; Schmidt et al., 2011).

1.2.2. Retinal glial cells

Glial cells are non-neuronal located within the CNS providing physical and metabolic assistance. They aid in neuronal communication, nutrient and oxygen supply, removal of neuronal waste and elimination of pathogens. Glia cells in the retina comprise astrocytes, Müller cells and microglia (Vecino et al., 2016).

1.2.2.1. Astrocytes

Astrocytes are present in the NFL and GCL. These glial cells provide structural support to retinal neurons and regulate the exchange of nutrients and waste products between blood vessels and neurons. They also contribute to the formation and maintenance of the BRB, protecting the retina from harmful substances (Chang et al., 2007; Sofroniew & Vinters, 2010).

Additionally, astrocytes help to regulate ion balance and neurotransmitter levels, modulating synaptic activity. In response to injury, astrocytes become activated and participate in glial (Cunha-Vaz et al., 2011; Vecino et al., 2016).

1.2.2.2. Müller cells

Müller cells are the predominant glial cells in the retina, and interact with all types of retinal cells, including neurons, contributing to the establishment of retinal architecture during development (Bringmann et al., 2006; Coughlin et al., 2017). They serve as a crucial support system, maintaining retinal integrity, and are responsible for the metabolic support of retinal neurons, regulating the transport of nutrients and molecules. These cells play a vital role in maintaining ion and water balance, essential for proper neuronal function and overall retinal health, as well as in the establishment of the BRB (J.-J. Wang, 2015).

Additionally, Müller cells act as protectors by swiftly removing neurotransmitters after synaptic transmission, preventing excitotoxicity and maintaining a clear signal-to-noise ratio. They produce and secrete neurotrophic factors, growth factors, and cytokines crucial for neuronal survival (Reichenbach & Bringmann, 2013). In response to retinal injury or disease,

Müller cells can become reactive and contribute to glial scar formation. While this response aims to limit damage spread, excessive scarring can negatively impact visual function and contribute to the pathogenesis of diseases such as glaucoma (Graca et al., 2018). Müller cells possess a unique property called "waveguiding", allowing them to guide light horizontally along their elongated processes, which enhances visual sensitivity (Ball et al., 2022; Shabahang et al., 2018).

1.2.2.3. Microglia

Microglia are the resident immune cells of the CNS and play a pivotal role in maintaining homeostasis in the brain and in the retina environment. Constantly surveying the surrounding microenvironment, microglia act as the first line of defence, rapidly responding to any sign of injury, infection, or neurodegeneration (Kim & Joh. 2006; Eyo & Wu, 2013). In a quiescent state, phagocytosis, surveillance, and capacity for releasing soluble factors are core properties through which microglia contribute to key biological functions for instance neurogenesis, synapse remodelling, tissue repair, myelination, and immune defence (Paolicelli et al., 2022).

When the neuronal tissue experiences injury or faces pathological stimuli, microglia react, performing phagocytosis and promoting the release of pro-inflammatory molecules, cytokines and chemokines, to clear pathogens, remove damaged cells, and initiate tissue repair. (Karlstetter et al., 2010; Silverman & Wong, 2018). However, in cases of chronic or dysregulated neuroinflammation, reactive microglia can contribute to neurotoxicity and damage of the neuronal tissue (Shao et al., 2022) leading to neurodegenerative diseases in the brain, Alzheimer’s disease (AD), Parkinson’s disease, amyotrophic lateral sclerosis (H. S. Kwon & Koh, 2020) and in the retina, glaucoma (H. L. Zeng & Shi, 2018a) and age-related macular degeneration (Guo et al., 2022).

1.3 Microglia in the central nervous system

1.3.1 Microglia contribution to neuroinflammation

Neuroinflammation is a complex immune response in the CNS triggered by injury, infection, or neurodegenerative disorders. The triggers of neuroinflammation activate the immune response within the CNS, leading to the release of pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS) (DiSabato et al., 2016; Williams et al., 2017). This immune response can recruit peripheral immune cells to the brain and retina, further exacerbating the inflammatory process (Sochocka et al., 2017). Neuroinflammation is a double-

edged sword with the potential to both protect and harm the CNS, depending on its context and duration (Ji et al., 2014; Schwartz & Baruch, 2014).

Microglia play a crucial role in neuroinflammation. When the CNS faces stress or damage, microglia react by undergoing morphologic, transcriptomic and proteomic alteration. (Paolicelli et al., 2022) exhibiting a pro-inflammatory response with released molecules, such as cytokines, chemokines, reactive oxygen species (ROS) and nitrite oxide (NO) (Long et al., 2022; Madeira, Boia, et al., 2016; Muzio et al., 2021; Zeng et al., 2023).

In acute situations, microglia's pro-inflammatory response is beneficial as it helps clear pathogens, remove damaged cells, and initiate tissue repair. However, these acute episodes can induce microglial priming, a long-lasting memory change, where microglia become more sensitive and reactive to subsequent inflammatory triggers. (Lima et al., 2022). During priming, microglia experience epigenetic modifications and changes in gene expression, leading to an altered immune response with heightened release of pro-inflammatory molecules upon encountering a secondary inflammatory stimulus (J.-W. Li et al., 2018; Niraula et al., 2017; Perry & Holmes, 2014). Persistent priming can lead to chronic neuroinflammation and contribute to the progression of neurodegenerative disorders such as AD, Parkinson's disease, and glaucoma (DeMaio et al., 2022; Ramirez et al., 2017).

1.3.2 Glaucoma

Glaucoma is an optic neuropathy that represents the leading cause of irreversible blindness affecting 57.5 million people worldwide (Allison et al., 2020). It is a chronic neurodegenerative disease characterized by the degeneration of RGCs which results in the excavation of the optic nerve head interfering with the normal circuit of transmission of visual information to the brain (Kang & Tanna, 2021; Pardue & Allen, 2018). Although glaucoma is a multifactorial disease, besides factors such as genetics, vascular abnormalities, metabolism or immune function, elevated intraocular pressure (IOP) has been described as a major risk factor for the disease onset and progression (Safa et al., 2022).

In most cases, glaucoma manifests as a silent disease that progresses without presenting symptoms until a late stage of neuronal damage and visual loss, therefore early interventions are essential to slow the progression of the disease (Weinreb et al., 2014). Although there is no cure, all current therapeutic approaches seek to reduce IOP through eyedrops, laser or surgery (Schuster et al., 2020).

While the molecular mechanisms that contribute to glaucoma onset and progression are yet to be understood, it has been described that elevated IOP exercises a mechanical strain on the optic nerve tissue, disrupting axonal transport (Zukerman et al., 2021). However, it is

important to better understand these molecular mechanisms in order to find new and more effective therapeutic targets.

1.3.3 Neuroinflammation in glaucoma

Chronic neuroinflammation has emerged as a critical factor in glaucoma. Among glaucoma patients, it has been revealed that the concentration of pro-inflammatory cytokines, such as IL-8 (Kuchtey et al., 2010), IL-1β and TNF-α (Baudouin et al., 2021), is elevated, supporting the existence of an inflammatory milieu within glaucomatous conditions (Aires & Santiago, 2021; Tezel, 2022). Moreover, elevated IOP, one of the major risk factors for glaucoma onset and progression, is sufficient to induce reactivity in microglia (Ramírez et al., 2020).

In response to elevated IOP and the mechanical strain that it imposes on optic nerve fibres, microglia shift from their quiescent to a reactive state characterized by the release of pro-inflammatory factors and phagocytosis enhancement (Aires et al., 2020; Madeira, Boia, et al., 2015b; Madeira, Elvas, et al., 2015a). This activation is prominently observed in both the retina and, notably, in the optic nerve head, resulting in damage and cellular death of the RGCs, the hallmark of glaucoma (Bosco et al., 2011; Howell et al., 2014)

To study the effect of IOP in microglia-induced neuroinflammation, *in vivo* and *in vitro* models have emerged as powerful strategies for delving into the complex molecular details of glaucoma offering new insights into disease mechanisms and potential therapeutic targets. An example of an *in vivo* model is laser photocoagulation-induced ocular hypertension. (Ortín-Martínez et al., 2015; Salinas-Navarro et al., 2010), while the most used *in vitro* model is the elevated hydrostatic pressure (EHP) model, which uses a pressure chamber, to elevate pressure (Aires et al., 2017, 2019, 2020).

In our group, it has already been shown that EHP exposure induces cell migration and proliferation, increases iNOS expression and enhances phagocytosis efficiency in BV-2 cells and in primary retinal microglia (Ferreira-Silva et al., 2020). Moreover, EHP exposure induces alterations of microglia morphology, increases pro-inflammatory cytokines (IL-1β and TNF-α) release and contributes to RGC death (Madeira, Elvas, et al., 2015). In animal models of glaucoma, laser photocoagulation-induced ocular hypertension activates microglia with MHCII induction, increases IL-1β and TNF-α and shows retinal and optic nerve RGC death (Madeira, Ortin-Martinez, et al., 2016).

1.3.4 Hypoxic conditions in microglia-induced glaucoma neuroinflammation

In the context of hypoxic conditions, hypoxia-inducible factor 1-alpha (HIF-1α) emerges as a pivotal transcription factor, central to the cellular response towards diminished oxygen levels. Activation of HIF-1α regulating cytochrome oxidase subunit expression by reducing respiratory capacity in order to reduce ROS production (Fukuda et al. 2007).

Notably, hypoxia can elicit the activation of microglia, it has been reported to increase pro-inflammatory cytokines and chemokines and enhance phagocytosis (X. Wang et al., 2022). Prolonged or chronic hypoxia has been associated to microglial priming, contributing to neuroinflammation, and potentially implicating it in the pathogenesis of neurodegenerative conditions (Kiernan et al., 2020).

In glaucomatous conditions, mice animal models after ocular hypertension inducion, show a hypoxic environment in retinal cells, RGCs, bipolar cells, and amacrine cells but also in glial cells, Müller cells and microglia (Jassim et al., 2022). This hypoxic state can be induced by IOP which upregulates the expression of HIF-1α observed in the retina and optic nerve head of glaucomatous eyes (Tezel G. & Martin B. 2004). Prolonged exposure to hypoxia, has been reported to induce RGCs damage contributing to the progression of glaucoma (Jassim & Inman, 2019).

1.4 Piezo1 mechanosensitive ionic channel

In 2010, Coste et al., revealed a novel family of mechanically activated cation channels in eukaryotes, the Piezo channels. These channels play important roles in touch sensing, pressure, respiration, angiogenesis, and stem cell differentiation (He et al., 2018; Nonomura et al., 2017; Yang et al., 2016). Piezo channels are classified into Piezo1 and Piezo2. Typically, Piezo 1 is mainly expressed in non-sensory tissues in neurons and in non-neuronal cell types, while Piezo2 channels are mainly expressed in the nociceptive system (Jäntti et al., 2022a). Piezo1 is expressed in mouse and human microglia (T. Zhu et al., 2023)

1.4.1 Piezo1 channel structure and activation

Structural data show that Piezo1 is a trimer with a trilobed topology that can be divided into two functional segments, the peripheral mechanotransduction and the central ion conduction pore. The peripheral portion encompasses the extracellular distal blades, peripheral helices (PHs), anchors within the transmembrane region, and intracellular beams. In contrast, the central component includes the C-terminal extracellular domains (CEDs), inner helices (IHs)

and outer helices (OHs) within the transmembrane region, and intracellular C-terminal domains (CTDs). Additionally, the combination of three CEDs forms an extracellular cap, while the intracellular beam links the PHs to the CTD. Within the transmembrane region, several transmembrane helical units (THUs) exist, roughly categorized as IHs, OHs, and PHs (De Vecchis et al., 2021; Xu et al., 2021) (Figure 3).

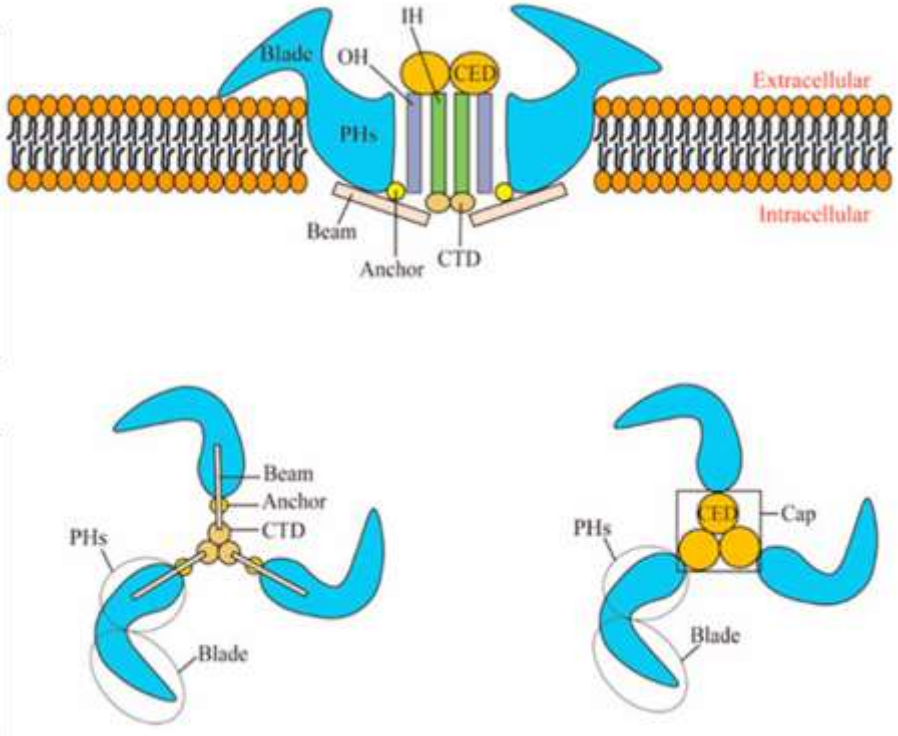

Figure 3 | Schematic representation of the Piezo1 channel structure. Piezo1 unit domains in three different perspectives: lateral, bottom and top view. C-terminal extracellular domain (CED); intracellular C-terminal domain (CTD); inner helix (IH); outer helix (OH); peripheral helices (PHs). Image from Xu et al., (2021)**.**

As a mechanosensitive ion channel, Piezo1 plays a vital role in converting mechanical forces, such as tension, pressure, or membrane stretching, into electrical signals within cells. When subjected to mechanical forces, Piezo1 undergoes deformation and opens, allowing the flow of calcium ions (Ca^{2+}), across the cell membrane (Fang et al., 2021; Yuan et al., 2023a). The influx of calcium through Piezo1 channels can trigger a cascade of intracellular events leading to changes in gene expression, cell differentiation, and various cellular responses (Huang et al., 2023; H. Liu et al., 2022).

1.4.2 Piezo1 role in the Hippo signalling pathway

The Hippo signalling pathway is a signalling pathway that plays a crucial role in regulating cell proliferation, tissue growth, and organ size. It is involved in maintaining tissue homeostasis and preventing the development of tumours (Boopathy & Hong, 2019; M. Fu et al., 2022a). One of the key downstream targets of the Hippo signalling pathway is the transcriptional coactivator Yes-associated protein (YAP) and its paralog, transcriptional coactivator with PDZ-binding motif (TAZ) (Y. Yu et al., 2020; Zhao et al., 2020).

When the Hippo signalling pathway is active, YAP and TAZ are phosphorylated by LATS1/2 kinases. Phosphorylated YAP/TAZ are sequestered in the cytoplasm, preventing them from entering the nucleus promoting cell death and inhibiting cell proliferation. However, when the Hippo signalling pathway is inactive, YAP and TAZ are dephosphorylated and can translocate to the nucleus, where they interact with transcription factors to promote cell growth and survival (H. Kwon et al., 2022; Meng et al., 2016; Plouffe et al., 2016) (Figure 4).

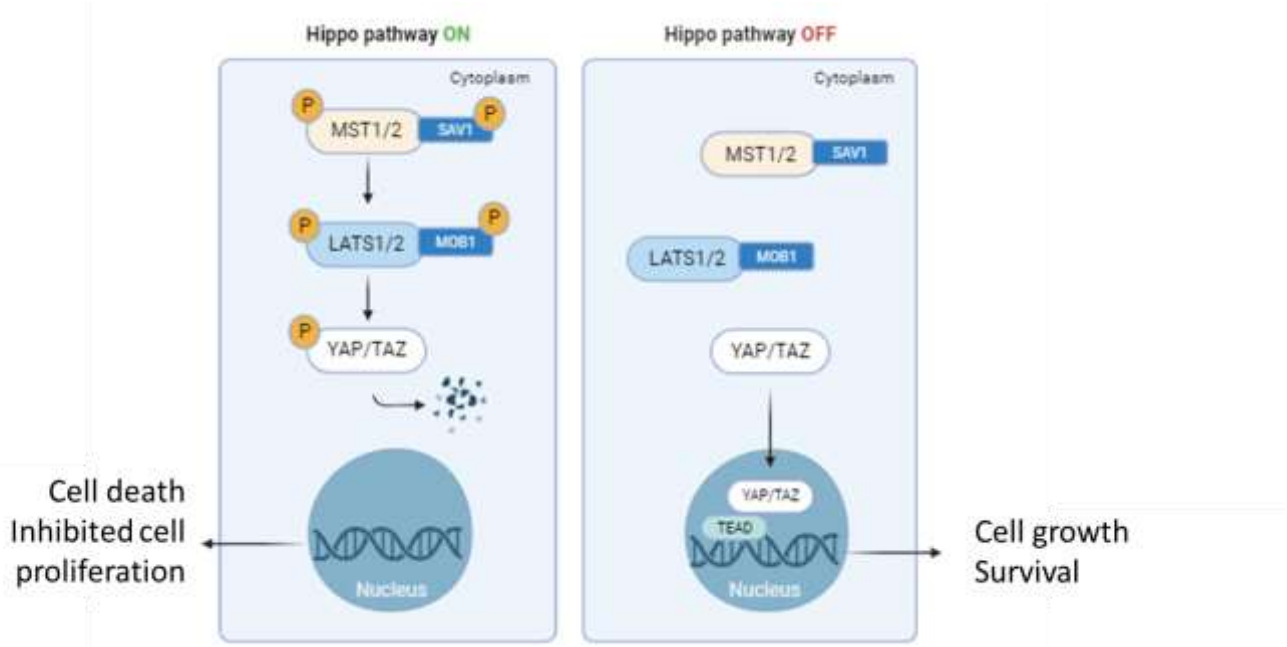

Figure 4 | Schematic representation of the Hippo signalling pathway. Activation of the Hippo signalling pathway (Left), YAP/TAZ proteins are phosphorylated by multiple upstream signals, promoting its cytoplasmic retention and proteolytic degradation. When the Hippo signalling pathway is deactivated (Right), YAP/TAZ are not phosphorylated and translocate to the nucleus, where they bind to the transcriptional enhanced associate domain (TEAD) and regulate genes required for cell proliferation and survival. Image from Biorender.com.

Piezo1 has been reported to deactivate the Hippo signalling pathway, promoting YAP and TAZ translocation to the nucleus inducing cell proliferation and tissue growth (Cobbaut et al., 2020; Y. Xiong et al., 2022a). In such conditions, Piezo1-induced YAP translocation to the nucleus can lead to uncontrolled cell proliferation and tumour growth, which has been reported in cancer cell migration and metastasis in cancers such as ovarian cancer (Y. Xiong et al., 2022a) cholangiocarcinoma (B. Zhu et al., 2021) and oral squamous cell carcinoma (Hasegawa et al., 2021). The interplay between Piezo1 and YAP has also been reported to be involved in microglia-induced neuroinflammation in neurodegenerative disorders including AD, where it may play a role in neuronal cell death and inflammation (Bruno et al., 2021).

1.4.3 Pharmacological regulation of the Piezo1 channel

1.4.3.1 Yoda1 a Piezo1 channel agonist

Besides mechanical stimuli, Piezo1 exhibits selective activation through Yoda1, a synthetic small molecule identified via high-throughput screening (Syeda et al., 2015). Yoda1 is recognized for its specific capability to activate the Piezo1 ion channel, as it has no modulatory effects on Piezo2 (Wijerathne et al., 2022).

Electrophysiology evidence has revealed that Yoda1 functions as a gating modifier for Piezo1 (Wijerathne et al., 2022). The rapid diffusion of hydrophobic Yoda1 from the solvent to the membrane and its binding site from the intracellular side of the membrane induces a twist-tilt-twist-like opening motion of the Piezo1 arm, reducing the mechanical activation threshold, changing the open/close equilibrium toward the open state (Botello-Smith et al., 2019) (Figure 5).

Numerous studies have indicated that effective concentrations for inducing a mechanical response typically range between 1 to 2 µM. However, at higher concentrations, above 10 µM, significant cytotoxicity has been observed in endothelial cells exposed to Yoda1 for more than 4h (Davies et al., 2019; Syeda et al., 2015). Furthermore, it is worth noting that the saturation point occurs at concentrations near 30 µM (Wijerathne et al., 2022).

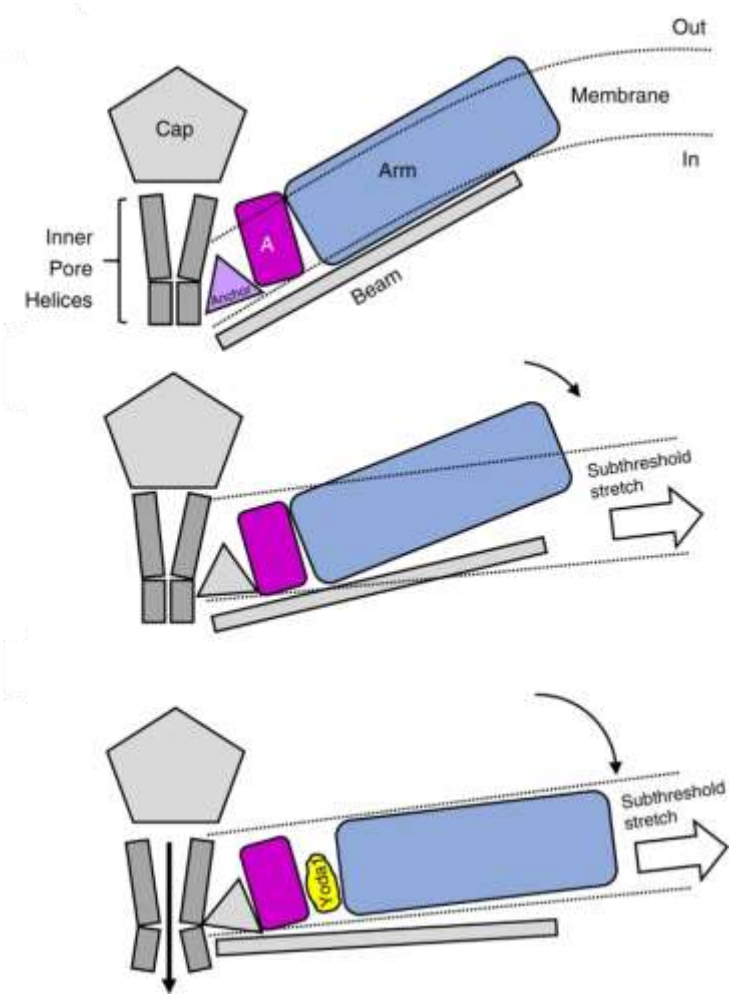

Figure 5 | Mechanism of action for Piezo1 activation through Yoda1. The Piezo1 arm (blue) is in a bent position in homeostatic conditions. In the presence of a stimulus that is not strong enough to trigger a response, the arm extends slightly because the lipid bilayer becomes flatter not sufficient to open the channel. Yoda1 binds between the arm and Repeat A (pink) acting like a wedge, separating these two domains. This wedge-like effect eventually results in the opening of the channel when sub-threshold stimuli are present. Adapted from (Botello-Smith et al., 2019).

1.4.3.2 GsMTx4 a Piezo1 channel inhibitor

GsMTx4, also referred to as Grammostola spatulata mechanotoxin 4, is a small, amphipathic peptide toxin derived from the venom of the chilean rose tarantula, *Grammostola spatulate* (Suchyna, 2017; Suchyna et al., 2000). This peptide stands out for its distinctive property of selectively inhibiting cation-permeable mechanosensitive channels including Piezo1 (Bae et al., 2011). Moreover, its inhibition is non-stereospecific, as both its enantiomers (L- and

D-form) demonstrate the ability to inhibit mechanosensitive channels (Gnanasambandam et al., 2017).

GsMTx4 acts on mechanosensitive channels like Piezo1 through direct interaction with the gating elements of the channel, rather than obstructing the actual channel pore. This interaction results in the adjustment of local membrane tension by modifying the mechanical properties of the surrounding lipid bilayer, thereby enhancing its stiffness. Consequently, this binding process restricts the occupation of deeper modes within relaxed bilayers, ultimately giving rise to an effect reminiscent of "tension clamping." Furthermore, GsMTx4's impact extends to the inhibition of whole-cell Piezo1 currents, primarily achieved through the stabilisation of the channel's closed state (Gnanasambandam et al., 2017).

The effectiveness of GsMTx4 in inhibiting the Piezo1 channel has generated varying recommendations from manufacturers and researchers. In the literature it has been reported that lower concentrations (500 nM) are sufficient for successful inhibition (H. Li et al., 2019), while others advocate for higher concentrations (1-10 μM) to achieve a full response (Miyamoto et al., 2014).

1.4.4 Piezo1 channel physiological role

As a mechanical force-sensing ion channel, Piezo1 is widely distributed across various tissues and cell types in the human body, including red blood cells, endothelial cells, sensory neurons, and more (Lai et al., 2022). This extensive distribution highlights its crucial role in governing various physiological processes, showcasing the intricate link between mechanical forces and cellular responses (Qin et al., 2021).

1.4.4.1 Piezo1 role in the central nervous system

Piezo1 plays a crucial role in mechanosensation within sensory neurons in the CNS. These neurons detect external mechanical stimuli like touch and pressure, and Piezo1 converts the mechanical stimulus into electrical signals for processing by the nervous system (H. Liu et al., 2022). Additionally, Piezo1 is essential for maintaining neuronal functions, including guiding axonal growth and promoting neuronal maturation during processes like axon formation and neurogenesis (Song et al., 2019).

Nonetheless, emerging evidence suggests that Piezo1 activation contributes to the pathogenesis of various neurological conditions, including neuropathic pain, neuroinflammation, and neurodegenerative disorders (Y. Y. Zhang et al., 2023). As a key regulator of neuronal excitability, any disruptions in Piezo1 activity hold the potential to either contribute to the initiation of epilepsy or influence the severity of the seizures (Tufail et al.,

2011). Furthermore, Piezo1 channels located within the endothelial cells lining cerebral blood vessels serve as sensors for detecting both shear stress and blood flow and can modulate the permeability of the blood-brain barrier (BBB), leading to neuroinflammation (Fels & Kusche-Vihrog, 2020; Zhou et al., 2014). This is a recurring characteristic observed in numerous neurological disorders both in the brain and in the retina (Zong et al., 2023).

Piezo1 is implicated in MS, where its activation inhibits axon regeneration and potentiates demyelination-induced axonal damage, contributing to MS progression (Yang et al., 2022; Zong et al., 2023). Furthermore, inhibiting Piezo1 has been associated with reduced demyelination-induced axonal damage and the application of Yoda1 has been observed to induce demyelination (Velasco-Estevez et al., 2020; Yang et al., 2022).

Recent studies shed light on Piezo1's potential role in the context of AD. The stiffened microenvironment induced by the amyloid plaques can hyperactivate the Piezo1 channel (Tortorella et al., 2022). As previously discussed Piezo1 activation deactivates the Hippo signalling pathway, promoting YAP nuclear translocation. Emerging studies also show that YAP-induced gene transcription may have implications for neuroinflammation and neurodegeneration in AD (Bruno et al., 2021; Velasco-Estevez et al., 2022).

In the retina, recent studies suggest Piezo1 as a potential contributor to glaucoma progression where elevated IOP is a major risk factor for disease progression, Piezo1 activation has been associated with the suppression of RGC neurite outgrowth, hindering their regeneration and connectivity, contributing to cell death (Morozumi et al., 2020). Additionally, Piezo1 has been linked to optic nerve head astrocyte reactivity (J. Liu, Yang, et al., 2021), indicating that Piezo1 plays a role in glaucoma.

1.4.4.2 Piezo1 channel activation in microglia

Besides biochemical cues, microglial activity is believed to be influenced by physical factors such as tissue stiffness and mechanical forces (Smolders et al., 2019). *In vitro* experiments have provided evidence that primary microglia can adapt to the stiffness of their environment showing a tendency to migrate toward areas characterized by higher stiffness and modifying their morphology, and actin cytoskeleton (Bollmann et al., 2015).

Piezo1 is expressed in murine and human microglia cells as well as in microglia cellular lines such as BV-2 This channel functions as a mechanosensor in microglial cell lines and plays a regulatory role in both microglial migration patterns and immune responses by modulating the pro-inflammatory response (T. Zhu et al., 2023).

Few studies have been performed in this field, for instance, Morozumi et al., 2020 showed that Piezo1 is upregulated in chronic elevated IOP model mouse retina. Additionally, enhanced stiffness, mediated by Piezo1, was also associated with increased pro-inflammatory cytokine production in microglia primary mouse microglia and BV-2 cells (T. Zhu et al., 2023)

Aims

Glaucoma manifests as RGCs neurodegeneration and optic nerve damage, where elevated IOP and advanced age are the principal risk factors. In our group, it has already been shown that microglial cells become reactive in EHP conditions and in glaucoma animal models with exhibit elevated IOP. However, the precise mechanism linking heightened pressure to microglial activation is yet to be elucidated. Our working hypothesis posits that Piezo1 within microglial cells are activated in response to increased pressure, thereby initiating a cascade of events characterized by calcium influx, ultimately culminating in the acquisition of a reactive phenotype by microglia.

In this work, the mechanisms triggered by Piezo1 activation in microglia when these cells are challenged with elevated pressure were studied. For that, this work was divided into two main tasks.

The first task focused on the effects of elevated pressure on Piezo1 activation and its effect on microglia reactivity. In the absence of mechanical stimulus, Yoda1 is able to open Piezo1 channels, moreover, the activation of Piezo1 can be inhibited by GsMTx4. Additionally, microglia cells were exposed to EHP and hEHP as mechanical stimuli, and microglia reactivity was determined. Microglia function was evaluated by assessing phagocytosis efficiency using fluorescent microbeads. Furthermore, the impact of Piezo1 activation on NO production was assessed through Griess Reaction assay, and by incubating BV-2 cells with the DAF-FM probe. Additionally, Western blot analysis was employed to assess the expression of iNOS.

The goal of the second task goal was to elucidate the molecular mechanisms following Piezo1 channel activation. Existing evidence indicates that calcium influx, resulting from Piezo1 activation, promotes the nuclear localization of mechanoreactive transcription YAP and TAZ. To achieve this, BV-2 cells were exposed to EHP and hEHP either alone or in the presence of GsMTx4. Additionally, BV-2 cells were stimulated with Yoda1. TAZ and YAP protein levels were assessed by Western blot in total protein lysates and in subcellular fractions.

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Chapter 2

Materials and Methods

2.1 BV-2 cell line

BV-2 cells, a murine microglia cell line was used. Cells were maintained in T-75 cm^2 culture flasks at a density of 1.5 x 10^6 cells/cm^2 with Roswell Park Memorial Institute (RPMI) medium 1640 (cat # 21875-034, Gibco, ThermoFisher Scientific, Paisley, UK) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (cat # 10270-106, Gibco, ThermoFisher Scientific, Paisley, UK), 1% (v/v) antibiotic solution of penicillin and streptomycin (cat # 15140-122, Gibco, ThermoFisher Scientific, Paisley, UK) and 2 mM L-Glutamine (cat # 25030-081, Gibco, ThermoFisher Scientific, Paisley, UK). Cells were kept in a humidified environment of 5% CO_2 at 37°C and cultures were passed when cells reached 80-90% confluence. For experiments, BV-2 cells were cultured in RPMI supplemented with 2% FBS and 1% (v/v) antibiotic solution of penicillin and streptomycin at a density of 1.9×10^4cells/cm^2 in 6-well plates or 1×10^4cells/cm^2 in 12-well plates.

2.2 Cell treatment

Cell cultures were maintained on a standard cell incubator or exposed to elevated hydrostatic pressure (EHP) using a pressure chamber (with 2% O_2 [hEHP] or 20% O_2 [EHP]) to elevate pressure 70 mmHg above normal atmospheric pressure for 4 or 24 h. Piezo1 mechanosensitive channel was pharmacologically manipulated by incubating cells with 1, 5 and 10 μM Yoda1 (cat # 5586, Tocris, Bristol, UK), a Piezo1 agonist for 4 h or with 0.5, 1 and 10 μM GsMTx4 (cat # ab141871, Abcam, UK), a Piezo1 inhibitor, one hour prior to the exposure to EHP.

2.3 Phagocytosis assay

Phagocytic efficiency in BV-2 cells was assessed by incubating cells with 0.0025% fluorescent latex microbeads (cat # L1030, Sigma-Aldrich, St Louis, MO) for 60 minutes prior to the end of cell treatment described above, at 37°C in a standard cell incubator or in a pressure chamber. In the end, cells were fixed using 4 % paraformaldehyde (PFA) with 4% sucrose at room temperature (RT) for 20 minutes. Fixed cells were permeabilized and blocked with a solution of 0.2% Triton X-100 (cat # 11869, Merck, Darmstadt, Germany) and 10% BSA (cat # A1391, PanReac Química S.L.U., Barcelona, Spain) for 5 minutes and stained with Phalloidin - Tetramethylrhodamine B isothiocyanate (1:500; cat # P1951 Sigma-Aldrich, St Louis, USA) a label that stains actin filaments in the cytoskeleton and 4',6-Diamidino-2-Phenylindole,

Dihydrochloride (DAPI) (1:2000; cat # P1306, Invitrogen, ThermoFisher Scientific, Massachusetts, USA) for 30 minutes protected from light. Coverslips were mounted using a fluorescence mounting medium (cat # 302380-2, Dako, Santa Clara, USA) and 10 random field images for each condition were acquired using an inverted fluorescence microscope (Zeiss Axio Observer.Z1 inverted microscope) with a 200x magnification (Plan-Apochromat 20x/0.8). Phagocytic efficiency was calculated with the following formula:

$$Phagocytosis\ Efficiency\ (\%) = \frac{(1 * \text{b1} + 2 * \text{b2} + 3 * \text{b3} \ldots + \text{n} * \text{bn})}{\text{Total number of cell}} * 100$$

Where bn represents the number of cells containing b beads (b=1, 2, 3 … up to a maximum of 6 points for more than 5 beads per cell) as previously described by our group (Aires et al., 2019b; Madeira, Boia, et al., 2016b).

2.4 Griess reaction assay

Griess reaction assay is a colourimetric assay used to assess the nitrite production (NO_2^-) in the culture medium that detects nitrite by the spontaneous oxidation of nitric oxide (NO) (D. Li et al., 2018). NO_2^- reacts under acidic conditions with sulfanilic acid to form a diazonium cation which subsequently couples to the aromatic amine 1-naphthylamine to produce a red-violet coloured, water-soluble azo dye that can be measured using a spectrophotometer(Tsikas, 2007). The culture medium was collected and centrifuged at 1000g for 5 min at 4°C to remove cell debris and the supernatant was collected. In a 96-well plate, the previous centrifuged culture medium was incubated with Griess mixture (1% (v/v) sulfanilamide in 5% (v/v) phosphoric acid (H_3PO_4) and with 0.1% (v/v) N-1-naphtyletilene diamine) in a proportion 1:1 for 30 minutes, protected from the light. The optical density was measured at 550 nm using a spectrophotometer (Synergy HT; Biotek, Winooski, USA) and nitrite concentration was determined by comparison to a 1 mM sodium nitrite standard curve (Aires et al., 2019b).

2.5 Nitrite oxide quantification

NO production was assessed using 4-amino-5-methylamino2',7'-difluororescein diacetate (DAF-FM diacetate) (cat # D23844, Invitrogen, ThermoFisher Scientific, Eugene, USA), a non-fluorescent reagent whose amino groups capture NO, de-acetylating the compound

to become DAF-FM, producing a green fluorescence that can be detected by fluorescence microscopy.

For this assay, cell medium was collected and stored. Cells were incubated with 8 µM DAF-FM-DA in Krebs-Henseleit Ringer Solution (KHR: 140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 3 mM KCl, 5 mM glucose, pH 7.4) for 60 minutes before the stimuli at 37°C in a humidified atmosphere of 5% CO_2. After incubation, the DAF-FM solution was replaced by the previously collected medium and cultures were exposed to the described treatments. In the end, it was performed cell staining as described above. Coverslips were mounted using glycergel (DAKO, Agilent, Santa Clara, CA, US) pre-heat at 56°C. Five images were randomly acquired for each condition using a confocal microscope (Zeiss LSM 710 laser-scanning confocal) with a 630x magnification (Plan-Apochromat 63x/1.4 Oil DIC).

Fluorescence was analysed using the ImageJ software and measured by means of the corrected total cell fluorescence (CTCF), calculated using the formula below as described in Madeira et al., 2015.

CTCF = Integrated density − (Area of selected cell × Mean fluorescence background reading)

2.6 Protein extraction

For total extracts, it was used a lysis buffer aimed for phosphorylated forms (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% (v/v) NP-40; 0.5% (v/v) sodium deoxycholate; 0.1% (v/v) SDS; 2 mM Na_3VO_4, 10 mM NaF, and a tablet of protease inhibitors (cOmplete mini, cat # 11836153001, Merck, Mannheim, Germany). The extracts for Piezo1 protein detection were obtained using radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl; 50 mM Tris-base; 5 mM EGTA, pH 7.5; 1% (v/v) Triton X-100; 0.5% (v/v) sodium deoxycholate; 0.1% (v/v) SDS; 1 mM DTT and protease inhibitors). The culture medium was removed, and cells were washed three times with ice-cold PBS, the respective buffers were added, and cells were scrapped manually and collected. Samples were sonicated with three pulses of 5s ON intercalated with 1s OFF with a 20% amplitude on ice and centrifuged at 3000 g for 10 min at 4°C. The supernatant was collected and stored at -80°C until further use.

The culture medium was removed for nuclear and cytosolic extracts, and cells were washed twice with ice-cold PBS. A hypertonic buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.4% (v/v) NP-40; 1 mM DTT; 1.5 mM Na_3VO_4; 50 mM NaF; 1 mM PMSF and protease inhibitors) was added for 10 minutes. Cells were scrapped manually, collected, and

kept on ice for 30 minutes with vortex each 10 min. Cells were centrifuged at 5690 g for 12 min at 4°C and the supernatant corresponding to the cytosolic part was collected and stored at -80°C until further use. The pellet was resuspended on a hypotonic buffer (20 mM HEPES, pH 7.9; 0.4 NaCl; 1 mM EDTA; 10% (v/v) glycerol; 1 mM DTT; 1.5 mM $NaVO_4$; 50 mM NaF; 1 mM PMSF and proteases inhibitors) and left on ice for one hour with vortex every 20 minutes. Samples were sonicated with three pulses of 1s ON intercalated with 1s OFF at an amplitude of 20% on ice and centrifuged at 28944 g for 20 min at 4°C. Supernatant corresponding to the nuclear fraction was collected and stored at -80°C until further use.

2.7 Protein quantification

Total extracts and the cytosolic fraction were quantified using the Pierce™ BCA Protein Assay kit (cat # 23225, ThermoFisher Scientific, Massachusetts, USA) following the manufacturer's instructions and 60 µg or 30 µg of protein from each sample were loaded in the gels, for total and cytosolic fractions, respectively.

For nuclear fraction, samples were quantified using the Bradford reagent prepared by dissolving 50 mg of Coomassie Brilliant Blue G-250 in 50 mL of methanol, adding 100 mL 85% (w/v) phosphoric acid (H_3PO_4) and filtered. The reagent was stored at 4°C protected from light (Bradford, 1976). In a 96-well plate it was added 5 µL of the samples to each well and 250 µL of Bradford reagent. To promote the reaction the plate was kept on a shaker for 30 seconds and incubated 10 min RT protected from light. Absorbance was measured using a spectrophotometer at an emission of 595 nm and protein quantification was assessed using a standard curve of BSA (1, 0.5, 0.25, 0.125 and 0 mg/mL).

2.8 Western Blot

Samples were denatured by dilution 1:6 in Sample Buffer 6x (0.35 M Tris-base, 0.4% SDS, pH 6.8; 30% Glycerol; 10% SDS; 0.012% Bromophenol blue) and then heated at 95°C for 5 minutes and separated in acrylamide gel (8%) by electrophoresis for approximately 1 hour at an initial voltage of 60 V for stacking separation and 140 V for resolving separation in an electrophoresis buffer (25 mM Tris-base; 190 mM Glycine; 0.1% (v/v) SDS; pH 8.8). Proteins were transferred to nitrocellulose membranes or polyvinylidene fluoride (PVDF) specifically for Piezo1 by wet transference at 4°C for 2 hours and 30 minutes at 70 V.

Before blockage, membranes were incubated for 5 minutes with Ponceau Staining (cat # P7170, Sigma, St. Louis, USA), washed with deionised water to remove the excess of the solution and revealed using a ChemiDoc MP Imaging System. Membranes were blocked in 5% BSA in TBS-T (Tris-base saline with 0.05% Tween-20) for phosphorylated proteins or 5% non-fat milk in TBS-T for non-phosphorylated. The incubation of the primary antibodies (Table 1) occurred overnight at 4°C and the secondary antibody (goat anti-rabbit IgG (H+L)-HRP conjugated; 1:10000 in 5% BSA/TBS-T, cat # 170-6515, Bio-Rad, California, USA) took place on the day after for 1 hour, followed by the revelation in ImageQuant LAS 500 chemiluminescence CCD camera using Western Bright Sirius HRP substrate (cat # K12043, Advansta, San Jose, USA) in a proportion 1:1.

Quantification was performed using ImageLab program version 6.1 and assessed the adjusted volume of each band of the sample. Normalization was valued by the sample volume ratio to the total protein volume of each lane using Ponceau Staining.

Table 1 | Western Blot primary antibodies.

Primary antibody	Host	Dilution	Catalogue number	Supplier
Anti-Piezo1	Rabbit	1:600	cat # PA5-77617	Invitrogen
Anti-TAZ	Rabbit	1:1000	cat # 4883	Cell Signaling
Anti-iNOS	Rabbit	1:500	cat # SC650	Santa Cruz
Anti-YAP	Rabbit	1:1000	cat # 4912	Cell Signaling
Anti-pYAP (S397)	Rabbit	1:1000	cat # 13619	Cell Signaling

2.9 Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The data were analysed firstly using the Shapiro-Wilk test to assess the normality of the sample distribution. In case of normal distribution, it was used one-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test. For non-normal distribution, it was performed a Kruskal-Wallis test followed by Dunn’s multiple comparison test. Statistical analysis was performed in Prism 5.0 Software (GraphPad Software) and p values less than 0.05 were taken as significant.

Chapter 3

Results

3.1 Effect of EHP on the protein levels of Piezo1 in BV-2 cells

It has been recently described that the Piezo1 channel is expressed in microglia as well as in BV-2 cells (T. Zhu et al., 2023). Thus, BV-2 cells were challenged with hEHP for 4h or 24h and protein levels of Piezo1 were assessed by Western blot (Figure 6).

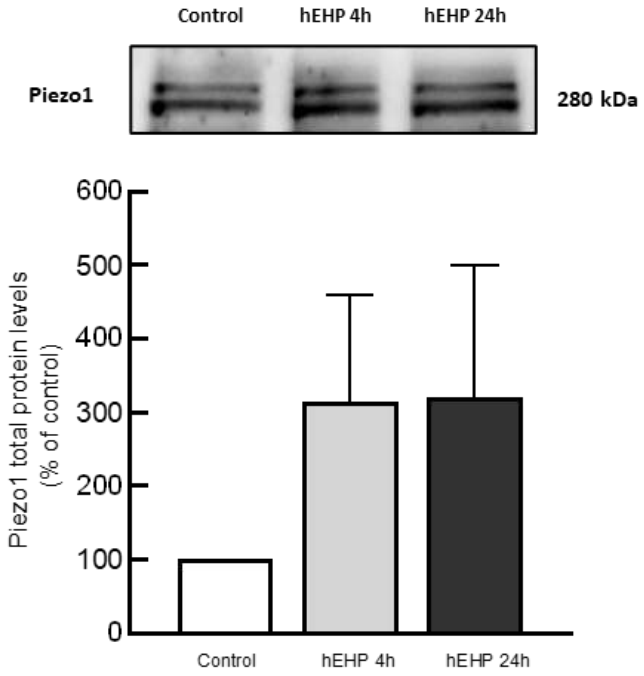
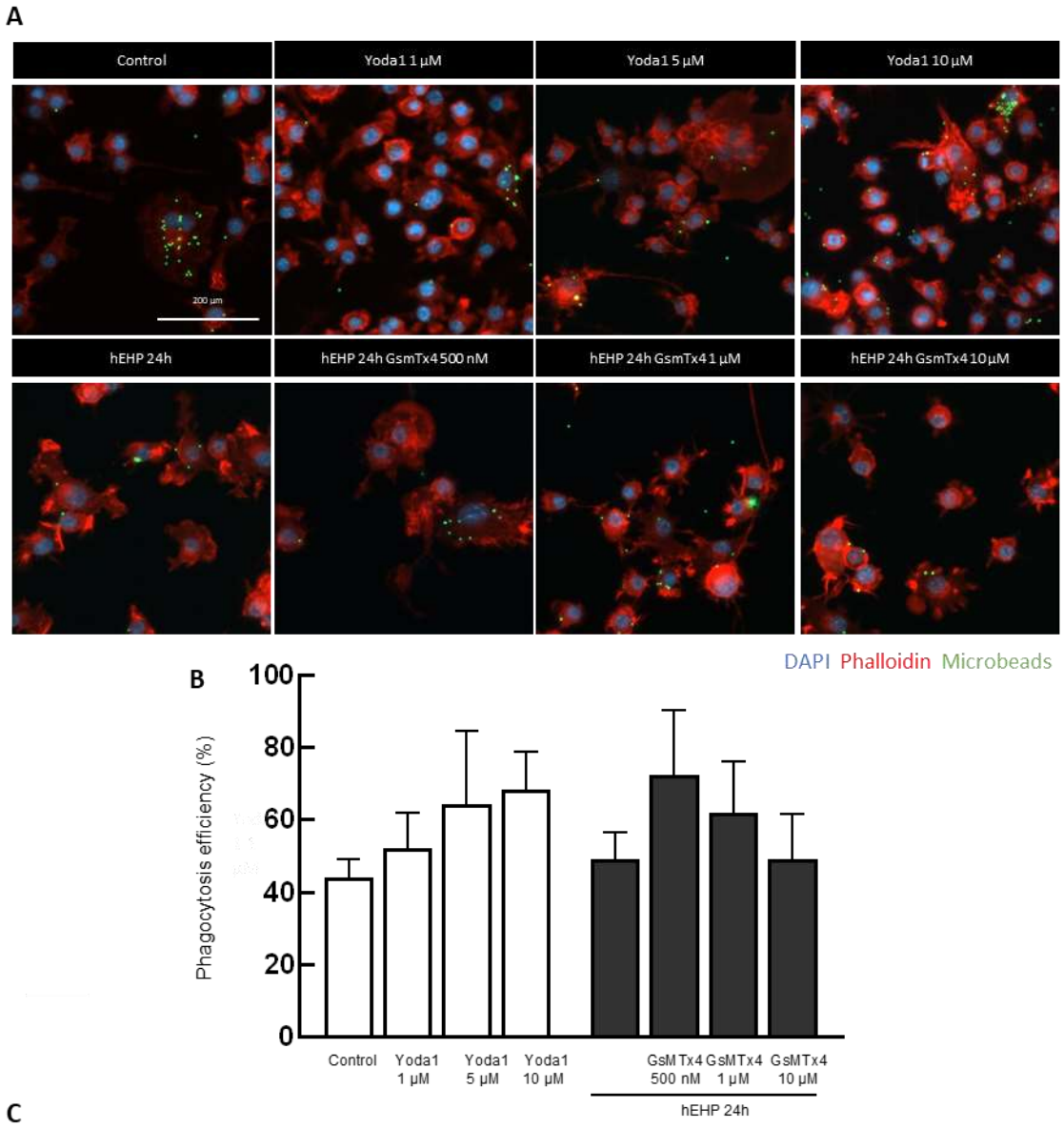


Figure 6 | Effect of EHP on the protein levels of Piezo1 in BV-2 cells. BV-2 cells were exposed to hEHP (2% O2) for 4 and 24h. Piezo1 protein levels were assessed by Western Blot. Results are expressed as mean ± SEM in percentage of the control and were obtained from 4 independent experiments.

The exposure of BV-2 cells to hEHP during 4h and 24h, slightly increased the Piezo1 protein levels to 314±145% and 321±180% of control, respectively.

3.2 Effect of Piezo1 activation in the phagocytic activity of BV-2 cells

Phagocytosis is one of the main features of microglia activation, to remove cell debris prior to cell regeneration, and altered phagocytosis can be involved in the pathogenesis of several CNS dysfunctions (R. Fu et al., 2014). Thus, phagocytic activity of BV-2 cells was assessed by analysing the incorporation of fluorescent latex microbeads (Figure 7).


A
Control
Yoda1 1 µM
Yoda1 5 µM
Yoda1 10 µM
200 µm
hEHP 24h
hEHP 24h GsmTx4 500 nM
hEHP 24h GsmTx4 1 µM
hEHP 24h GsmTx4 10 µM
DAPI Phalloidin Microbeads
B
Phagocytosis efficiency (%)
100
80
60
40
20
0
Control
Yoda1 1 µM
Yoda1 5 µM
Yoda1 10 µM
GsMTx4 500 nM
GsMTx4 1 µM
GsMTx4 10 µM
hEHP 24h
C
Control
Yoda1 1 µM
Yoda1 5 µM
Yoda1 10 µM
200 µm
EHP 24h
EHP 24h GsmTx4 500 nM
EHP 24h GsmTx4 1 µM
EHP 24h GsmTx4 10 µM
DAPI Phalloidin Microbeads

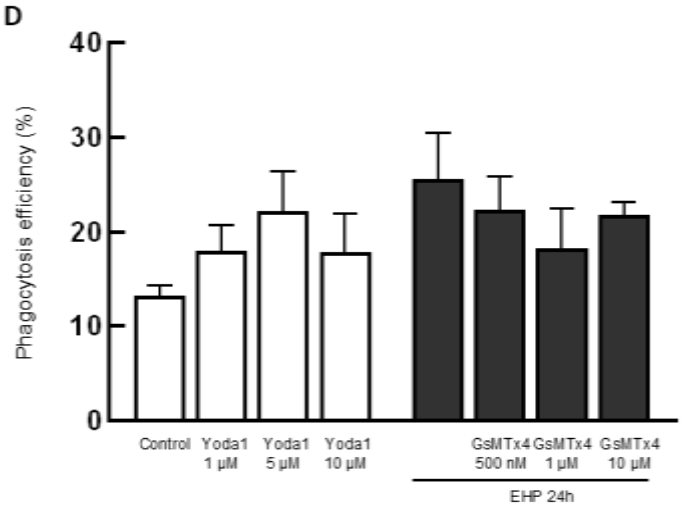


Figure 7 | Effect of Piezo1 activation in the phagocytic activity of BV-2 cells. BV-2 cells were incubated with Yoda1 (1 µM, 5 µM and 10 µM) or exposed to hEHP (2% O2) or EHP (20% O2) during 24h in the presence or absence of GsMTx4 (500 nM, 1 µM and 10 µM). Phagocytosis was assessed after incubating cells with fluorescent microbeads (green). Cytoskeleton was labelled with phalloidin-TRITC (red) and nuclei were stained with DAPI (blue). Cells were visualized in a Zeiss fluorescence microscope with an objective of 20x. Scale 200 µm. (A) Representative images for each condition and respective (B) Phagocytic activity determined using the formula indicated in the Materials and Methods section, after acquiring 10 images per condition. Results are presented as mean ± SEM and were obtained from 4-6 independent experiments.

To evaluate the effect of Piezo1 activation in phagocytic activity, BV-2 cells were exposed to the channel agonist (Yoda1 at 1, 5 and 10 µM). Moreover, BV-2 cells were challenged with hEHP (2% O_2) and EHP (20% O_2) for 24h in the presence or absence of the Piezo1 inhibitor (GsmTx4 at 500 nM, 1 and 10 µM).

Figure 7B shows that when BV-2 cells were incubated with Yoda1 10 µM, there was a slightly increased phagocytic efficiency to 68±11% when compared to the control (44±5%). Exposure to hEHP did not change the phagocytic efficiency (49±7%) compared to control conditions. Interestingly, pre-treatment with GsMTx4 (500 nM and 1 µM) in cells exposed to hEHP for 24h tended to increase the phagocytic efficiency to 72±18% and 62±14%, respectively, however, the incubation with GsMTx4 1 µM showed no alterations (49±13%) in comparison to the control.

Regarding Figure 7D, Yoda1 incubation showed a slight increase in phagocytosis efficiency in a concentration of 5 µM (22±4%) when compared to control conditions (13±1). The exposure to 24h of EHP also showed a slight increase in phagocytosis efficiency (26±5% of the control). Pre-incubation incubation with GsMTx4 1 µM in cells exposed to 24h EHP showed a slight decrease (18±4% of control) when compared to EHP. At concentrations of 500 nM and 10 µM, GsMTx4 incubation showed a slight increase in phagocytosis efficiency when compared to control conditions (22±5% and 22±1%, respectively).

3.3 Effect of Piezo1 activation in nitrative stress

Microglia activation contributes to oxidative and nitrative stress (Cobb & Cole, 2015). To study the effect of Piezo1 activation in nitrative stress, BV-2 cells were incubated with the channel agonist (Yoda1 at 10 µM). Furthermore, BV-2 cells were exposed to hEHP (2% O_2) for 4h or 24h with or without Piezo1 inhibitor (GsMTx4 at 1 µM). Thus, nitrite concentration was evaluated by the Griess reaction assay (Figure 8), as an indirect method to determine NO production, which was also evaluated by fluorescence microscopy using the DAF-FM probe (Figure 9). The iNOS protein levels were evaluated by Western blot (Figure 10).

At a cellular level, nitrate (NO_3^-), nitrite (NO_2^-) and nitric oxide (NO) exist in an equilibrium of oxidation/reduction reactions (Falls et al., 2017). When the state of equilibrium is disrupted, it gives rise to the generation of peroxynitrite (ONOO-), a profoundly toxic reactive species capable of inducing lipid peroxidation and causing oxidative damage to proteins (Iizumi et al., 2016). The production of NO_2^- was quantified using the Griess reaction in the cell supernatant (Figure 8).

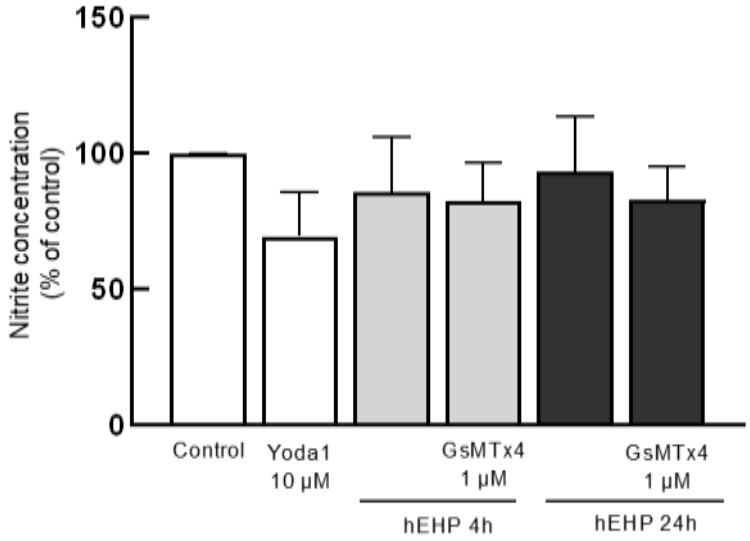
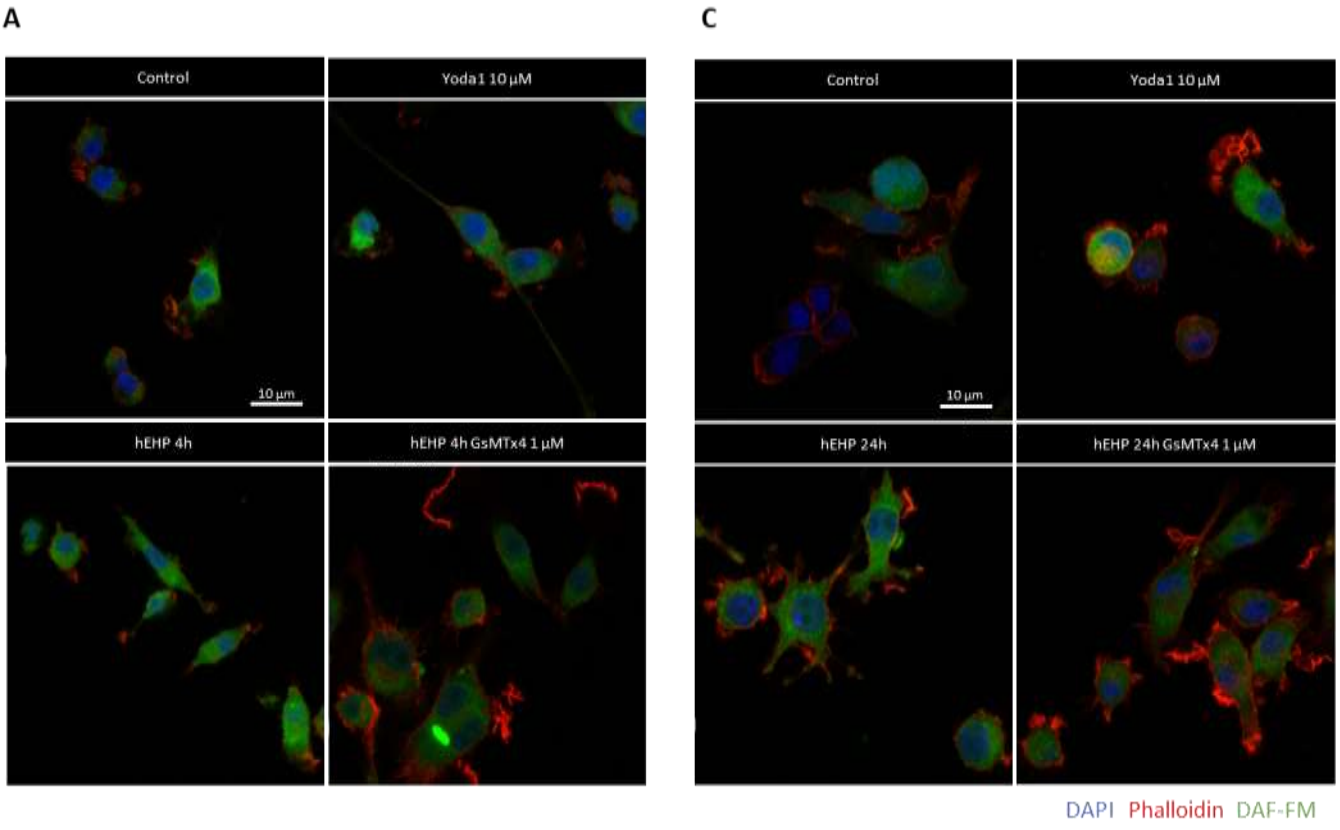


Figure 8 | Effect of Piezo1 activation in the nitrite concentration. BV-2 cells were incubated with 10 µM Yoda1 or exposed to hEHP (2% O2) for 4h and 24h, in the presence or absence of 1 µM GsMTx4. Nitrite levels were determined in the culture medium supernatant using the Griess reaction method. The results are presented as mean ± SEM in percentage of control and were obtained from 3-5 independent experiments.

BV-2 cells incubated with Piezo1 agonist (Yoda1 at 10 µM) showed a slight decrease in nitrite concentration to 70±16% of the control. When BV-2 cells were exposed to hEHP for 4h and 24h no significant alterations were detected in nitrite concentration (86±20% and 94±20% of control, respectively). Additionally, pre-treatment with 1µM GsMTx4 did not show alterations (82±14% and 83±13%, respectively).

A more direct and accurate method to quantify NO production is by using the probe DAF-FM a probe that when in contact with NO forms a fluorescent benzotriazole (Nasuno et al., 2020), (Sheng et al., 2005). BV-2 cells were incubated with DAF-FM for 1h prior to exposure to the described treatments and its green fluorescence was measured by mea corrected total cell fluorescence (CTCF) (Figure 9).



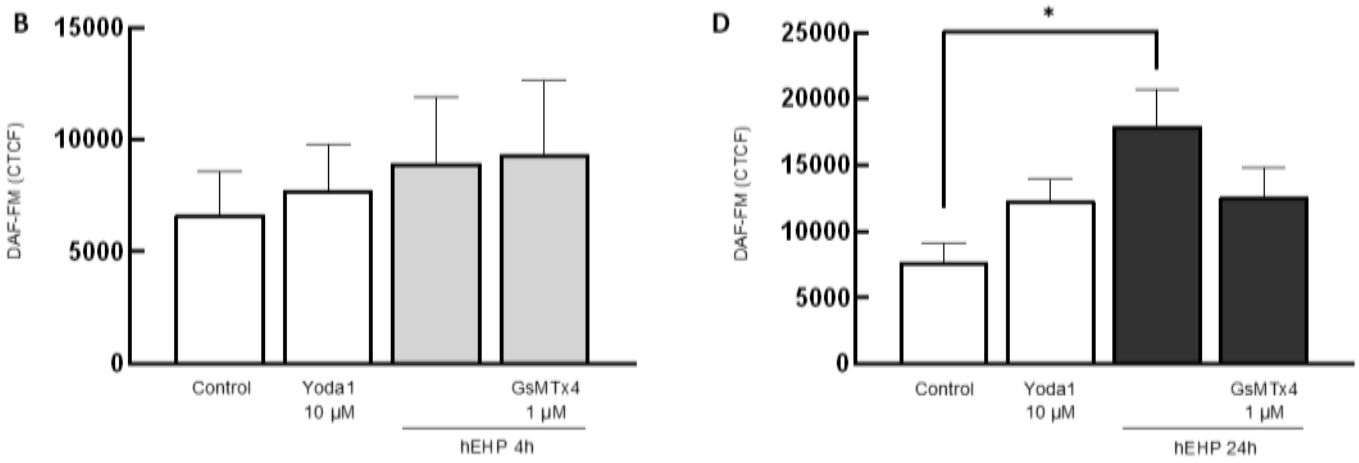


Figure 9 | Effect of Piezo1 activation in the production of NO. BV-2 cells were incubated with the NO-sensitive probe DAF-FM-DA (green) one hour prior to the incubation with 10 µM Yoda1 or exposure to hEHP (2% O2) for 4 and 24h in the presence and absence of 1 µM GsMTx4. Nuclei were stained with DAPI (blue) and cytoskeleton with phalloidin-TRITC (red). The preparations were visualised using a Zeiss LSM 710 confocal microscope with an oil objective of 63x and were acquired 5 images per condition. Scale 10 µm. (A) Representative images for 4h exposure to hEHP from 3 independent experiments are depicted and (B) its respective calculated CTCF. (C) Representative images for 24h of hEHP exposure and (D) its respective calculated CTCF, n=4, $p<0.05$ one-way ANOVA followed by Sidak's multiple comparisons test.

The incubation with Yoda1 10 µM showed no significant alterations in DAF-FM CTCF measurement (7720±2035) when compared to control conditions (6664±1906). The exposure to hEHP did not reveal alterations in NO-induced fluorescence in CTCF when compared to the control (8926±2959). Pre-incubation with GsMTx4 1 µM in cells exposed to 4h hEHP revealed no significant alterations in NO-induced fluorescence in CTCF when compared to control conditions (9340±3287) (Figure 9 B).

The graph presented in Figure 9 D, shows that the incubation of BV-2 cells with Yoda1 10 µM slightly increased the NO-induced fluorescence in CTCF to 12353±1560 when compared to control conditions (7653±1472). The exposure to hEHP for 24h significantly increased the NO-induced fluorescence in CTCF to 17934±2826% ($p<0.05$) when compared to the control. The pre-treatment with GsMTx4 (1 µM) in cells exposed to 24h of hEHP slightly decreased NO-induced fluorescence in CTCF (12629±2194% of control) when compared to 24h hEHP.

NO is synthesised by NO synthases (NOS). Although there are three isotypes of these enzymes, the inducible isoform of NOS (iNOS) is expressed in microglia and is known to contribute to the response of microglia upon injury (Sierra et al., 2014). To study the effect of Piezo1 activation on iNOS protein levels, BV-2 cells were exposed to the mentioned treatments and the total protein content was collected and assessed by western blot (Figure 10).

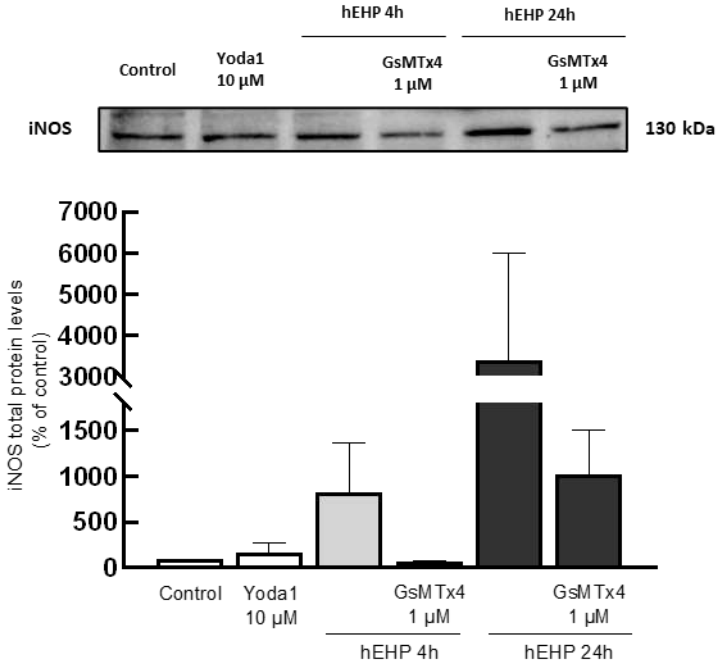


Figure 10 | Effect of Piezo1 activation in the protein levels of iNOS. BV-2 cells were incubated with 10 µM Yoda1 or exposed to hEHP (2% O2) for 4 and 24h with and without 1 µM GsMTx4. iNOS protein levels were assessed using Western Blot. Results are presented as mean ± SEM and are expressed in percentage of the control, from 4 independent experiments.

No alterations were observed in iNOS protein levels when BV-2 cells were incubated with Yoda1 (10 µM) (170±97% of control) compared with the control. In cells exposed to hEHP for 4h, iNOS protein levels were 823±547% of control. The pre-treatment with 1 µM GsMTx4 showed a tendency to decrease iNOS protein levels (60±17% of control) when compared to 4h hEHP (p=0.09). After 24h exposure to hEHP, there seems to be a high increase in iNOS protein expression to 3400±2595% when compared to control conditions, however, statistically non-significant. The pre-treatment with GsMtx4 1 µM slightly increased iNOS protein levels to 1023±479% of control.

3.4 Effect of EHP and Piezo1 activation in the protein levels of TAZ, YAP and p-YAP (S397)

The activation and translocation of mechanosensory protein YAP and its homologous protein TAZ to the nucleus are critical steps in their role as transcriptional coactivators, and they are tightly controlled by the Hippo signalling pathway that controls its cellular location (M. Fu et al., 2022b). Furthermore, emerging evidence indicates that Piezo1 deactivates the Hippo/YAP signalling pathway, promoting YAP and TAZ translocation to the nucleus. (B. Zhu et al., 2021)

To assess the effect of Piezo1 activation in the expression of TAZ and YAP proteins, as well as the phosphorylated form of YAP, p-YAP (S397), BV-2 cells were incubated with the channel agonist (Yoda1 at 10 µM). BV-2 cells were also exposed to hEHP (2% O_2) for 4h or 24h with or without Piezo1 inhibitor (GsMTx4 at 1 µM). Afterwards, the protein content was isolated and evaluated by western blot (Figure 11).

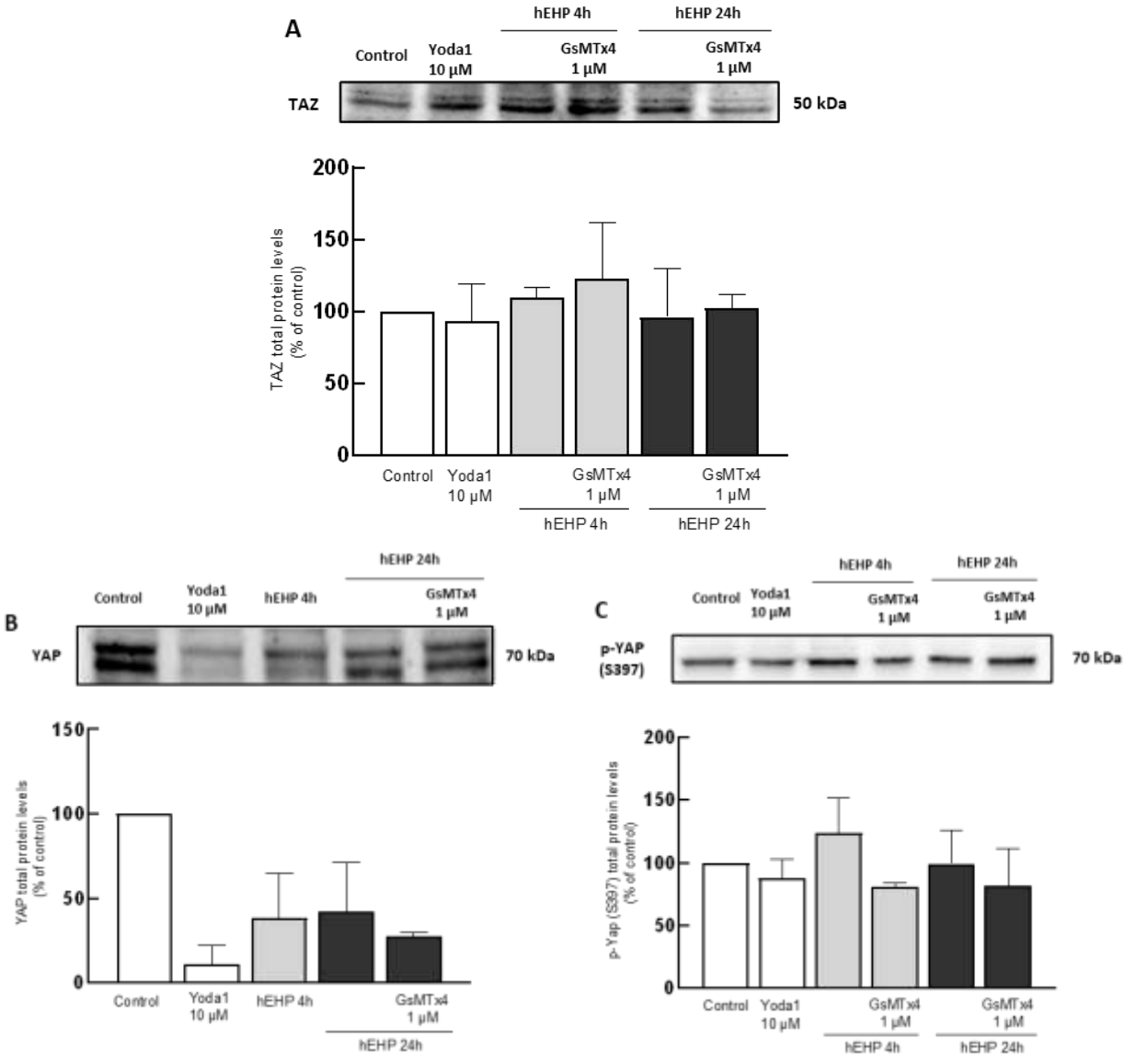


Figure 11 | Effect of EHP and Piezo1 activation in the protein levels of TAZ, YAP and p-YAP (S397). BV-2 cells were incubated with 10 µM Yoda1 or exposed to hEHP for 4 and 24h in the presence or absence of 1 µM GsMTx4. The total protein levels of (A) TAZ, (B) YAP, and (C) p-YAP (S397) were assessed by Western Blot. Results are presented as mean ± SEM and are expressed in percentage of the control and were obtained from 3 (A), 2 (B), and 4 (C) independent experiments.

Regarding TAZ total protein levels (Figure 11 A) there are no alterations when BV-2 cells were incubated with Yoda1 10 µM (94±26% of control). The same was observed for BV-2 cells exposed to hEHP for 4h or 24h (110±7% and 96±34%, respectively) and pre-treatment with GsMTx4 had no effect on TAZ protein levels in cells exposed to hEHP for 4h or 24h (123±40% and 103±4%, respectively).

In YAP total protein levels (Figure 11 B), the incubation of BV-2 cells with Yoda1 10 µM showed a decrease in YAP protein levels to 11±11% of control although not statistically significant. There was a slight decrease in the total protein levels in BV-2 cells exposed to hEHP for 4h or 24h (38.5±26.5% and 42±29%, respectively). Although non-significant, the pre-treatment with GsMTx4 in cells exposed to hEHP for 24h decreased YAP total protein levels (26±3%) in comparison to control conditions.

For p-YAP (S397) (Figure 11 C), there were no significant alterations in the total protein levels after Yoda1 10 µM incubation (88±15% of control) when compared to control conditions. The exposure of BV-2 cells to hEHP for 4h or 24h also showed no alterations (124±28% and 99±27%, respectively) and the pre-treatment with GsMTx4 1 µM did not affect p-YAP(S397) total protein levels (81±3% and 82±30%, respectively).

Considering that it is necessary that the YAP/TAZ complex translocate to the nucleus to promote gene transcription, after assessing the protein levels in all cellular content we assessed both nuclear and cytosolic fractions of each protein (Figure 12).

No alterations were observed in nuclear TAZ protein levels when BV-2 cells were incubated with Yoda1 10 µM (115±20% of control). The exposure to EHP (20% O_2) for 4h or 24h also showed no differences in TAZ nuclear protein levels (114±21% and 115±25%, respectively), which did not change with the pre-treatment with GsmTx4 1 µM (99±20% and 105±16%, respectively) (Figure 12A).

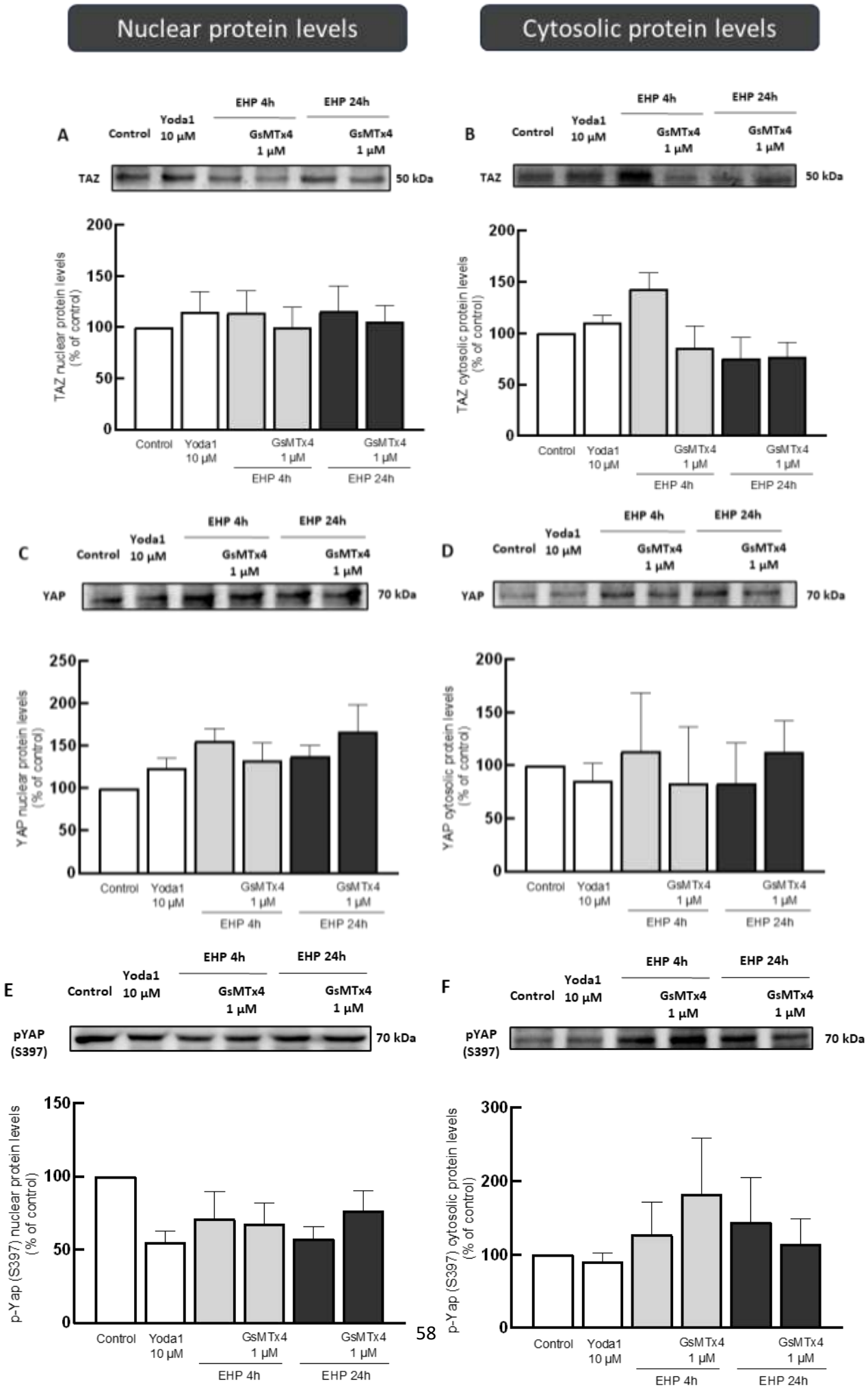

Nuclear protein levels
Cytosolic protein levels
A
B
Control
Yoda1 10 µM
EHP 4h
EHP 24h
GsMTx4 1 µM
TAZ
50 kDa
TAZ nuclear protein levels (% of control)
TAZ cytosolic protein levels (% of control)
C
D
YAP
70 kDa
YAP nuclear protein levels (% of control)
YAP cytosolic protein levels (% of control)
E
F
pYAP (S397)
70 kDa
p-Yap (S397) nuclear protein levels (% of control)
p-Yap (S397) cytosolic protein levels (% of control)

Figure 12 | Effect of EHP and Piezo1 activation in the protein levels of TAZ, YAP, and p-YAP (S397) in the cytosolic and nuclear fraction of BV-2 cells. BV-2 cells were incubated with 10 µM Yoda1 or exposed to EHP for 4 and 24h in the presence or absence of 1 µM GsMTx4. The protein levels in the nuclear and cytosolic fractions of TAZ (A and B), YAP (C and D), and p-YAP (S397) (E and F) were assessed by Western Blot. Results are expressed as mean ± SEM in percentage of the control and were obtained from 2-4 independent experiments.

Regarding the cytosolic fraction of TAZ (Figure 12 B), the incubation with Yoda1 10 µM had no effect on TAZ cytosolic protein levels (110±7%) when compared to control conditions (100%). BV-2 cells exposed to 4h of EHP showed a slight increase to 143±16% of control, an effect that was diminished by the pre-treatment with GsMTx4 1 µM (86±21%), although not statistically significant. The exposure to EHP for 24h in the absence and presence of GsMTx4 1 µM had no alterations on TAZ cytosolic protein levels (76±21% and 77±14%, respectively).

For YAP nuclear protein levels (Figure 12 C), no alterations were observed when BV-2 cells were incubated with Yoda1 10 µM (124±12% of control). The exposure to EHP for 4h slightly increased protein levels to 155±15%, an effect that had no alterations with the pre-treatment with GsMTx4 1 µM (133±21%). The exposure to 24h of EHP slightly did not affect nuclear protein levels of YAP (137±13%), and the pre-treatment with GsMTx4 1 µM showed a slight increase to 166±32% when compared to the control.

For YAP cytosolic fraction (Figure 12 D), incubation with Yoda1 10 µM had no alterations in protein levels (85±17%) when compared to control conditions. The same was observed for BV-2 cells exposed to EHP for 4h or 24h (113±55% and 83±39%, respectively), an effect that had no alterations with the pre-treatment with GsMTx4 1 µM (83±53% and 113±30%, respectively).

For p-YAP (S397) nuclear protein levels (Figure 12 E) there was a slight decrease when cells were incubated with Yoda1 10 µM to 55±8% of control. The exposure to EHP for 4h did not affect p-YAP (S397) nuclear protein levels (71±18%), however, 24h exposure to EHP showed a decrease (68±14%) when compared to control conditions, although not statistically significant. Pre-treatment with GsMTx4 1 µM in cells exposed to 4h or 24h of EHP had no effect on p-YAP (S397) nuclear protein levels (68±14% and 76±14%, respectively).

Regarding p-YAP (S397) cytosolic fraction (Figure 12 F), Yoda1 10 µM incubation had no effect on the protein levels (90±12%) when compared to control conditions (100%). Exposure to EHP for 4h did not alter the protein levels (127±44%), however, pre-treatment with GsMTx4 1 µM slightly increased cytosolic protein levels to 182±77% of control. The exposure

for 24h to EHP did not change the cytosolic protein levels of p-YAP(S397) (145±60%) when compared to the control, an effect that had no alteration with the pre-treatment with GsMTx4 1 μM (114±35%).

Chapter 4

Discussion

In glaucoma, a neurodegenerative disease characterized by RGC loss and optic nerve damage, microglia are central contributors to neuroinflammation (H. L. Zeng & Shi, 2018b). In response to injury such as elevated IOP or ischemia, microglia release proinflammatory mediators, driving a neuroinflammatory cascade (Fernández-Albarral et al., 2022). While initially mobilized as neuroprotective agents to clear debris and promote repair, sustained activation can lead to a shift towards a neurotoxic phenotype, exacerbating retinal damage and neuronal loss (J. Liu, Liu, et al., 2021). In the retina, the receptor in microglia that responds to conditions of ocular hypertension that might be responsible for triggering the inflammatory phenotype remains elusive. In our hypothesis, we postulated that the mechanosensitive ion channel Piezo1 is involved in the process by which microglial cells detect mechanical pressure, subsequently triggering mechanotransduction and inducing inflammation (Tang et al., 2023). In this work, we studied the contribution of the Piezo1 mechanosensitive channel in elevated pressure-induced microglia reactivity.

In the realm of glaucoma research, EHP models are used to mimic conditions of elevated IOP, enabling the possibility to study the cellular and molecular mechanisms that may contribute to disease onset and progression (Aires et al., 2017). In our group, several studies have been conducted using BV-2 cells showing that exposure to EHP is sufficient to induce microglia reactivity (Madeira, Elvas, et al., 2015a) with an enhancement of motility, migration, phagocytic activity, and proliferation (Ferreira-Silva et al., 2020). Moreover, microglia cells release extracellular vesicles when exposed to EHP, showing that BV-2 cells detect pressure changes (Aires et al., 2020).

Oxygen homeostasis is vital for mammalian health, with disruptions in oxygen availability, known as hypoxia, impacting cellular behaviour, and triggering various physiological responses (Samanta et al., 2017). Additionally, hypoxia-induced microglia reactivity promotes neuroinflammation in the developing brain and the retina (Kaur et al., 2013). In response to oxygen deprivation, a critical cellular event occurs through the stabilization and activation of HIF-1α. HIF-1α operates as a transcription factor capable of exerting a direct influence on microglial reactivity, by promoting the transcription of pro-inflammatory genes, including cytokines and chemokines (Hashimoto et al., 2023; Palazon et al., 2014). It has also been reported that IOP promotes a hypoxic environment that induces RGCs damage contributing to the progression of glaucoma (Jassim & Inman, 2019)

In the first part of the current work, the effect of hEHP in BV-2 cells was studied. The exposure to hEHP showed a slight increase in the protein levels of Piezo1 in BV-2 cells. It has been reported that the Piezo1 channel is upregulated under pressure conditions in lung endothelial cells in animal models of pulmonary hypertension (Ziyi Wang et al., 2021) and in

mice cardiomyocytes (Z.-Y. Yu et al., 2022). Moreover, low oxygen levels potentially mediate Piezo1 upregulation as reported in some cell types and tissues, such as endothelial cells (Z. Wang et al., 2021) and lung epithelial cells (H. Xiong et al., 2022). A possible mechanism might be through HIF-1α activation in response to hypoxia that binds directly to hypoxia-response elements within the promoter region of target genes (Yfantis et al., 2023), which might include Piezo1, however, no information has been reported in the literature so far.

Phagocytosis is the process by which microglia engulf and remove dead cells, debris, and potentially harmful substances, contributing to the maintenance of neuronal tissue homeostasis (Galloway et al., 2019; Ha et al., 2021). Piezo1 activation did not significantly alter phagocytosis efficiency. Nevertheless, the effect of Yoda1 in phagocytosis seems to be concentration-dependent, and we can speculate that higher concentrations or incubation time could result in a significant effect. Piezo1 activation enhances phagocytosis efficiency in murine microglia cells in AD (Jäntti et al., 2022b). The exposure to hEHP did not affect phagocytosis efficiency. Hypoxia exposure is also reported to also enhance phagocytosis in RAW264.7 cells, a macrophage-like cell line and in primary peritoneal macrophages demonstrating that hypoxia-induced overexpression of HIF-1α is sufficient to lead to an increase in phagocytosis (Anand et al., 2007). It has been also reported that the phagocytosis activity of pulmonary macrophages is impaired in intracellular hyperoxic conditions (Patel et al., 2016). Hypoxia disrupts the efficient flow of electrons through the mitochondrial electron transport chain. This inefficiency occurs because oxygen, the terminal electron acceptor, becomes less accessible, consequently generating ROS within the cell. Therefore, despite the external oxygen deficiency, this ROS accumulation creates an intracellular hyperoxic condition (Kung-Chun Chiu et al., 2019). Piezo1 activation is also reported to increase ROS in cells such as cardiomyocytes (Yuan et al., 2023b) and in macrophages (Atcha, Jairaman, et al., 2021b). Taking this into consideration, our results showing no alterations in phagocytosis efficiency when cells were exposed to hEHP might indicate that Piezo1 activation in conditions of hypoxia leads to an exacerbated ROS production, creating an intracellular hyperoxic environment that impairs phagocytosis efficiency.

When evaluating the impact of EHP on phagocytosis efficiency, our findings exhibited a tendency to increase phagocytosis efficiency when compared with control, according to previous findings (Aires et al., 2019c). Yoda1 slightly increased phagocytosis efficiency while Piezo1 inhibition with pre-incubation of 1 µM GsMTx4 attenuated the EHP effect on phagocytosis efficiency. Even though GsMTx4 can inhibit Piezo1 opening, it is not Piezo1 specific and might be acting in other mechanosensitive channels, not fully inhibiting Piezo1, this might suggest that Piezo1 might play a role in modulating microglia phagocytosis. Taking

into consideration, these results might suggest that Piezo1 activation enhances phagocytosis efficiency in BV-2 cells while prolonged low levels of oxygen under EHP conditions might impair it. Nevertheless, additional research is necessary to draw comprehensive conclusions.

Microglia-induced nitrosative stress assumes a pivotal role in the intricate pathogenesis of glaucoma, wherein the intricate orchestration of NO extends its influence on the regulation of blood flow within the optic nerve head and the modulation of intraocular pressure (Schmidl et al., 2013). Dysregulation of NO production and signalling pathways can incite vascular dysfunction culminating in the dysfunction and damage in RGCs (Garhöfer & Schmetterer, 2019; Wareham et al., 2018). To explore the effect of Piezo1 activation in nitrosative stress NO production was assessed by the Griess reaction assay, an indirect method that measures the concentrations of nitrites in the culture medium. No alterations were observed in the nitrite concentration, which might be due to the fact that the Griess reaction assay is relatively sensitive but might not be suitable for detecting low levels of NO production (Csonka et al., 2015; Hunter et al., 2013). When assessing NO production directly with the DAF-FM probe, our results showed that Piezo1 activation for 4 h had no effect on NO production even in cells exposed to hEHP, while 24h exposure increased NO production. According to the literature, Piezo1 activation enhances NO production in red blood cells (Kuck et al., 2022) and endothelial cells (Lhomme et al., 2019). The same is reported for macrophages (Jackson et al., 2007) in hypoxic conditions. Both stimuli, Piezo1 activation and hypoxia exposure have been reported to have a time-dependent component. Regarding Piezo1 activation, (Swain & Liddle, 2021) showed in a smaller time scale (5s - 1 min) that prolonged Yoda1 incubation (1 min) induces a higher NO production. NO production has also been reported to be a calcium-dependent process (Kuck et al., 2022). Considering that Yoda1 leads to a partial opening of the channel, limiting the extent of Ca^{2+} influx, and given that alterations in intracellular Ca^{2+} levels are known to impact NO production in endothelial cells (Silva & Ballejo, 2019) and in astrocytes (Nianzhen Li et al., 2003), our findings imply that the Ca^{2+} influx triggered by Yoda1 may elicit a slight increase in NO production, falling short of achieving full channel activation. Hypoxia-induced NO production is time-dependent. Min et al., (2006) showed that hypoxia (1% O_2) increases HIF-1α expression and NO production with a maximum effect after 24h exposure in bovine aortic endothelial cells, explaining the lack of significant alteration when exposed to hEHP for 4h. Moreover, with these results, it is not possible to conclude whether this effect was due to Piezo1 activation, hypoxia exposure or even a cumulative effect of the two factors, however, the slight decrease of NO production with the pre-incubation with GsMTx4 might suggest a predominant role of Piezo1.

Upregulation in iNOS expression has been correlated with an increase in NO production, evident both within a hypoxic milieu (Stachon et al., 2020; Thompson et al., 2009) and also following Piezo1 channel activation (Cai et al., 2023; X. Zhang et al., 2022). iNOS protein levels slightly increased when cells were exposed to hEHP for 4h, but the increase was higher after 24 h exposure, suggesting the possibility of time-dependent effect. Furthermore, the slight decrease in iNOS expression following a 24 h exposure to hEHP after pre-incubation with the channel inhibitor GsMTx4 in comparison to the respective hEHP conditions might suggest that Piezo1 activation might enhance iNOS expression, however, the slight increase when compared to the control might suggest a cumulative effect of hypoxia in the upregulation of iNOS.

Piezo1 has been described to influence YAP and TAZ subcellular localization through modulation of the Hippo signalling pathway (B. Zhu et al., 2021). Upon Piezo1 activation, the Hippo signalling pathway is deactivated consequently promoting the nuclear translocation of YAP and TAZ. Within the nucleus, YAP and TAZ engage in gene transcription processes (M. Fu et al., 2022; R. Liu & Persson, 2004). Regarding the Hippo signalling pathway elements, our results showed that Piezo1 activation demonstrates no significant impact on the total levels of TAZ. In hypoxia, the activation of hypoxia-inducible factors (HIFs) also deactivates the Hippo signalling pathway, promoting the nuclear translocation of YAP and TAZ, and enhancing their transcriptional activities in cancer cell lines (Ma et al., 2015). When assessing TAZ, YAP and p-YAP (S397) total protein levels, our results revealed only a slight decrease in YAP total protein levels. Yan et al., (2014b) showed that hypoxia (1% O_2 for 24 h) reduces YAP total protein levels in ovarian cancer cell lines, however, it is also reported a reduction in p-YAP total protein levels and a modest increase of total TAZ. Regarding the effect of Piezo1 activation, Y. Xiong et al., (2022) showed that in ovarian cancer cell lines YAP total protein levels after incubation with Yoda1 (50 μM) showed no alterations in total protein levels, while p-YAP total protein levels showed a decrease. To have a better understanding of Piezo1's effect on TAZ, YAP and p-YAP (S397) total protein levels, further experiments must be performed using EHP as a mechanical stimulus in normoxic conditions.

When examining the subcellular distribution at both nuclear and cytosolic levels, Piezo1 activation had no effect on either TAZ or YAP protein levels. According to the literature, incubation with 50 μM Yoda1 has no effect on YAP cytosolic protein levels in A-1847 and 3AO cells, cellular lines of ovarian cancer, showing an increase in its nuclear localization (Y. Xiong et al., 2022b), which was not evident in our results. This disparity could be attributed to the limited number of repetitions, fostering heightened variability. The lack of changes in YAP's cytoplasmic levels could potentially be attributed to the documented direct impact of Piezo1

activation on the Hippo signalling pathway's upstream regulation of YAP, particularly targeting LATS1 (Y. Xiong et al., 2022b). This kinase is responsible for YAP/TAZ phosphorylation and retention in the cytoplasm when Hippo signalling pathway is activated, it has been reported that phosphorylation of LATS1 may be inhibited by intracellular calcium (Wei & Li, 2021). Our analysis of p-YAP (S397), an inactive YAP form, indicates a minor reduction in nuclear and a slight decrease in cytosolic levels upon Piezo1 activation. These align with existing literature that suggests Piezo1 activation suppresses the Hippo signalling pathway, thereby retaining YAP's phosphorylated form in the cytoplasm for further degradation (B. Zhu et al., 2021). However, the absence of statistical significance underscores these findings.

Taken together, our results show that Piezo1 might be upregulated in hEHP conditions and its activation in BV-2 cells might enhance phagocytosis efficiency, which might be impaired due to the hypoxia exposure. hEHP exposure increases NO production and might be a time-dependent stimulus, however, more experiments must be performed in normoxic conditions to have a better understanding of the Piezo1 activation effect in NO production. Piezo1 activation effect in YAP/TAP cellular dynamics is more comprehended when assessing their subcellular location which revealed a slight increase in YAP nuclear levels as well as a slight increase in p-YAP (S397) cytosolic protein levels.

Chapter 5

Conclusions and Future Perspetives

In this study, we demonstrated that BV-2 cells express Piezo1, and in conditions of hEHP there was a slight increase in the protein levels of Piezo1. Concerning the role of Piezo1 activation on microglia phagocytosis, Yoda1 incubation and EHP exposure slightly increased phagocytosis efficiency, an effect that was attenuated when Piezo1 was inhibited through pre-incubated with 1 μM GsMTx4. Exposure to hEHP did not affect phagocytosis efficiency.

Regarding the Piezo1 activation effect on nitrosative stress, when directly assessing the NO production, the cumulative effect of Piezo1 activation through 24 h exposure to EHP in a hypoxic environment significantly enhanced NO production and highly increased iNOS expression.

To investigate the influence of Piezo1 on the Hippo signalling pathway, we analysed the protein levels of TAZ, YAP, and p-YAP (S397). No alterations were detected in these proteins in hypoxic conditions. When examining the subcellular location of these proteins, Piezo1 activation caused a tendency to decrease cytoplasmic levels of p-YAP (S397).

Further experiments are needed to elucidate the role of the Piezo1 channel in elevated pressure-induced microglia reactivity. On a first analysis, it would be interesting to complete the Western blot results on the EHP effect on Piezo1 expression levels in BV-2 cells, by immunolabeling and qPCR to assess mRNA levels. Additionally, we plan to enhance our understanding of the cellular localization of YAP and TAZ by utilizing confocal imaging techniques. To expand our investigation on the impact of Piezo1 activation on microglia reactivity, we will analyse other aspects of microglia function beyond phagocytosis, for instance, cell migration through techniques such as scratch wound assays and the Boyden chamber migration assay as well as the production of pro-inflammatory cytokines (IL-1β and TNF) using ELISA kits.

For future research and more in-depth analysis, several intriguing avenues could be explored. One approach would be to silence the channel, possibly through techniques like CRISPR-Cas9, in order to better understand its impact. Additionally, investigating the role of Piezo1 in relation to LATS1 could provide valuable insights into Piezo1 activation's direct effect on the Hippo signalling pathway. Furthermore, conducting a genetic study involving glaucoma patients to determine whether Piezo1 is upregulated in the disease could offer valuable clinical relevance and shed light on its potential significance in the context of glaucoma.

Chapter 6

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