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ENDOCANNABINOID SIGNALLING IN OTOTOXICITY

Uncovering novel biomolecular clues in *in vitro* and *in vivo* models

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Abbreviations

2-AG - 2-Arachidonyl glycerol

AA - Arachidonic acid

ABHD - α/β Hydrolase Domain Containing Protein

ABR - Auditory Brainstem Response

AEA - Arachidonoyl ethanolamide

ALA - Alpha-linolenic acid

ANOVA - Analysis of Variance

ASC - Apoptosis-associated speck-like protein

BC - Basal cell layer

cAMP - cyclic adenosine monophosphate

CB₁R - Cannabinoid Receptor 1

CB₂R - Cannabinoid Receptor 2

CIS - Cisplatin

CNS - Central nervous system

COPD-Chronic obstructive pulmonary disease

DAG - Diacylglycerol

DAGL - Diacylglycerol lipase

DHA - Docosahexaenoic acid

DHEA - Docosahexaenoyl Ethanolamide

DMSO - Dimethyl sulfoxide

eCB - Endocannabinoid

ECS - Endocannabinoid system

ELISA - Enzyme linked immunosorbent assay

EP - Endocochlear potential

EPEA - Eicosapentaenoyl ethanolamide

ESI - Electrospray ionization

FAAH - Fatty acid amide hydrolase

FDR - False discovery rate

FUR - Furosemide

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GPCR - G-protein coupled receptor

GSDMD - Gasdermin D

HC - Hair cell

IC - Intermediate cell layer

IC₅₀ - Half-maximal inhibitory concentration

IF - Immunofluorescence

IHC - Inner hair cell

IL - Interleukin

iNOS - Inducible nitric oxide synthase

KAN - Kanamycin

LEA - Linoleoyl ethanolamide

LFC - Log₂ fold change

MAGL - Monoacylglycerol lipase

MAG - Monoacyl glycerol

MC - Marginal cell layer

MET - Mechanoelectrical transduction channel

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Myo7a - Myosin 7a

NAAA - *N*-acylethanolamine acid amidase

NAE - *N*-acylethanolamine

NAPE-PLD - *N*-acyl-phosphatidylethanolamine phospholipase D

NF-κB - Nuclear-factor kappa-light-chain-enhancer of activated B cells

NH - Normal hearing

NLRP3 - Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3

NRF2 - Nuclear factor erythroid 2-related factor 2

NSAID - Non-steroidal anti inflammatory drug

OC - Organ of Corti

OCT - Organic cation transporter

OEA - Oleoylethanolamide

OHC - Outer hair cell

PCA - Principal component analysis

PE - Phosphatidylethanolamine

PEA - Palmitoylethanolamide

PHARC - polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract

PIP2 - Phosphatidylinositol 4,5 bisphosphate

PLA₂ - Phospholipase A₂

PLC - Phospholipase C

POEA - Palmitoleoyl ethanolamide

PPAR - Peroxisome Proliferator-Activated Receptor

ROS - Reactive oxygen species

SC - Supporting cell

SEA - Steroylethanolamide

SEM - Standard error of mean

SG - Spiral ganglia

SGN - Spiral ganglion neurons

SNHL - Sensorineural hearing loss

SPM - Specialized pro-resolving lipid mediator

STS - sodium thiosulfate

SV - Stria vascularis

TNF α - Tumor necrosis factor alpha

TPM - Transcripts per million

TRPV1 - Transient receptor potential Vanilloid 1

UHPLC-MS/MS - Ultrahigh-performance liquid chromatography-tandem mass spectrometry

VEH - Vehicle

Δ -9-THC - (-)-*trans*- Δ^9 -tetrahydrocannabinol

Abstract

Cisplatin-induced ototoxicity is a debilitating side effect of chemotherapy which represents a leading cause of sensorineural hearing loss (SNHL) and remains a significant clinical burden to date. Cisplatin is known to primarily target sensory hair cells (HCs) in cochlea resulting in HC damage/loss leading to hearing impairment. At present, no effective treatments exist to prevent/counteract these adverse effects. Therefore, the lack of viable pharmacological approaches necessitates the need for better understanding of the molecular mechanisms underlying cisplatin-induced hearing loss to identify new potential targets. In this context, recent preliminary studies have pointed out promising therapeutic roles for bioactive lipids such as endocannabinoids (eCBs) in ototoxicity. However, despite its emerging interest, the endocannabinoid system (ECS) remains poorly investigated in the context of auditory health and disease. To this end, firstly, a systematic molecular characterization of the ECS in a cochlear HC-like cell line was performed. We demonstrated for the first time the presence of bioactive eCBs and eCB-like lipids (through targeted lipidomics) along with key receptor targets and metabolic enzymes at the protein level in the cell line. Afterwards, potential alterations of the ECS were explored in an *in vitro* model of cisplatin-induced ototoxicity. Interestingly, novel biochemical perturbations in the ECS were observed, such as decrease in the protein expressions of Cannabinoid receptor 2 (CB₂R), Diacylglycerol lipase β (DAGL β) and α/β Hydrolase Domain-Containing Protein 6 (ABHD6), revealing HC-related targets in the context of drug-induced hearing trauma. Following the characterization of the ECS and its modulation upon ototoxicity *in vitro*, we extended our investigation to an *in vivo* model for a more physiological and translational relevance. To this end, we investigated the ECS components that were found to be altered *in vitro* (CB₂R, DAGL β and ABHD6) within the organ of Corti (OC) of an *in vivo* model of cisplatin-induced ototoxicity. Intriguingly, the analysis revealed similar alterations *in vivo* thereby strengthening our findings. Furthermore, we demonstrated that antagonism of CB₂R through SR144528 ameliorates cisplatin-induced HC death through reduction of cleaved-caspase 3 levels, thereby providing mechanistic insights into the role of CB₂R in ototoxicity. Taken together, the work described in the present thesis provided a comprehensive characterization of the ECS elements in cochlear physiology using HC-like cell line. Furthermore, the study uncovered novel biomolecular targets within the ECS that may hold potential therapeutic relevance for cisplatin-induced SNHL.

1. Introduction

1.1 The Cochlea

The cochlea is a fluid-filled, spiral-shaped organ encased in the bony labyrinth, located in the inner ear, which is responsible for hearing function. The ability of this specialized organ to perform such a complex function lies in its cytoarchitecture, which comprises of multiple structures and cell types [1]. The cochlea is divided into three fluid-filled spaces, namely the scala vestibuli, the scala media/cochlear duct and the scala tympani [2]. The cochlear fluids (endolymph and perilymph) in these spaces are critical in maintaining the endocochlear potential (EP). The scala media filled with endolymph is bordered above by the Reissner's membrane and below by the basilar membrane, which hosts the sensory apparatus - the organ of Corti (OC). The OC houses specialized mechanoreceptor cells called Hair cells (HCs) which convert mechanical sound vibrations to neurochemical signals, thanks to their stereocilia. The stereocilia are tiny projections on the apical region of HCs, whose deflection by sound-induced mechanical stimulus triggers ion influx and neurotransmitter release onto the auditory nerve fibers. The other two fluid-filled spaces, scala vestibuli and the scala tympani contain the perilymph and intersect at the apical helicotrema [3]. The lateral wall of the cochlear duct is occupied by the spiral ligament laterally and by the stria vascularis (SV) medially. The spiral ligament is a fibrous structure that serves several critical functions in the cochlea. They include housing the capillary bed for supply and drainage in the cochlea, providing mechanical support to SV and anchoring the OC to the membrane [4]. The SV, on the other hand, is a highly vascularized region and has a significant role in maintaining the EP, ensuring balance of the ionic cochlear fluids [5]. EP is critical for normal hearing process as EP drives the electrochemical force that allows HCs to efficiently transduce mechanical sound energy to neural impulses. In humans, the cochlea is organized as a spiral canal which makes 2.5 turns around the modiolus - a spongy central core containing the spiral ganglion (SG). The SG houses a cluster of nerve cell bodies referred to as the spiral ganglion neurons (SGNs) [1]. SGNs are the primary neurons that transmit the neural signals from the HCs to the brain. The cell bodies of the SGNs are located in the Rosenthal's spiral canal, each of which gives rise to peripheral processes extending toward the OC. The central processes of the SGN fibers connect to form the auditory nerve and innervate the auditory processing centers in the brain.

The organ of Corti (OC)

The OC is a long-band shaped epithelium coiled around the modiolus in the cochlea. It consists of spatially organized sensory HCs and non-sensory supporting cells (SCs). The sensory HCs are of two types, namely the inner hair cells (IHCs) and outer hair cells (OHCs) which form a line of one row and three rows respectively from the base to the apex of the cochlea. The stereocilia are arranged on the apical surface of the HCs which is in contact with the tectorial membrane, a gelatinous extracellular membrane that covers the auditory epithelium [4,6]. The IHCs are called “true sensory cells” since they send neural impulses along the auditory nerve, whereas the OHCs are termed as “cochlear amplifiers” due to their role in enhancing and modulating the function of the IHCs. Moreover, OHCs are essential in tuning the sensitivity and selectivity of the sound in the cochlea.

The range of hearing frequencies is distributed tonotopically across the cochlea from the base to the apex, where the base transduces high frequencies and the apical end transduces low frequencies [4]. The ability of the organ to transduce such wide range of frequencies is due to the sophisticated mechanical features of the cell structures as well as its biomechanics, which involve ultra-fast ion channels and an inbuilt cochlear amplifier [6]. Sensory transduction is initiated by sound vibrations travelling down the cochlear fluids which causes the basilar membrane to vibrate, leading to deflection of the stereocilia atop the HCs. The deflection of stereocilia opens the mechano-electric transduction (MET) channels which causes HCs to depolarize. This process results in transduction of mechanical energy to neural impulses which are transmitted via the ribbon synapses to the auditory nerve and then to central nervous system (CNS), where they are perceived as sound [7]. Ribbon synapses are specialized synapses that organize synaptic vesicles near voltage gated calcium channels, allowing HCs to transmit auditory information with speed and precision.

HCs are surrounded by SCs, which are highly differentiated and polarized epithelial cells. Five different types of SCs are organized from the outer to the inner edge of the organ: (i) Hensen’s cells, (ii) Deiters’ cells, (iii) Pillar cells, (iv) Inner phalangeal and (v) Border cells. They offer a wide range of auxiliary functions such as modulation of extracellular matrices, homeostasis of ions and molecules, and structural support of the sensory epithelium. Moreover, in non-mammal cochleae, they can also renew into new HCs after damage, thereby serving as stem cell-like cells [8]. The detailed structure of the OC is provided in Figure 1.

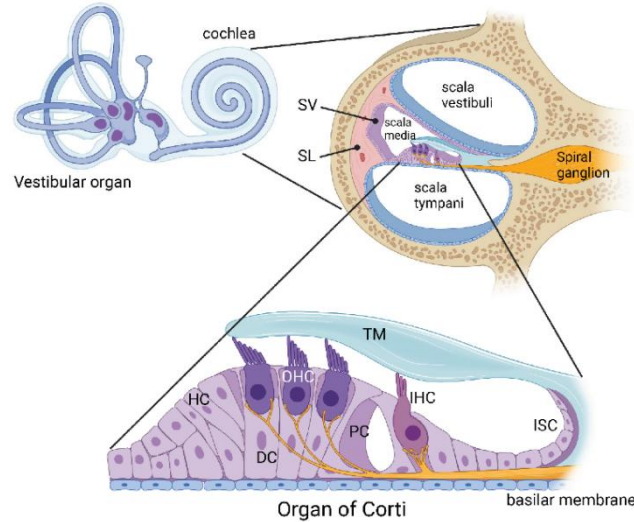


Figure 1. The cochlea and the OC structures [9]

1.2 Sensorineural Hearing loss (SNHL)

Sensorineural hearing loss (SNHL) is the most common and complex type of hearing impairment at a global scale, accounting for over 90% of all cases. SNHL refers to hearing loss that results from injury to the cochlea, auditory nerve or the auditory processing centers in the CNS. The majority of SNHL cases in humans are due to structural damage and degeneration of the sensory HCs [10,11]. Unfortunately, the precise pathological mechanisms underlying SNHL are not yet fully known but growing body of evidence suggests a complex interplay of genetic, molecular and cellular factors such as disturbances in ion homeostasis, oxidative stress, metabolic abnormalities, inflammation, cell apoptosis and mitochondrial impairment among others [11,12].

Currently, emerging research in pharmaceuticals, gene therapies, regenerative medicine and cell therapies hold promise for future therapeutic interventions but they are still preliminary and limited to preclinical stages [13,14]. Therefore, to date prosthetics such as hearing aids and cochlear implants are still the most common interventions for SNHL because the underlying damage is usually permanent and cannot be treated with medications/surgical interventions. Prosthetic devices help amplify sounds (in case of hearing aids) or directly stimulate the auditory nerve (through cochlear implants). However, the efficacy of prosthetics may vary due to several factors. Firstly, the effectiveness of these devices largely depends on the extent and the location of the damage. For instance, severe degeneration of HCs or cochlear nerve can limit the stimulation provided by the devices, which indeed require healthy nerves to transmit the signals. Furthermore, prosthetics do not recapitulate the natural hearing quality [15]. This calls for a better understanding

of the molecular mechanisms of SNHL to identify promising pharmacological targets as new and more efficient alternatives to mitigate SNHL.

1.3 Ototoxicity

Among various etiologies of acquired SNHL, ototoxicity is recognized as one of the leading causes [16]. It is defined as damage to the structures of the inner ear (cochlear and vestibular) owing to the exposure of chemicals, pharmaceuticals and radiation [17]. Symptoms of ototoxicity include SNHL, aural fullness, dizziness and vertigo. The onset of these symptoms can occur immediately or can also begin from weeks after exposure. Moreover, the risk is markedly exacerbated by several factors such as accompanying comorbid conditions, aging, noise exposure and complex drug interactions [16,17].

Many drugs on the market have been associated with ototoxic side effects. Some classic examples include platinum-based chemotherapeutic agents (such as cisplatin and carboplatin) aminoglycoside antibiotics (such as kanamycin, gentamycin), macrolides (such as azithromycin) [18], loop diuretics (such as furosemide) [19], antimalarial drugs (such as chloroquine and hydroxychloroquine) [20] and non-steroidal anti-inflammatory drugs (NSAIDs) [21]. In addition, some immunosuppressants [22], antiviral drugs [23] and antiparasitic drugs [23] have also been identified to be potentially cochleotoxic.

Among these, the most studied include the aminoglycoside antibiotics and platinum-based therapeutics because of their wide usage in clinical settings. Aminoglycoside antibiotics are known for their efficacy against gram negative bacterial infections, whereas platinum-based compounds are widely used for treating solid malignant tumors. Recent investigations into these drug classes have revealed some of the mechanisms underlying their ototoxic effects. For instance, both aminoglycosides and platinum-based therapeutics are known to cause damage to OHCs at the basal turn of the cochlea before targeting the apical cells and IHCs. This pattern of vulnerability can be attributed to OHC's higher metabolic demand, larger MET current and subsequent calcium overload with respect to OHCs located in low-frequency apical regions and IHCs [24,25]. Other suggested mechanisms of ototoxicity include DNA and mitochondrial damage, reactive oxygen species (ROS) formation and inflammasome activation. Nonetheless, ototoxicity remains poorly understood due to the unknown effects of these drugs and interplay of multiple signaling pathways highlighting the need for further mechanistic investigations.

1.3.1 Cisplatin-induced Ototoxicity

Cisplatin (*cis*-diamminedichloroplatinum II) - a platinum-based compound - is a potent and one of the most used chemotherapeutic agents to treat a wide variety of solid tumors. This drug combats tumors through the formation of covalent DNA crosslinks, leading to apoptotic and necrotic cell death [26]. The main reported side effects of cisplatin include nephrotoxicity, neurotoxicity and ototoxicity. Despite its adverse effects, it remains a cornerstone in oncology owing to its high efficacy. Even though some less toxic alternatives to cisplatin have been explored for cancer therapy, its exceptional effectiveness makes it difficult to replace with alternatives [16].

Previous studies have addressed some of the molecular mechanisms underlying cisplatin-induced ototoxicity. For instance, in the OC, spiral ligament and SV, cisplatin treatment induced the activation of nuclear-factor kappa B (NF- κ B) [27,28] and inducible nitric oxide synthase (iNOS) *in vitro* and *in vivo* [29]. These molecular responses initiate downstream inflammatory cascades that cause cochlear injury. Building on this, cisplatin treatment has also shown increased expression of pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNF α), Interleukins 1 and 6 (IL-1, IL-6) in HC-like cells and in animal models [30]. Beyond inflammation, cisplatin is reported to cause direct cellular damage through apoptosis, nuclear DNA damage-induced metabolic abnormalities, mitochondrial damage and subsequent ROS production in cochlear cells as observed in several cases of systemic cisplatin administration [31]. Furthermore, autophagy dependent cell death has also been recognized as a key mechanism in this context. Together these interconnected mechanisms highlight the complexity of cisplatin mediated cochlear damage. While much knowledge has been gained, the exact mechanisms of cisplatin-induced ototoxicity remain unclear due to the complex, multi-factorial nature of the damage caused by cisplatin involving multiple cell types and interconnected molecular pathways. This missing information poses a critical challenge in the development of effective therapies. Indeed, to date the only novel intervention approved by the FDA is a sodium thiosulfate (STS) formulation for children aged 1 month and older [32,33]. However, although it appears to be a significant milestone, 39-44% were not protected (Clinical trial ID: NCT00652132, NCT00716976). Moreover, alternative pharmacological strategies to mitigate cisplatin-induced ototoxicity have not reached clinical applications yet, largely due to the unknown effects of drugs on the antineoplastic activity of cisplatin. For instance, antioxidants - such as biological extracts [34], statins [35] and alpha lipoeic acid [36] - have shown potential in mitigating ototoxicity but they are reported to

interfere with the anti-cancer function of cisplatin. Furthermore, MET channel [37] and organic cation transporter (OCT) [38] channel inhibitors have been explored; however, these agents have been demonstrated to have limited effectiveness. Besides these, apoptotic pathways, (involving Bcl2, p53, AKT) [39,40] autophagy [41], nuclear factor erythroid 2-related factor 2 (NRF2) signalling pathways [42] among others have also been extensively targeted but have so far shown limited efficacy. Overall, the available literature highlights the need for a better understanding of the molecular, biochemical, and biophysical aspects of ototoxic trauma caused by cisplatin in order to identify novel targets and thereby effective treatments.

1.4 Bioactive lipids in health and disease

Endogenous bioactive lipids are a highly complex network of molecules involved in multiple biological processes. Although lipid signalling was traditionally thought to be limited to cell membrane processes, emerging lipidomic studies have demonstrated their role in other cellular and molecular processes as well [43]. The endogenous bioactive lipids can be categorized principally into four classes: eicosanoids, specialized pro-resolving lipid mediators (SPMs), lysophospholipids and endocannabinoids (eCBs) [44]. Emerging evidence has highlighted the importance of these bioactive lipids in different health and disease conditions. Under physiological state, they contribute to maintaining homeostasis of key cellular processes such as intracellular signalling and communication, gene expression and immune responses [43,45]. Indeed, dysregulated lipid signalling is reported in several pathological conditions such as cancer, autoimmune diseases, inflammatory processes, neurodegeneration, among others [44]. For instance, eicosanoids are well-known for their prominent roles in promoting inflammation and have been implicated in various inflammatory diseases such as rheumatoid arthritis and atherosclerosis [46,47]. Differently, SPMs act to counterbalance inflammation and foster tissue repair, thereby promoting resolution of inflammation. Their dysregulation is indeed involved in a broad range of inflammatory and immune-related conditions, including cardiovascular, metabolic, neurodegenerative diseases among others [44,48]. Furthermore, the aberrant regulation of eCB signalling has also been observed in several metabolic disorders [49] and neurodegenerative pathologies [45]. Given the key role of lipids in diverse disease conditions, therapeutic strategies targeting lipid classes are currently being investigated in many of the above-mentioned pathophysiological conditions [50–55].

In the context of auditory pathologies, although there are limited studies on lipid signalling, existing data offers promising insights into its potential role in auditory health [56,57]. For instance, preclinical and clinical studies have pointed out the role for precursors of ω -3 fatty acid such as alpha-linolenic acid (ALA) and docosahexaenoic acid (DHA) in auditory nerve conduction [58]. Recent studies have also linked the involvement of eCBs [59], sphingolipids [60] and SPMs [61] in ototoxicity. Hence, the available literature suggests lipid signalling as a critical regulatory network underlying the complex mechanisms driving auditory pathologies.

Among the large family of bioactive lipids, the endocannabinoid system (ECS) represents a key regulatory network and despite its well-established roles in various pathological conditions, its role in inner ear physiology and related auditory disorders has received limited attention. Given the growing recognition of ECS in several processes, the ECS may represent a promising yet unexplored target for understanding the mechanisms underlying cochlear pathologies.

1.4.1 The Endocannabinoid system (ECS)

The ECS has emerged as an important ubiquitously expressed lipid system, known for its widespread regulation of various physiological processes. Much of our knowledge on the ECS stems from extensive studies focusing on their role in the context of neuromodulatory and inflammatory functions. The ECS comprises the eCB lipids, their receptor targets and metabolic enzymes (Figure 2) [62–64]. eCBs are derived from ω -6 polyunsaturated fatty acids, and they are produced “on demand”. eCB lipids exhibit effects that partially resemble those of psychoactive cannabinoids such as (–)-*trans*- Δ^9 -tetrahydrocannabinol (Δ -9-THC) [65]. The main eCBs include Arachidonylethanolamide or anandamide (AEA) and 2-Arachidonylglycerol (2-AG) which are synthesized by distinct metabolic routes. eCBs are produced from their foundational phospholipid precursors, phosphatidylethanolamine (PE) and Phosphatidylinositol 4,5 bisphosphate (PIP2) present in the lipid bilayers. These phospholipid molecules are enzymatically transformed to generate specialized precursors such as *N*-acyl-phosphatidylethanolamine (NAPE) for AEA and Diacylglycerol (DAG) for 2-AG [66]. The metabolic pathway for AEA and 2-AG is detailed in the paragraphs below.

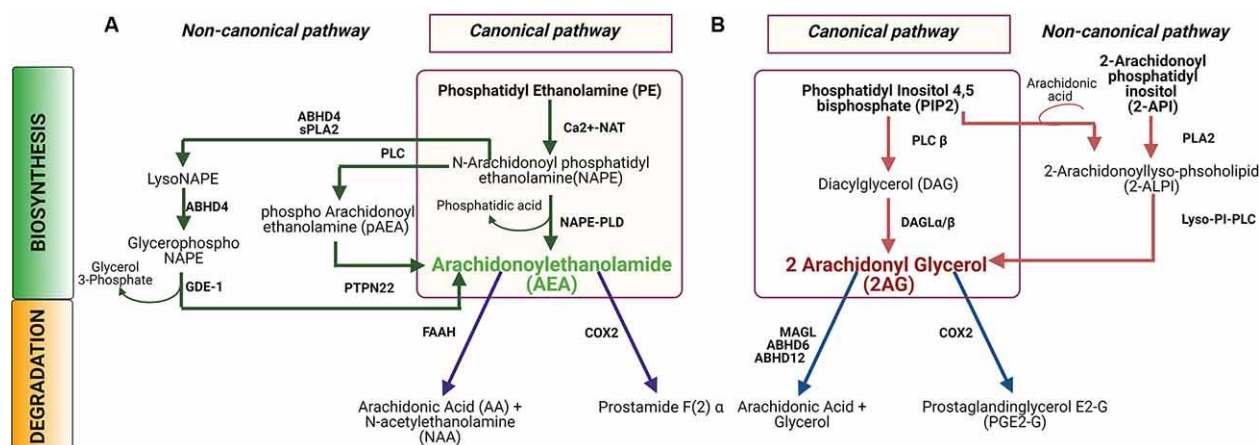


Figure 2. The biochemical signalling pathway of the ECS [59]

Main eCBs and eCB-like lipids

The main eCBs are AEA and 2-AG. AEA is structurally a fatty acid amide which contains arachidonic (AA) - an ω -6 polyunsaturated fatty acid - linked to an ethanolamine group through an amide bond. AEA is classified as an unsaturated *N*-acylethanolamine (NAE) due to the presence of multiple double bonds within the hydrocarbon chain of its AA component. Differently, 2-AG is a monoacylglycerol lipid composed of an AA moiety esterified at the *sn*2 position of its glycerol backbone [67]. Both AEA and 2-AG have been detected in the brain and other peripheral tissues, but AEA levels are generally reported to be lower compared to 2-AG in most organs. In addition to classical eCBs, several structurally related eCB-like lipids have also been identified, expanding the complexity of the ECS. They influence the ECS through allosteric modulation of the eCB receptors [68] or by competing with the degradative enzymatic machinery and hence indirectly enhancing eCB signalling. As a result of these effects, they are referred to as eCB-like lipids [63]. Among the growing family of eCB lipids, AEA and 2-AG were the first to be discovered and therefore the signalling of AEA and 2-AG, as well as subsequent downstream pathways, are relatively well-characterized with respect to the repertoire of other eCB-like congeners. Moreover, eCB-like lipids have received relatively less attention compared to AEA and 2-AG owing to the difficulty in isolating them from cells/tissues. Among the eCB-like lipids, the most studied members are NAEs such as *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA) [69] due to their relatively high tissue abundance which makes them more physiologically relevant and easily detectable for experimental purposes. Additionally, they are documented to have relatively clear biological effects because of their known interactions with classical eCB receptors

such as peroxisome proliferator-activated receptors α (PPAR α) and Transient receptor potential vanilloid 1 (TRPV1), which play established roles in metabolism, inflammation and mediating analgesic effects. Other eCB-like lipids, such as *N*-stearoylethanolamine (SEA) and *N*-linoleoylethanolamide (LEA), remain poorly explored due to the lack of established receptor targets which makes it challenging to elucidate their precise biological effects. Besides eCB-like NAEs, other *n*-3/*n*-6 eCB derivatives have been identified but their biochemistry is not yet fully understood [69].

AEA metabolism

AEA is currently known to be synthesized *via* four routes. The principal biosynthetic pathway involves one-step hydrolysis of AA-containing *N*-acyl-phosphatidylethanolamines (NAPEs) by NAPE-specific phospholipase D (NAPE-PLD). Importantly, studies in rodents have shown that the deletion of NAPE-PLD leads to a marked reduction in AEA, underscoring its key role in AEA biosynthesis [63,70]. The second pathway entails the hydrolysis of NAPE by phospholipase C (PLC) to phospho-AEA, which is later dephosphorylated by several phosphatases to yield AEA. The third pathway involves the generation of lyso-NAPE by Phospholipase A₂ (PLA₂), followed by hydrolysis of lyso-PLD to AEA. Finally, the last pathway employs the hydrolysis of lyso-PLD through the α/β Hydrolase Domain-Containing Protein 4 (ABHD4) enzyme to produce AEA.

On the other hand, AEA degradation involves only a limited number of enzymes. The primary enzyme responsible for AEA degradation is the fatty acid amide hydrolase (FAAH), often also named FAAH-1, which breaks down AEA into ethanolamine and AA. Consistently, FAAH-deficient mice show ~15 folds increase in AEA, highlighting the important contribution of FAAH in the degradation of AEA [71]. Another isoform of FAAH, known as FAAH-2, is documented to be expressed in humans but not in mice. Similar to FAAH-1, FAAH-2 has been reported to directly hydrolyze AEA. Intriguingly, FAAH-2 seems to primarily promote the degradation of other NAEs rather than that of AEA. Lastly, *N*-acylethanolamine acid amidase (NAAA) has been identified as a significant contributor in hydrolyzing AEA and it is known to have higher activity toward saturated NAEs (e.g. PEA) rather than unsaturated NAEs (e.g. AEA) [63,70].

2-AG metabolism

The biosynthesis of 2-AG primarily occurs through the hydrolysis of DAGs which are in turn obtained either by PLC mediated hydrolysis of glyceryl-phosphatidyl-inositol or through the enzymatic breakdown of phosphatidylcholines via PLD/phosphatidic acid phosphatase pathway. The resulting DAGs are then hydrolyzed through DAG lipases (DAGL), namely DAGL α/β . An alternative pathway involves the enzymatic activity of lysophosphatidic acid phosphatase on arachidonoyl-lysophosphatidic acid to generate 2-AG [63,70]. Regarding the degradation of 2-AG, in the brain 85% of 2-AG is hydrolyzed by monoacylglycerol lipases (MAGLs), whereas ABHD6 and ABHD12 account for about 9% and 4% of 2-AG degradation respectively [72]. The hydrolysis of 2-AG leads to the production of AA and glycerol [73]. Apart from these classical pathways, in human leukocytes, a novel biosynthetic pathway for 2-AG has recently emerged: it involves the acylation of AA into phospholipids [74]. This new pathway has been documented to synthesize other unsaturated monoacylglycerols (MAGs), but the existence and function of this pathway in other cells and tissues remain unexplored.

eCB receptors

eCBs bind to selective receptors to exert their biological effects. The key eCB receptors include Cannabinoid receptor 1 (CB₁R), Cannabinoid receptor 2 (CB₂R), TRPV1 and nuclear PPAR $\alpha/\delta/\gamma$. Among these, CB₁R and CB₂R are known to be the principal eCB receptors. eCBs AEA and 2-AG are both ligands for CB₁R and CB₂R but they exhibit different binding properties. AEA acts as a full agonist of CB₁R and a partial agonist of CB₂R [75], whereas 2-AG is a full agonist of both the receptors and is widely regarded as the primary eCB responsible for activating canonical receptors CB₁R and CB₂R [76]. CB_{1/2}R belong to a large family of heptahelical G-protein coupled receptors (GPCRs) that activate G_{i/o} proteins. Upon activation, these GPCRs inhibit adenylyl cyclase, leading to the reduction of cyclic adenosine monophosphate (cAMP) levels. This initiates various downstream signalling processes such as gene transcription, cell motility, synaptic functions and other important cellular and molecular processes [77]. Moreover, the expression of canonical eCB receptors vary within the different tissues. CB₁R is mainly found in CNS, whereas CB₂R is largely distributed in the peripheral and immune system [62]. However, growing evidence has pointed out critical roles for these receptors that extend beyond these conventional locations [78]. Besides CB_{1/2}R, non-selective cation channels known as TRP channels have been recognized as additional

eCB receptor targets. Among them, TRPV1 is the most studied receptor. This is because TRPV1 receptor's role in pain and inflammatory responses had been well established before it was discovered as an eCB receptor target. Structurally, TRPV1 is a six transmembrane domain channel containing pore-forming hydrophobic chains between the fifth and the sixth domains. This structural configuration enables TRPV1 to act as a sensor, allowing it to be activated by capsaicin, noxious stimuli such as heat, low P_H and various lipids [79]. TRPV1 is predominantly expressed in peripheral nerve endings where it contributes to the thermal and nociceptive sensations. Additionally, TRPV1 was found to be detected in various cell types such as immune cells, myocytes, adipocytes, pancreatic cells where they are primarily involved in regulating inflammation and metabolic functions. Activation of TRPV1 leads to downstream influx of second messengers (such as calcium) to the intracellular space, contributing to multiple intracellular processes [80]. Other eCB receptors include PPAR α , PPAR β/δ and PPAR γ , which belong to the PPARs family of heterodimeric transcription factors [81]. When activated, the ligand-receptor complex binds to specific DNA sequences called peroxisome proliferating response elements (PPRE) within the promotor regions of the target genes. Consequently, PPARs are known to be implicated in immunity, metabolism and other cellular processes. Many exo-and endogenous lipid molecules including eCBs, phytocannabinoids and other eCB-like congeners are known to bind PPARs. This complex crosstalk controls important biological processes in the human body [82]. PPAR isoforms are either co-expressed or selectively expressed in certain tissues which enable them to regulate various signalling processes according to tissue-specific physiological contexts. For example, PPAR α is present at higher levels in liver, adipocytes, endocrine, cardiac and muscle tissues where its primary function is to mediate fatty acid oxidation and lipid catabolism [83,84]. PPAR γ is abundantly expressed in tissues with high metabolism such as adipocytes but its presence was also noted in other tissues such as heart, lung and brain, where it plays a key role in adipogenesis and glucose metabolism [85]. PPAR β/δ is reported to be expressed ubiquitously and its expression is generally found to be higher in the brain due to its extensive role in energy expenditure and fatty acid metabolism [84].

1.5 The ECS in cochlear physiology and pathology

To date, the role of the ECS in cochlear physiology and pathology remains poorly investigated. In fact, most of the studies are focused only on a few eCB receptors, particularly CB $_1$ R, CB $_2$ R and TRPV1, which are reported to be expressed in both postnatal and adult mouse cochlea [59,86–88].

Figure 3 shows the currently known distribution of ECS elements in adult cochlea from gene and protein expression studies. The presence of the ECS receptors within the cochlea is fundamental for correct auditory processing. Indeed, CB₁R knock-out mice exhibited impaired auditory brain stem responses (ABRs) [89] and the genetic ablation of CB₂R led to loss of ribbon synapses in mice [90]. Within auditory pathological contexts, such as tinnitus and drug-induced ototoxicity, the ECS seems to play a critical role. In fact, it was demonstrated that the activation of cannabinoid receptors exerts detrimental effects and may even worsen tinnitus in rodents [91,92]. Conversely, another study showed that administration of dronabinol, the (-)-*trans* isomer of tetrahydrocannabinol (Δ -9-THC) rescued tinnitus pathology [93]. These studies indicate that eCB receptor activation may be implicated in tinnitus; however, the data are inconclusive highlighting the need for further research to clarify the role of ECS in tinnitus. With regard to drug-induced ototoxicity, eCB receptors CB₂R and TRPV1 have emerged as primary targets of investigation. Recent studies demonstrated that selective targeting of CB₂R and TRPV1 in rats prevented cisplatin-induced hearing loss [94,95]. These preliminary data reveal eCB receptors as promising pharmacological targets to treat cisplatin-induced hearing loss for which currently no effective clinical treatments exist. Furthermore, following kanamycin exposure, gene and protein levels of TRPV1 and TRPV4 channels were found to be altered [96]. Additionally, PPAR receptor agonists have also been reported to show otoprotective effects against gentamicin-induced ototoxicity [97]. These studies suggest that both canonical and non-canonical receptors may serve as potential targets for mitigating drug-induced ototoxicity. However, the direct effects of ototoxic drugs on these receptors' expression and localization are not adequately addressed. Moreover, the role of receptors such as CB₂R in otoprotection is unclear as literature reports contradicting data on their involvement in ototoxicity [88,94]. Hence these conflicting findings underscore the need for further investigation to clarify the role of eCB receptors in ototoxicity.

Compared to the ECS receptors, metabolic enzymes are underexplored in the context of auditory physiology and pathology. Gene expression of some major eCB metabolic enzymes in the inner ear have been demonstrated; however, the studies have been performed only in embryonic otic vesicles and early postnatal stages (P7) of the sensory epithelia. AEA and 2-AG metabolic enzymes like *faah*, *magl*, *dagl* and *abhd* class of enzymes are expressed in different cell types of the cochlea [59]. It is important to note that at these stages the cochlea is not fully developed. While the transcript expression has been observed at these pre-mature cochlear stages, it remains unclear

whether their expression persists in adulthood. Among the metabolic enzymes, *abhd12* has received significant attention. This is because genetic mutations in this enzyme were first linked to neurodegenerative disorder polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC) syndrome [98]. Furthermore, *abhd12* knockout mice models showed aberrant ABR latencies and an abnormal response in acoustic startle tests thereby delineating the importance of *abhd12* gene in normal auditory processing [98]. Besides these studies, no functional data is available regarding metabolic enzymes of the ECS.

Given the abovementioned limitations, there is a critical need for comprehensive, cell type specific and mechanistically resolved studies on the ECS in auditory physiology and pathology, particularly within mature cochlea.

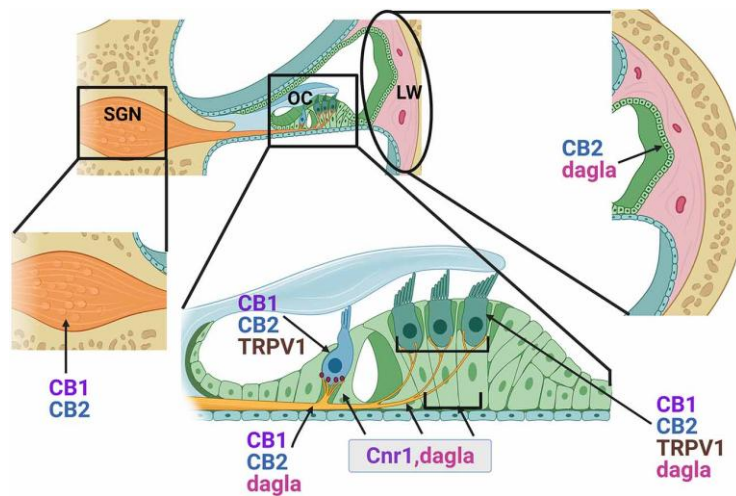


Figure 3. The distribution of the known ECS elements in the adult mouse cochlea [59]

2. Objectives of the study

Current evidence from literature suggests that targeting ECS may serve as a promising therapeutic approach in cisplatin-induced ototoxicity; however, the available data are limited to a few fragmented studies. Importantly, to critically investigate the role of the ECS in pathological scenarios, it is crucial to dissect its presence and relevance in physiological conditions, an aspect that remains unaddressed to date. In light of these knowledge gaps, the aim of the present thesis is to carry-out a comprehensive characterization of the ECS in cochlear HCs and evaluate the effects of ototoxic compounds on the ECS using *in vitro* and *in vivo* models. The main objectives of the thesis are listed below:

1. To identify and characterize ECS receptors, metabolic enzymes and lipids in the auditory UB/OC1 HC-like cell line using western blotting and targeted lipidomics;
2. To dissect the potential alterations of the ECS upon cisplatin-induced ototoxicity *in vitro* using UB/OC1 cells through western blotting and targeted lipidomics;
3. To investigate potential alterations of the ECS *in vivo* using a C57BL/6 cisplatin-furosemide mouse model, through immunohistochemistry.

3. Materials and Methods

3.1 Cell culture and treatments

Immortalized mouse OC-derived UB/OC1 cells were purchased from Cancertools.org. The cells were cultured in minimal essential medium (MEM) with Earl's salts which were supplemented with 10% fetal calf serum (Cytiva), 1% glutamine (Sial, cat. no. SIAL-L-Glu), 50 U/ml γ -interferon (Pepro Tech, cat. no. 315-05). The cells were grown at 33°C in an incubator with 5% CO₂. The cells were sub-cultured using Trypsin 1X (Euroclone, cat. no. ECB3052D).

3.2 *In vitro* model of cisplatin-induced ototoxicity

To establish the *in vitro* model of cisplatin-induced ototoxicity, cells were seeded at a density of 25000 cells/cm² in a 96-well plate for 24 hours and then treated with a range of concentrations (2.5, 6.25, 12.5, 25 and 50 μ M) of cisplatin (Sigma Aldrich, cat. no. P4394). 0.2 mg/ml of cisplatin solution was first prepared as a stock in physiological solution (0.9% sodium chloride) and then was diluted to the desired concentrations in complete culture medium. The concentrations and the treatment duration were decided based on previously established studies on the same cell line [39,99–101].

3.3 Cell viability assays

To evaluate cell viability, classical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. To this end, cells were seeded on a 96-well plate at a density of 25000 cells/cm² and cultured for 24 hours. Cells were then exposed to cisplatin/vehicle treatments. 5 mg/ml of MTT (Merck, cat. no. M2128-1G) in 0.1 M phosphate buffered saline (PBS1X) was added to the cells, and they were incubated for 2 hours at 33°C. The reaction was stopped using 1:1 ratio of acidified isopropanol (2-propanol-0.04 N HCl) to cells, and the plate was immediately read at 570 nm and 630 nm (reference absorbance) with a TECAN Magellan Pro v7.4 microplate reader (Promega; cat. no. 30172786). Cell viability was then measured by subtracting the 630 nm background OD from the 570 nm and then expressed as folds over control. Half-maximal inhibitory concentration (IC₅₀) of cisplatin at 30 μ M was then extrapolated by linear regression analysis of MTT data and was used as a suitable dose for further experiments. Since the MTT assay measures the metabolic activity of cells, it is considered an indirect indicator of cell viability because the reduction of MTT to formazan crystals occurs only in metabolically active cells with functional mitochondria. Therefore, Trypan blue (Sigma-Aldrich, cat no. T8154) dye exclusion

test was performed to accurately assess the number of live or dead cells. Briefly, 0.1% trypan blue was used at a ratio of 1:2.5 to stain cell suspensions; then, the counting of live and dead cells was performed manually using a hemocytometer. Trypan blue positive cells were counted as dead cells, while unstained cells were counted as live cells. Cell viability was expressed as folds over vehicle.

3.4 Animals

Adult males and females aged 8-12 week C57BL/6J mice (Charles River Laboratories) (RRID:SCR_003792) were used for the study. Animals were housed in individually ventilated cages (IVC) with food and water under standard laboratory conditions at UMC Utrecht. All animal experimental procedures were approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD: AVD11500202418278). The number of males and females was randomized for the study. All investigations were conducted in compliance with the ARRIVE guidelines including the 3R principle (Replacement, reduction and refinement).

3.5 Animal model of cisplatin-induced ototoxicity

The mouse model of cisplatin-induced ototoxicity was established and adapted from [102], by performing a dose-response protocol and different routes of administration. A pilot study was established for optimizing dosage and routes of administration to find a right balance between ototoxic threshold *versus* overall survival, which is critical due to systemic toxicity of cisplatin [103]. Hydration support was provided by administering lactated ringers' solution for 3 days after cisplatin treatment. This supportive care was given to maintain electrolyte balance and support renal functions. Animals were weighed throughout the experiment to monitor their health. 19 mice in total were included in the study and randomly assigned to the following experimental groups (Figure 4).

Group 1- 4 mice were injected with cisplatin 1 mg/kg (intraperitoneally i.p.) and furosemide 100 mg/kg (intravenously i.v.);

Group 2- 4 mice were injected with cisplatin 1 mg/kg (i.p.) and furosemide 200 mg/kg (i.p.);

Group 3- 4 mice were injected with cisplatin 2 mg/kg (i.p.) and furosemide 100 mg/kg (i.v.);

Group 4- 7 mice were injected with cisplatin 2 mg/kg (i.p.) and furosemide 200 mg/kg (i.p.)

Group 4 was selected as the best model for subsequent investigations, as detailed in the “Results” section.

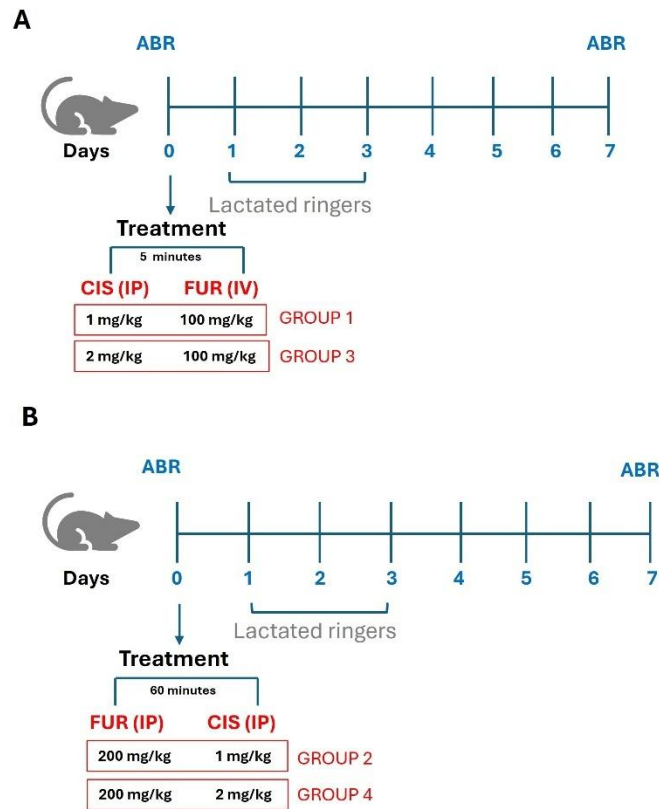


Figure 4. Schematic illustration of the experimental design established to induce cisplatin-induced ototoxicity in mice. Schematic representation of the dosage regimen for groups 1 and 3 **(A)**. Schematic representation of the dosage regimen for groups 2 and 4 **(B)**. Abbreviations: ABR: Auditory brainstem response; CIS: cisplatin; FUR: Furosemide; IP: intraperitoneal; IV: intravenous

3.6 Click-evoked auditory brainstem responses (ABRs)

ABRs were performed as described previously [104] to confirm normal hearing (NH) before the start of the experiments and at endpoint of the ototoxicity protocols. Briefly, animals were maintained under anesthesia using isoflurane in a soundproof and an electrically shielded box (52 × 34 × 28 cm). The animals were placed in a heating pad throughout the procedure. Subdermal needle electrodes (27 GA needle, 13mm) were placed behind the right pinna (active), on the skull (reference) and hind limb (ground). A TDT3 system (Multi-I/O processor RZ6; Tucker-Davis Technologies, Alachua, FL, USA) was used to generate acoustic stimuli of 20 μ s-monophasic clicks. The acoustic stimuli were presented using a speaker (Bowers and Wilkins, CCM683; 8 Ω ; 25–130 W) kept at 5 cm from the left ear. The signals were pre-amplified using a 5113-pre-

amplifier (Princeton Applied Research, Oak Ridge, TN, USA) with an amplification $\times 5,000$ and a band pass filter of 0.1–10 kHz. The digitized signals from the TDT3 system were then used for analyses (100 kHz sampling rate, 24-bit sigma-delta converter). The responses were then analyzed using MATLAB software. Stimulus intensity was applied in 10 dB attenuation steps starting with maximum sound level at approximately 105 dB peak equivalent SPL (equals 0 dB in attenuation) and ABRs were recorded until there were no responses. The threshold criteria were determined by the interpolated sound level, where the amplitude of the largest wave measured was 0.3 μV . ABR waveforms were identified by characteristic peak-trough pattern. Amplitudes and latencies of waves I and II were measured since they were the most prominent and consistent waveforms in our measurement [105]. In addition, they are commonly used to assess hearing impairments in animal models [106,107]. A threshold shift of >10 dB was employed as the criterion for indicating the onset of functional hearing impairment, as previously described [101,108]. Responders and non-responders of the ototoxic drugs in the cohort were classified based on this criterion. All ABR analyses were blinded, and groups were disclosed only after the completion of the analyses. Representative ABRs recordings from NH animals are provided below in Figure 5.

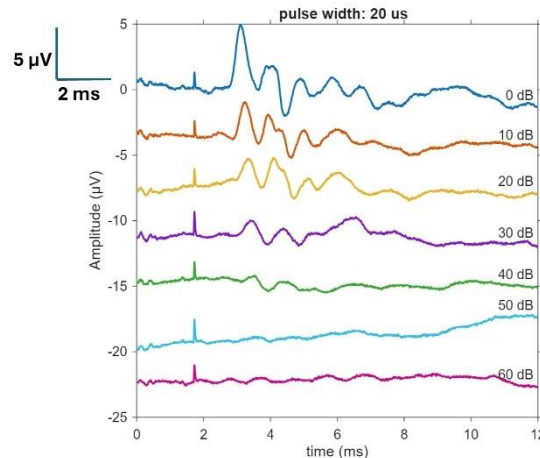


Figure 5. Representative ABR waveforms of NH animals. Abbreviations: ABR: Auditory brainstem response; NH: normal hearing.

3.7 Protein extraction and Western blotting

Western blotting was used to quantify proteins of interest in the *in vitro* experiments. To this end, cells were cultured in 75 cm² culture flasks. Cell pellets were lysed using a lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.1% SDS, EDTA 5 Mm, Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific, Waltham, MA, USA and QS dH₂O). The lysis was

carried out on ice for 30 minutes and then centrifuged at 13200 rpm for 20 minutes at 4°C. The supernatant (proteins) was then stored at –80°C. Quantification of the proteins was performed using the Bradford (BioRad, cat. no. 5000006) assay. Tissues of mouse brain, liver, and heart were used as positive controls for appropriate proteins, and the extraction of these proteins was performed as mentioned above.

Afterwards, 60 µg of proteins from vehicle and cisplatin treated cell lysates were loaded on Bolt 4-12% Bis-Tris Plus gels (ThermoFisher Scientific, cat. no. NW04120BOX) and was transferred using iBlot™ 3 Transfer Stacks, midi, PVDF (Thermo Fisher Scientific, cat. no. IB34001) on an iBlot™ 3 dry transfer system. Blocking was performed using 5% non-fat dry milk. The membranes were then incubated with suitable primary antibodies (listed in Table 1) in 5% non-fat dry milk (PanReac AppliChem ITW Reagents, cat. no. A0830), except for proteins PPAR γ , DAGL β , NF- κ B, Lamin A/C, NLRP3, GSDMD, ASC and caspase-1 for which 5% Bovine serum albumin (BSA) was used. Afterwards, membranes were incubated with specific HRP-conjugated secondary antibodies (anti-rabbit, anti-mouse or anti-goat; Table 2), diluted in 5% non-fat dry milk in TBST. Super Signal West Pico Plus (Thermo Fisher Scientific Thermo Fisher, cat. no. 34580) or Super Signal West Atto Ultimate Sensitivity (Thermo Fisher Scientific Thermo Fisher, cat. no.38555) chemiluminescent substrates were used to develop the membranes and the detection was performed through a ChemiDoc XRS plus imaging system (Bio-Rad Laboratories; RRID:SCR_019690). Image J software (RRID:SCR_003070) was then used to quantify bands' densitometry.

PRIMARY ANTIBODY	SUPPLIER AND CATALOG NUMBER	HOST SPECIES, DILUTION AND DILUTION MEDIUM	CHEMILUMINESCENT SUBSTRATE USED
CB ₁ R	Abcam; ab259323	Rabbit; 1:1000	Super Signal West Pico Plus
CB ₂ R	Cayman Chemical; 101550	Rabbit; 1:200	Super Signal West Pico Plus
TRPV1	OriGene; TA336871	Rabbit; 1:1000	Super Signal West Pico Plus
PPAR α	Sigma Aldrich; SAB4502260	Rabbit; 1:500	Super Signal West Pico Plus
PPAR δ	Invitrogen; PA1-823A	Rabbit; 1:500	Super Signal West Atto Ultimate Sensitivity
PPAR γ	Cell Signaling; 2443	Rabbit; 1:500	Super Signal West Atto Ultimate Sensitivity
NAPE-PLD	Cayman Chemical; 10305	Rabbit; 1:200	Super Signal West Atto Ultimate Sensitivity
ABHD 4	Invitrogen; PA5-75867	Rabbit; 1:500	Super Signal West Pico Plus
FAAH	Abcam; Ab-54615	Mouse; 1:500	Super Signal West Atto Ultimate Sensitivity
NAAA	Invitrogen; PA5-48061	Goat; 1:500	Super Signal West Atto Ultimate Sensitivity
DAGL α	Santa Cruz; SC-390409	Mouse; 1:100	Super Signal West Atto Ultimate Sensitivity
DAGL β	Cell Signaling; 12574 (D4P7C)	Rabbit; 1:1000	Super Signal West Atto Ultimate Sensitivity
MAGL	Abcam; Ab24701	Rabbit; 1:200	Super Signal West Atto Ultimate Sensitivity
ABHD 6	Invitrogen; PA5-101396	Rabbit; 1:500	Super Signal West Atto Ultimate Sensitivity
ABHD12	Antibodies.com; ABIN3183122	Rabbit; 1:500	Super Signal West Atto Ultimate Sensitivity
GAPDH	Invitrogen; MA1-16757	Mouse; 1:1500	Super Signal West Pico Plus
Myosin VII A (Myo7a)	Proteus Biosciences; PTS-25-6790-C050	Rabbit; 1:200	Super Signal West Atto Ultimate Sensitivity
NF- κ B	Cell Signaling; 8242	Rabbit; 1:1000	Super Signal West Pico Plus

α/β Tubulin	Cell Signaling; 2148S	Rabbit; 1:1000	Super Signal West Pico Plus
NLRP3	Cell Signaling; 15101	Rabbit; 1:500	Super Signal West Atto Ultimate Sensitivity
ASC	Cell Signaling; 67824 (D2W8U)	Rabbit; 1:1000	Super Signal West Atto Ultimate Sensitivity
GSDMD	Cell Signaling; 39754 (E9S1X)	Rabbit; 1:1000	Super Signal West Atto Ultimate Sensitivity
Caspase-1	Cell Signaling; 83383	Rabbit; 1:500	Super Signal West Atto Ultimate Sensitivity
Lamin A/C	Cell Signaling; 4777S	Mouse; 1:1000	Super Signal West Pico Plus
Caspase-3	Cell Signaling; 9662S	Rabbit; 1:1000	Super Signal West Pico Plus

Table 1. List of primary antibodies used for Western blotting

SECONDARY ANTIBODY	SUPPLIER AND CATALOG NUMBER	HOST SPECIES AND DILUTION
Anti-Rabbit IgG (H+L) HRP	Invitrogen; 31460	Goat 1:10000
Anti-Mouse IgG (H+L) HRP	Invitrogen; 31430	Goat 1:10000
Anti-goat HRP	Antibodies.com; AI7358	Rabbit 1:5000

Table 2. List of secondary antibodies used for Western blotting

3.8 Nuclear/cytoplasmic fractionation

Nuclear/cytoplasmic protein pools were extracted from cell pellets to evaluate the activation status of NF- κ B [109]. Briefly, fresh cell pellets from cisplatin and vehicle treated cells were collected and the nuclear/cytoplasmic protein pools were separated using NE-PER kit (ThermoFisher, cat. no. 78833) following the manufacturer's instructions. 30 μ g of these protein extracts were then subjected to Western blotting as described above. α/β tubulin and Lamin A/C markers were used as cytoplasmic and nuclear housekeeping proteins respectively.

3.9 Interleukin (IL)-1 β Enzyme-linked immunosorbent (ELISA) assay

Released IL-1 β was measured *in vitro* using a mouse IL-1 β ELISA kit (Invitrogen, cat. no. 88-7013-22). The absorbance was read at 450 nm and 570 nm (reference absorbance) using a TECAN Magellan Pro v7.4 microplate reader (Promega, cat. no. 30172786). The final absorbance of the

samples was calculated by subtracting the absorbance values obtained at 450 nm from the reference absorbance. Then, the standard absorbances were plotted as a logarithmic plot using a curve fitting software CurveExpert 1.4 (Hyams Development).

3.10 Ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS)

Targeted lipidomic of eCBs and eCB-like lipids was performed using a new UHPLC-MS/MS method with slight modifications [110]. To have sufficient sample for reliable lipid detection, 1×10^6 cells were used. Briefly, samples were homogenized and extracted by adding 125 μ L of MeOH and 200 mM formic acid to a Precellys 24 tissue homogenizer (Bertin, Montigny-le-Bretonneux, France). To standardize the analysis, internal standards (AEA-d4, PEA-d4, 2-AG-d8) were spiked into the extraction solvent at a final concentration of 5 ng/mL. Before UHPLC-MS/MS analysis, a sample clean-up step was performed employing OMIX C18 tips from Agilent Technologies (Santa Clara, CA, USA). The instrumentation used for UHPLC-MS/MS was a Waters UPLC Acquity H-Class (Waters, Milford, MA, USA), coupled with a Qtrap 4500 from Sciex (Toronto, ON, Canada). Electrospray ionization mode (ESI) was achieved using a Turbo V source working as an ESI source and operating in a positive mode (ESI+). The analytes were separated by using a Waters Acquity BEH C18 column (100×2.1 mm, packed with 1.8μ m particles), with H₂O and acetonitrile:methanol 50:50 (v:v) - both containing 0.01 % formic acid - in mobile phase. Lipid levels were then expressed as pmol/ 10^6 cells.

3.11 Tissue processing and histology

For histological analysis of cochlear tissues, the otic capsule containing the cochlea was collected immediately after euthanizing the animal and fixed with paraformaldehyde (PFA) 4% (Sigma-Aldrich, cat. no. 100496) for 48 hours at 4°C. Once the fixation was complete, the samples were transferred to a decalcification solution containing 0.12 M EDTA (Invitrogen Cat.no 15575020) in PBS1X and maintained in slow agitation for 7-9 days at 4°C. Cryopreservation of the specimens was performed by stepwise sucrose (Sigma-Aldrich, cat. no. S7903) gradients of 5%, 15% and 30% for one day each. Afterwards, they were processed for mid-modiolar cryosectioning by freezing the decalcified tissue embedded Tissue Tek OCT (VWR, cat. no. 361603E) and stored at -20°C until sectioning. Mid-modiolar sections of 10 μ m were obtained using a Leica CM1850 cryostat (Nussloch, GmbH, Germany) and mounted on coated Superfrost-PLUS slides for immunostaining.

3.12 Immunofluorescence staining and confocal imaging

Cells

UB/OC1 cells were seeded on cover slips in a 24 well plate for 24 hours. Cisplatin and vehicle treatments were performed as described above. After treatments, cells were fixed with PFA 4% (Sigma-Aldrich, cat. no. 100496). The blocking step was performed using BSA 5% for 1 hour at RT. Afterwards, cells were incubated with primary antibodies (Table 3) overnight at 4°C.

PRIMARY ANTIBODY	SUPPLIER AND CATALOG NUMBER	DILUTION
Myosin VII A (Myo7a)	Proteus Biosciences; PTS-25-6790-C050	1:200
CB ₂ R	Cayman Chemical; 101550	1:50
DAGL β	Invitrogen; PA5-26331	1:50
ABHD 6	Invitrogen; PA5-101396	1:100

Table 3. List of primary antibodies used for immunofluorescence of UB/OC1 cells

The following day, cells were washed 3 times with PBS 1X and subsequently incubated with the secondary antibodies (anti-rabbit IgG conjugated to red fluorescent dyes, 1:300) (Alexa Fluor goat-anti Rabbit 594, Invitrogen, cat. no. A11012) at 37°C for 2 hours. The nuclei were counterstained using Bisbenzimidazole nuclear dye (Hoechst). The coverslips were mounted on microscope slides using gelatin. The slides were then visualized using a TCS SP5 confocal microscope (Leica). Negative controls consisted of samples that underwent the same protocol as described above but were not incubated with primary antibodies. The parameters of gain and offset were set the same for treated and untreated conditions, and the red fluorescence was measured on manually selected regions of interest (ROI) and represented as an intensity profile. The ROI parameters were kept the same for both untreated and treated conditions.

Tissues

Immunofluorescence staining was performed to evaluate the alterations of the elements CB₂R, DAGL β and ABHD6 in the OC of the cochlea in control and cisplatin-treated animals. To this end, mid-modiolar sections were first allowed to thaw at RT for 20 minutes. Then, the sections were washed with PBS 1X for 5 minutes. Afterwards, unspecific bindings were blocked with suitable blocking buffers for each protein (see Table 4). After the blocking step, slides were incubated overnight with primary antibodies at 4°C (detailed in Table 4). The following day, slides were

washed and incubated with secondary antibodies (Alexafluor 488, Molecular Probes, Invitrogen, Carlsbad, CA, USA) (detailed in Table 4). The nuclei were counterstained using Bisbenzimidazole nuclear dye (Hoechst). Sections following the same procedure but incubated only with secondary antibodies were considered as negative controls. Slides were then imaged using a Stellaris 8 STED confocal microscope. A z-stack containing ~ 4-6 planes with a step size of 0.5 μm was set. The fluorescence intensity within the OC was assessed by selecting the Region of interest (ROI) and measuring the mean gray value using Image J software.

PROTEIN	BLOCKING SOLUTION	PRIMARY ANTIBODIES AND DILUTIONS	SECONDARY ANTIBODIES AND DILUTIONS
CB ₂ R	BSA (5%) + PBS-Triton (0.01%)	Anti-CB ₂ R, Cayman chemicals; 101550, 1:50	Goat anti-Rabbit Alexafluor 488, 1:300
DAGL β	Goat serum 10% +PBS-Triton (0.1%)	Anti-DAGL β , Invitrogen; #PA5-26331, 1:100	Goat anti-rabbit Alexafluor 488, 1:500
ABHD6	Goat serum 10% +PBS-Triton (0.1%)	Anti-ABHD6, Invitrogen; # PA5-101396, 1:100	Goat anti-rabbit Alexafluor 488, 1:500

Table 4. Blocking buffers, primary antibodies and secondary antibodies used in immunofluorescence staining on cochlear tissues

3.13 SiR-8 live cell confocal imaging

Sub-cellular localization of CB₂R in UB/OC1 cells was observed by using a CB₂R selective fluorescent probe (SiR-8) and live cell confocal imaging [111]. To this end, cells were seeded in an 8-well IBIDI chamber with 10000 cells/well for 24 hours. Cisplatin and vehicle treatments were performed as described above. After treatments, cells were immediately exposed to 0.8 μM of SiR-8 and a time-lapse imaging was performed using a TCS SP5 confocal microscope (Leica). Specifically, images were acquired for 20 minutes at an interval of 15 seconds each, maintaining the same parameters of gain and offset for untreated and treated conditions. The fluorescence intensity profile was obtained as described in Paragraph 3.12.

3.14 SR144528 treatment in UB/OC1 cells

Cells were seeded at a density of 25000 cells/cm² for 24 hours and treated with 2 μ M of SR144528 (Sigma Aldrich cat. no. SML1899) for 1 hour in culture medium without FCS [112,113]. A stock solution of 8 mM was first prepared in 100% Dimethyl sulfoxide (DMSO) and then was diluted to the desired concentration. The final DMSO concentration was 0.025%. Afterwards, cells were treated with IC₅₀ of cisplatin as previously described.

3.15 Data Visualization and Statistical analysis

All the obtained data were first tested for normality using Shapiro-Wilk test. Statistical analysis was performed using two-tailed paired/unpaired *t*-tests, one-way/two-way Analysis of Variance (ANOVA). Specific details of statistical approaches used for each experiment are provided in the respective figure legends. The data are reported as mean $\pm$ Standard error of mean (SEM). The number of animals used in the study was estimated using G*Power 3.1 software. All the analyses were performed with GraphPad prism 8 (GraphPad Software Inc., San Diego, CA, USA). Principal component analysis (PCA) was performed using MetaboAnalyst 6.0. Prior to analysis, data was log₁₀-transformed to reduce skewness and stabilize variance. Subsequently autoscaling (mean-centering and division by standard deviation) was performed to normalize the samples and ensure all samples contributed equally to the analysis. Statistical significance was considered as *p*<0.05. Any outliers in the data were determined through Grubb's test.

4. Results of the *in vitro* study

4.1 Identification of eCB receptors, metabolic enzymes and lipids in UB/OC1 cells

To perform an extensive characterization of the ECS in UB/OC1 cells, we profiled the expression of key receptor targets and metabolic enzymes at the protein level along with the levels of eCB and eCB-like lipids in the cell line. Western blot analysis revealed the presence of all principal receptor targets: CB₁R (Figure 6A), CB₂R (Figure 6B), TRPV1 (Figure 6C) and PPAR family of nuclear receptors (PPAR α / γ / δ) (Figure 6D, E, F). Subsequently, the expression levels of the enzymes involved in the metabolic pathways of AEA and 2-AG were investigated. The principal AEA biosynthetic enzyme NAPE-PLD was not detected in the cell line (Figure 6G), instead the secondary biosynthetic enzyme ABHD4 (Figure 6H) as well as the degradative enzymes FAAH and NAAA (Figure 6I, J) were found to be expressed. Regarding 2-AG pathway, both biosynthetic enzymes DAGL α / β (Figure 6K, L) were observed along with the degradative enzymes ABHD6

and ABHD12 (Figure 6N, O). However, the principal 2-AG degradative enzyme MAGL was absent (Figure 6M). All the above-mentioned components were investigated using mouse brain (B), liver (L) or heart (H) tissues as suitable positive controls for the ECS elements. The ECS elements which were not found to be expressed in the cell line (MAGL and NAPE-PLD) were not included in the subsequent analysis.

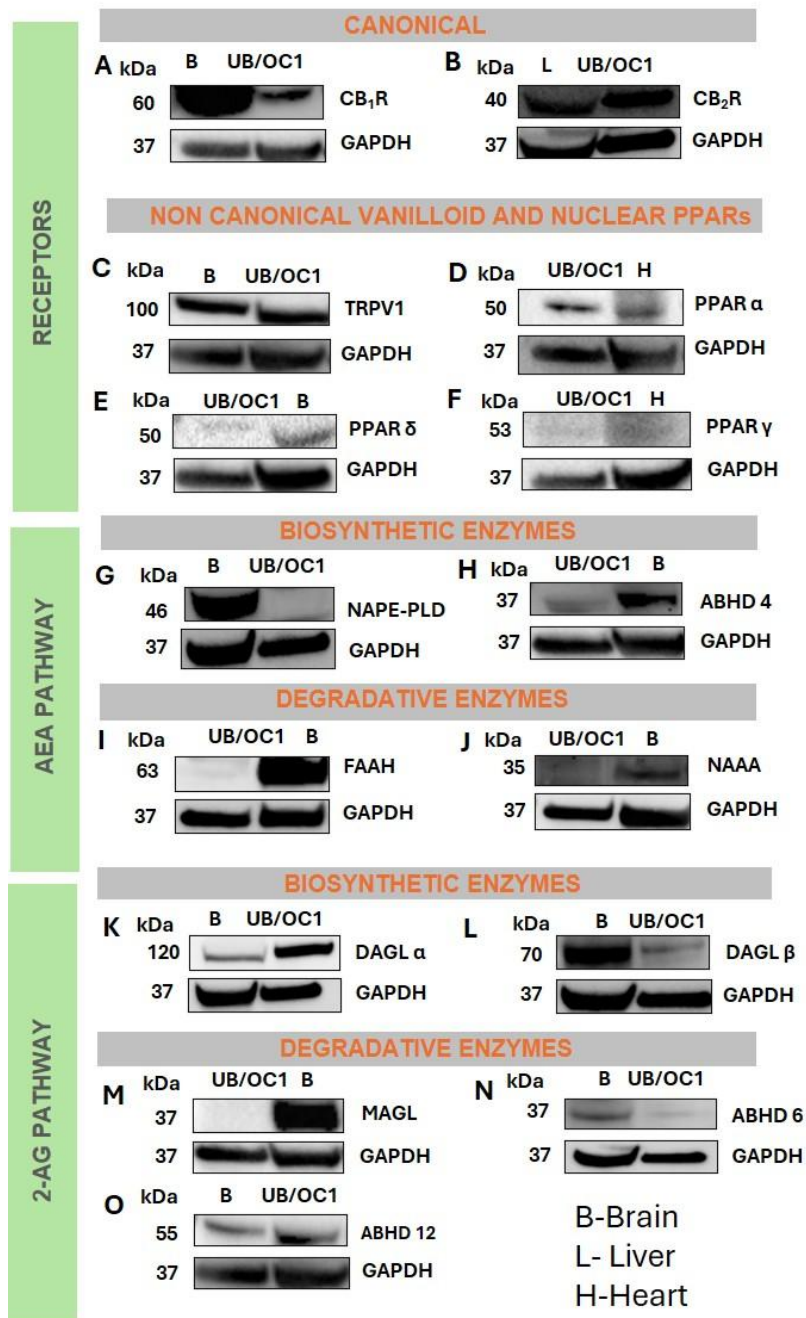


Figure 6. Identification of ECS receptors and metabolic enzymes at the protein level in UB/OC1 cells. Western blot bands of eCB receptors CB₁R (A), CB₂R (B), TRPV1 (C), PPAR α (D), PPAR δ (E), and PPAR γ (F). Western blot of the AEA biosynthetic enzymes NAPE-PLD (G) and ABHD4 (H), and of AEA degradative enzymes FAAH (I) and NAAA (J). Western blot bands of the 2-AG metabolic enzymes DAGL α (K), DAGL β (L) and MAGL (M), ABHD6 (N) and ABHD12 (O). Each protein is shown alongside positive controls with respective GAPDH housekeeping. Abbreviations: ABHD4/6/12: α/β hydrolase domain-containing protein; B: Brain; CB₁R: cannabinoid receptor 1; CB₂R: cannabinoid receptor 2; DAGL α/β : diacylglycerol lipases α and β ; FAAH: fatty acid amide hydrolase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; H: heart; L: Liver; MAGL: monoacylglycerol lipase; NAPE-PLD: *N*-acyl-phosphatidylethanolamines-specific phospholipase D; NAAA: *N*-acylethanolamine acid amidase; PPAR $\alpha/\gamma/\delta$: peroxisome proliferator-activated nuclear receptors α , γ , δ ; TRPV1: transient receptor potential vanilloid receptor 1. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

Upon characterization of eCB receptors and enzymes, the endogenous lipids were quantified using targeted UHPLC-MS/MS based lipidomics. Both major eCBs - AEA (~ 0.302 pmol/ 10^6 cells) and 2-AG (~ 1503.027 pmol/ 10^6 cells) (Figure 7) - were identified in the cell line along with some eCB-like lipids (Figure 8), such as PEA (~ 7.0160 pmol/ 10^6 cells), LEA (~ 2.301 pmol/ 10^6 cells), OEA (~ 10.722 pmol/ 10^6 cells), SEA (~ 4.403 pmol/ 10^6 cells), Palmitoleoyl ethanolamide (POEA) (~ 0.477 pmol/ 10^6 cells), and Eicosapentaenoyl ethanolamide (EPEA) (~ 0.353 pmol/ 10^6 cells). However, Docosahexaenoyl ethanolamide (DHEA) was not detected. Among all the lipids analyzed, 2-AG was found to be the most abundant. The individual levels of lipids in all four biological replicates are expressed as a heatmap in Figures 7B and 8B.

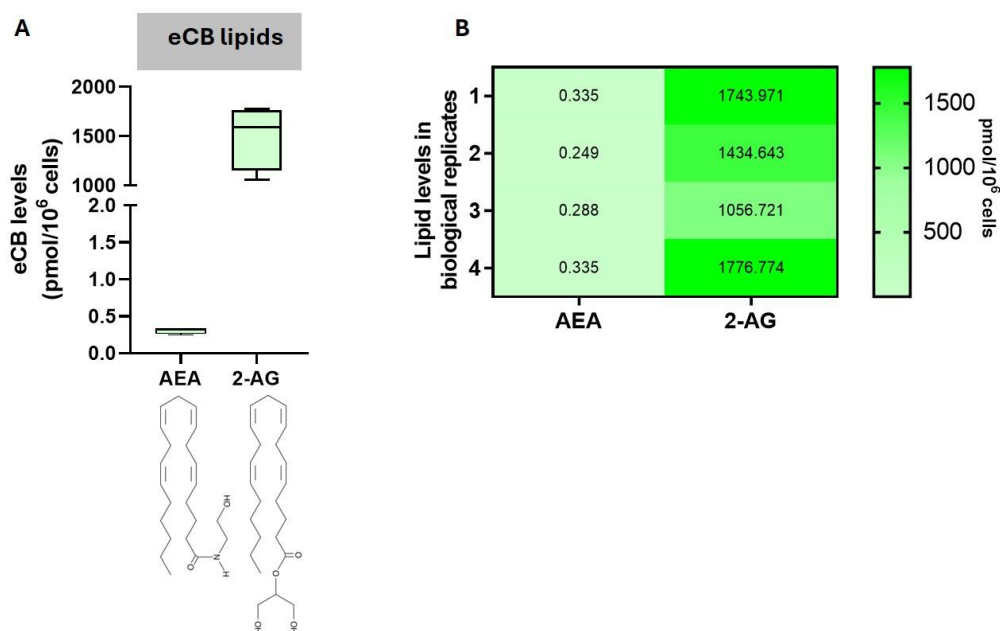


Figure 7. Quantification of AEA and 2-AG in UB/OC1 cells. Endogenous levels of AEA and 2-AG quantified by UHPLC-MS/MS. The data is presented as a box plot with whiskers ranging from min to max values; each eCB is shown with its chemical structure. **(A)**. Individual levels of eCBs in UB/OC1 cells for 4 biological replicates are represented as a heatmap **(B)**. Abbreviations: 2-AG: 2-arachidonoylglycerol; AEA: *N*-arachidonylethanolamine; eCBs: endocannabinoids. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0)

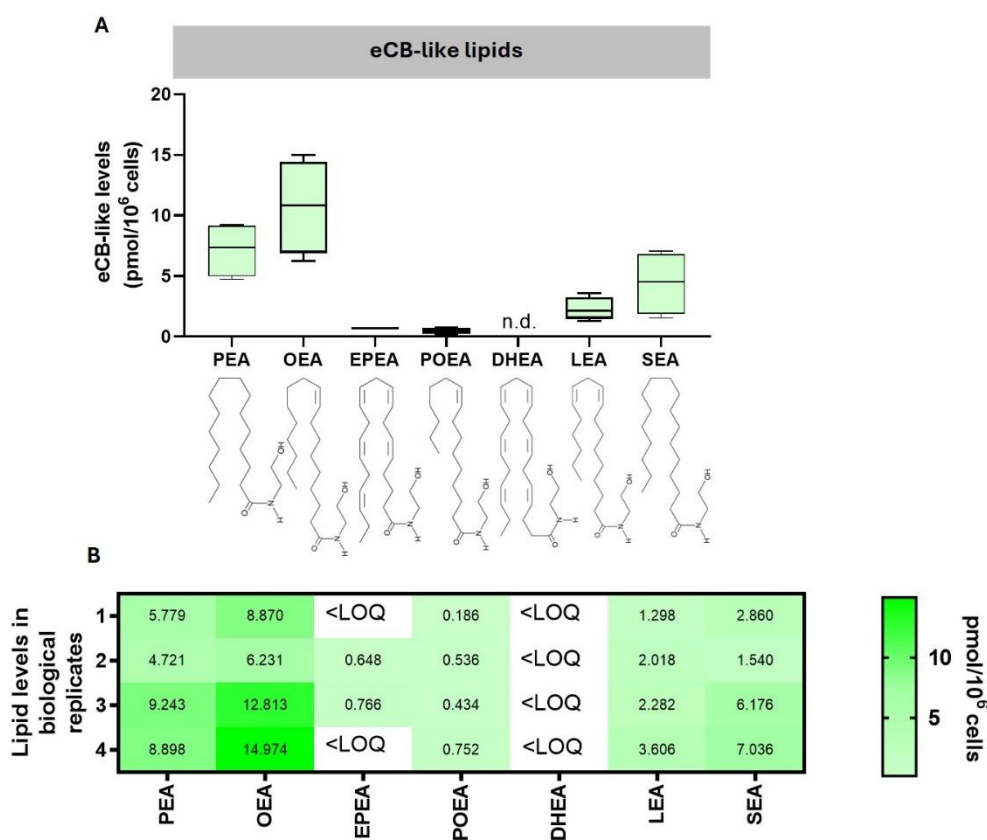


Figure 8. Quantification of eCB-like lipids in UB/OC1 cells. Levels of eCB-like lipids PEA, OEA, EPEA, POEA, DHEA, LEA and SEA quantified by UHPLC-MS/MS. The data is presented as a box plot with whiskers ranging from min to max values; each lipid is shown with its chemical structure. **(A)**. Individual levels of eCB-like lipids in UB/OC1 cells for 4 biological replicates are represented as a heatmap **(B)**. Samples in which the lipids were not detectable below the limit of quantification (LOQ) are represented as <LOQ. Abbreviations: DHEA: docosahexaenoylethanolamine; eCBs: Endocannabinoids; EPEA: N-epoxyeicosatetraenoylethanolamine; LEA: N-linoleoylethanolamine; OEA: N-oleoylethanolamine; PEA: N-palmitoylethanolamine; POEA: N-palmitoleoylethanolamine; SEA: N-stearoylethanolamine. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

4.2 Establishment of the *in vitro* model of cisplatin-induced ototoxicity

To investigate the effects of cisplatin on the ECS, we established an *in vitro* model of ototoxicity. MTT assay and trypan blue dye exclusion tests were used to determine cell viability. To this end, cells were treated with the following cisplatin concentrations for 24 hours: 2.5 μ M, 6.25 μ M, 12.5 μ M, 25 μ M and 50 μ M. Cells which were untreated are denoted as control and cells which were treated with physiological solution are denoted as vehicle. After cisplatin treatment, a significant dose-dependent decrease in the metabolic activity of UB/OC1 cells was observed with respect to control and vehicle-treated cells through MTT, as shown in Figure 9A. No differences in metabolic activity between control and vehicle-treated cells were observed and hence vehicle was chosen as the appropriate control for the study. The half-maximal inhibitory concentration (IC_{50}) of cisplatin was determined using MTT assay and was found to be 30 μ M (Figure 9B). The IC_{50} concentration was then used as the appropriate dose for further studies. Subsequently, the trypan blue exclusion test reported a significant decrease (0.5-fold over VEH; $p<0.0001$) in the number of live cells and a significant increase (3-fold over VEH; $p=0.0166$) in the number of dead cells in cisplatin-treated conditions compared to vehicle, complementing the MTT assay (Figure 9C). In support of the cytotoxic effect of cisplatin on UB/OC1 cells, Figure 9D reports phase contrast images of the cells treated with cisplatin showing abnormal morphology and pycnotic appearance compared to untreated cells.

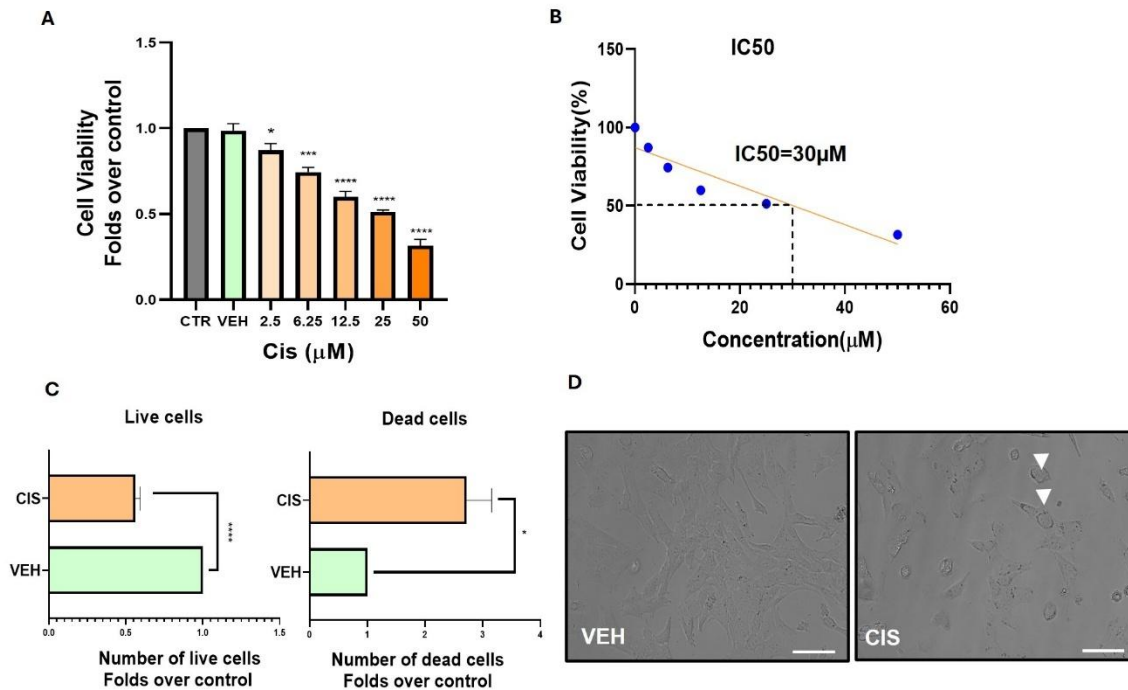


Figure 9. Cell viability assays upon cisplatin treatment in UB/OC1 cells. Cell viability of UB/OC1 cells after treatment with increasing concentrations of cisplatin measured by MTT assay. Statistical analysis was performed by one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs CTR). (A). IC₅₀ determination of cisplatin from MTT assay using linear regression analysis (B). Live and dead cell counts using trypan blue exclusion test in VEH and CIS - treated cells. Statistical analysis was performed using two-tailed unpaired Student's *t*-test (* $p < 0.05$, **** $p < 0.0001$ vs VEH) (C, D). Representative phase-contrast images of VEH and CIS treated cells (E), scale bar: 100 μm. Data is presented as mean ± SEM for three independent experiments. Abbreviations: CIS: cisplatin; CTR: control; IC₅₀: half-maximal inhibitory concentration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

Afterwards, to further strengthen the robustness of the established experimental paradigm as an *in vitro* model of ototoxicity, the selective HC marker Myosin 7a (Myo7a) was investigated. First, we confirmed the expression of Myo7a in the cell line using both western blotting and immunofluorescence staining (Figure 10A, B). Furthermore, Myo7a protein levels were found to be significantly reduced after treatment with cisplatin compared to vehicle (0.5-fold over VEH; $p = 0.0205$) (Figure 10A) which was also confirmed using immunofluorescence staining (Figure 10B).

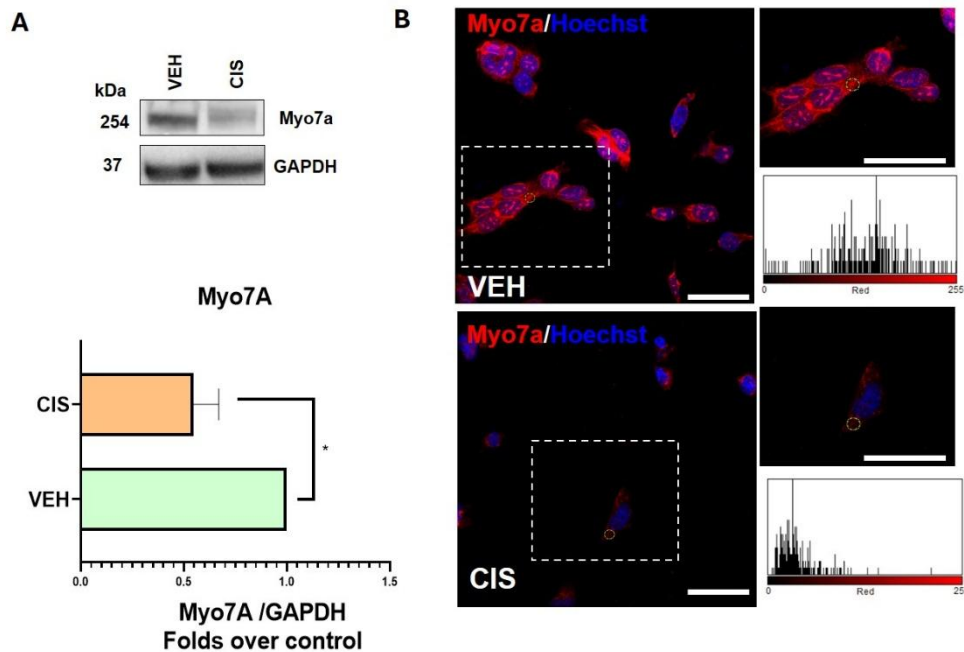


Figure 10. Assessment of Myo7a levels upon cisplatin-induced ototoxicity in UB/OC1 cells. Western blot bands of Myo7a with respective GAPDH housekeeping in VEH- and CIS-treated cells and their densitometric quantification. Statistical analysis was performed using two-tailed unpaired student's *t*-test (* $p < 0.05$ vs VEH) (A). Representative confocal microscopy images of Myo7a (red) counterstained with Hoechst nuclear dye (blue), scale bar: 50 μ m. White dotted boxes show zoomed-in views, and the corresponding histograms are representative of the circled areas. Data is presented as mean $\pm$ SEM for three independent experiments (B). Abbreviations: CIS: cisplatin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Myo7a: myosin 7a; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

4.3 Cisplatin induced the activation of NF- κ B and its downstream effector caspase-3 in UB/OC1 cells

Upon establishment of the *in vitro* model, possible cellular events characteristic of ototoxicity were assessed. To this end, we selected NF- κ B activation as a key marker. NF- κ B is a transcription factor involved in a plethora of processes including inflammation, cell survival and proliferation [115,116]. Moreover, NF- κ B activation is one of the key pathways involved in cisplatin-induced ototoxicity [117]. Canonical NF- κ B activation is indicated by the translocation of NF- κ B p65 from the cytoplasm to the nuclear compartment of the cells. Hence, the NF- κ B activation was evaluated

by analyzing its localization through immunofluorescence (Figure 11A) and western blotting (Figure 11B, C, D). Nuclear translocation of NF- κ B was observed only in cisplatin-treated cells using both techniques, suggesting the activation of NF- κ B-mediated signalling pathways in our model.

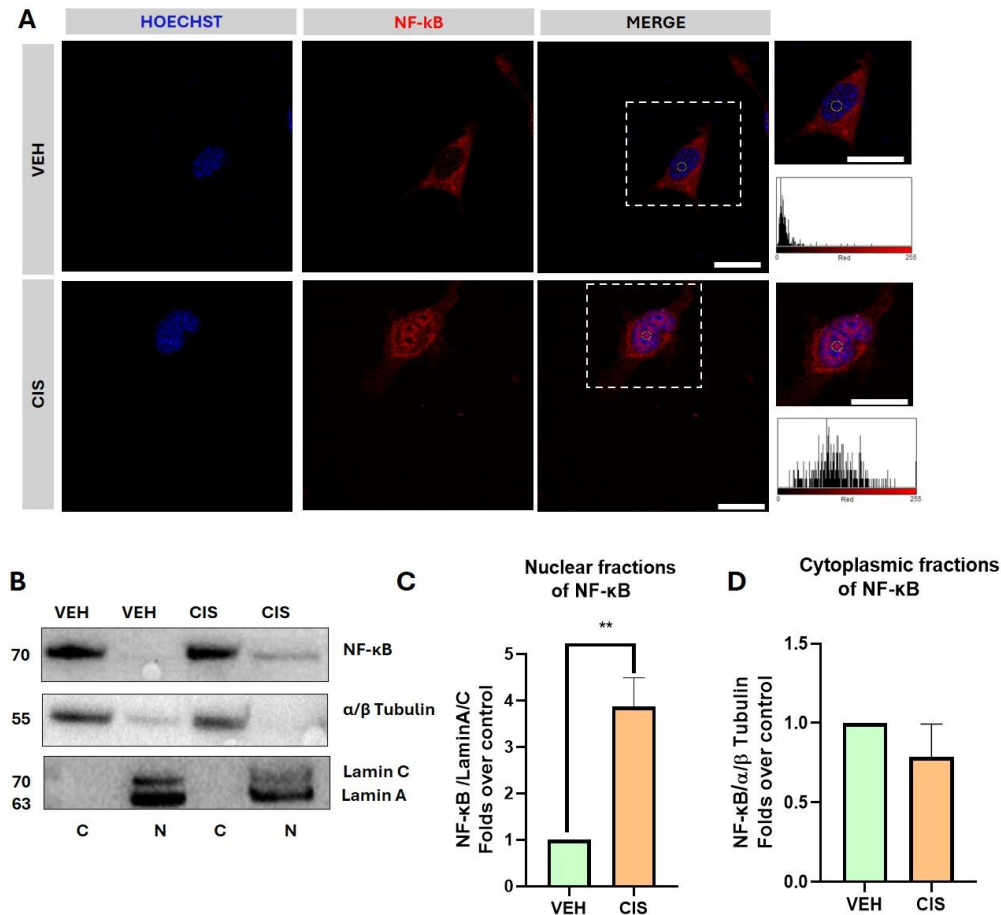


Figure 11. NF- κ B activation in UB/OC1 cells upon cisplatin-induced ototoxicity. Representative confocal microscopy images of NF- κ B (red) counterstained with Hoechst nuclear dye (blue) in VEH and CIS treated cells; scale bar: 25 μ m (A). White dotted boxes show zoomed-in views, and the corresponding fluorescence intensity histograms are representative of the circled areas. Representative western blot images of NF- κ B in cytoplasmic and nuclear compartments with α/β tubulin as a cytoplasmic housekeeping marker and Lamin A/C as a nuclear housekeeping marker (B). Densitometric quantification of the western blot bands of NF- κ B in nuclear (C) and cytoplasmic fractions (D). Statistical analysis was performed using two-tailed unpaired Student's *t*-test (** p <0.01 vs VEH). Abbreviations: C: cytoplasmic fraction; CIS: cisplatin; N: nuclear fraction; NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B-cells; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

On this basis, we proceeded to investigate downstream targets of NF- κ B activation such as Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome complex and caspase-3-mediated apoptosis. Inflammasomes are multiprotein complexes and are found to play a vital role in initiation and propagation of inflammation, of which NLRP3 is one of the most common complexes found to be implicated in *in vivo* models of cisplatin-induced hearing loss [118]. Intriguingly, recent evidence points out the existence of the NF- κ B-NLRP3 inflammatory axis for the initiation and regulation of inflammatory responses [119]. The inflammasome complex proteins namely NLRP3 (Figure 12A), apoptosis-associated speck-like protein (ASC) (Figure 12B) and Gasdermin (GSDMD) (Figure 12C) were found to be expressed in the cell line but we failed to demonstrate the expression of Caspase-1 (Figure 12E) in the cells. Furthermore, after cisplatin treatment, no significant differences in this protein complex were observed for both the full-length and cleaved forms (Figure 12A-F). Consequently, no released IL-1 β - the final effector the inflammasome pathway - was observed for both vehicle and cisplatin treated cells (Figure 12G, H). Overall, these data indicate that NF- κ B /NLRP3 axis was not activated in our experimental conditions. We also examined other inflammatory cytokines such as TNF α and IL-6, however these were not detected in the cells (data not shown).

After exploring the inflammatory events we proceeded to investigate caspase-3, a key executioner of apoptotic cell death [120] . Caspase-3 mediated apoptosis has been shown to play a central role in cisplatin induced cochlear HC death [121,122]. We found Pro-caspase-3 to be detected in both vehicle- and cisplatin-treated cells (Figure 12I,J) whereas cleaved caspase-3 was only detected in cisplatin-treated cells ($p=0.0070$) (Figure 12I, K) indicating the activation of apoptotic events in our ototoxic model.

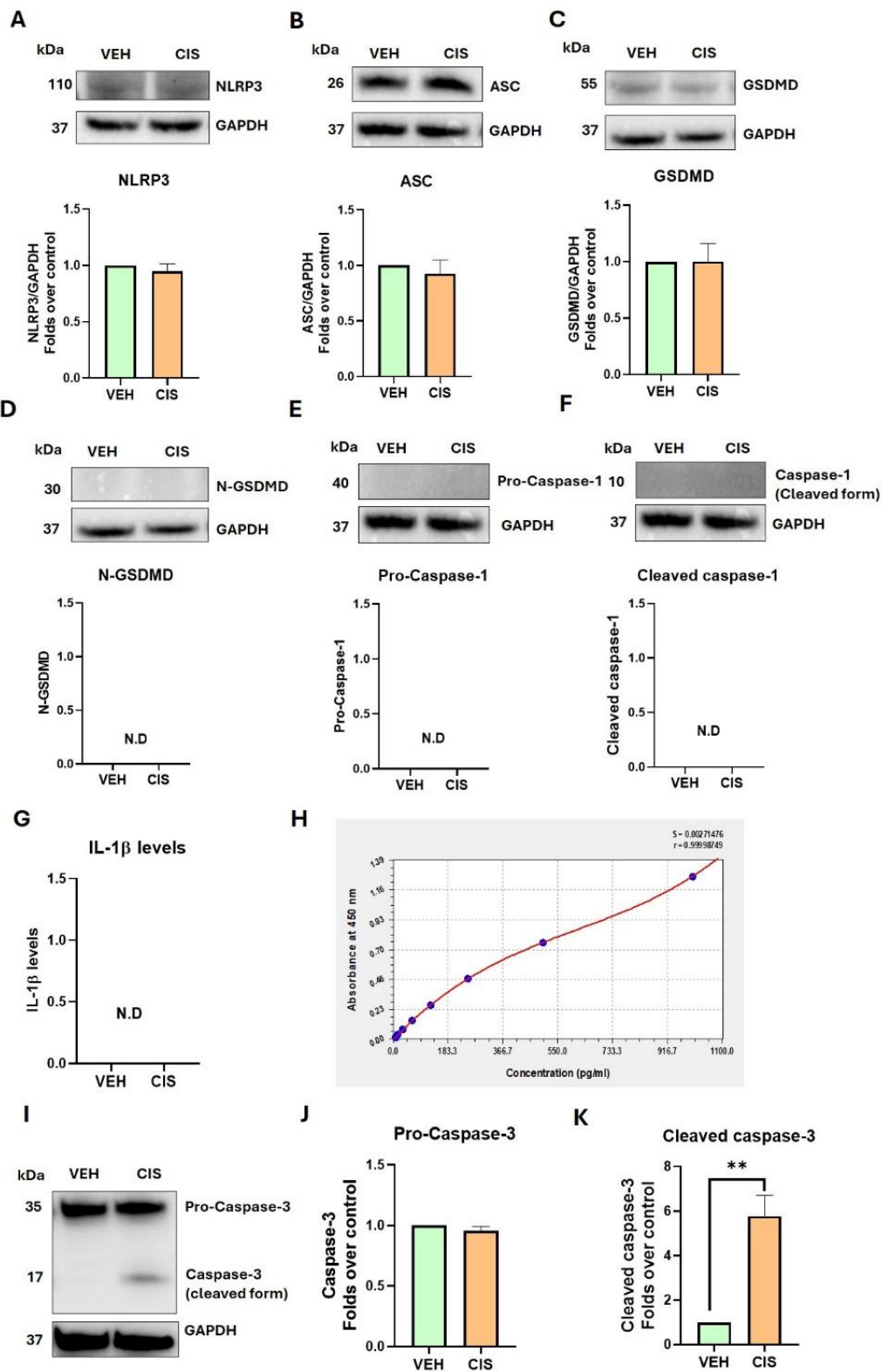


Figure 12. Downstream signaling components of NF- κ B in UB/OC1 cells. Representative Western blot bands and densitometric quantification of proteins NLRP3 (A), ASC (B), GSDMD (C), N-GSDMD (D), pro-caspase-1 (E) and cleaved caspase-1 (F) in VEH and CIS-treated UB/OC1 cells. IL-1 β levels quantified by ELISA in VEH and CIS-treated cells (G). Standard curve for IL-1 β assay (H). Representative Western blot bands of proteins pro-caspase-3 and cleaved caspase-3 in VEH and CIS-treated cells (I) Densitometric quantification of pro-caspase -3 (J) and cleaved caspase-3 (K) in VEH and CIS treated cells. Statistical analysis was performed using two-tailed unpaired Student's *t*-test (** p <0.01 vs VEH). Data is presented as mean $\pm$ SEM for three independent experiments. Abbreviations: ASC: apoptosis-associated speck-like protein; CIS: cisplatin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GSDMD: gasdermin-D; IL: Interleukin; NLRP3: pyrin domain-containing-3; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

4.4 Cisplatin downregulates CB₂R expression but does not affect its subcellular localization pattern

Following the establishment of the *in vitro* ototoxic model, we investigated the impact of cisplatin on the primary eCB receptors CB₁R (Figure 13A), CB₂R (Figure 13B), TRPV1 (Figure 13C) and PPAR $\alpha/\delta/\gamma$ (Figure 13D-F). Interestingly, CB₂R was found to be significantly downregulated ($p=0.0006$) in cisplatin-treated cells compared to vehicle-treated controls (Figure 13B), whereas no changes in the other proteins were observed, indicating a selective involvement of CB₂R post-cisplatin damage (Figure 13A, C, D, E, F).

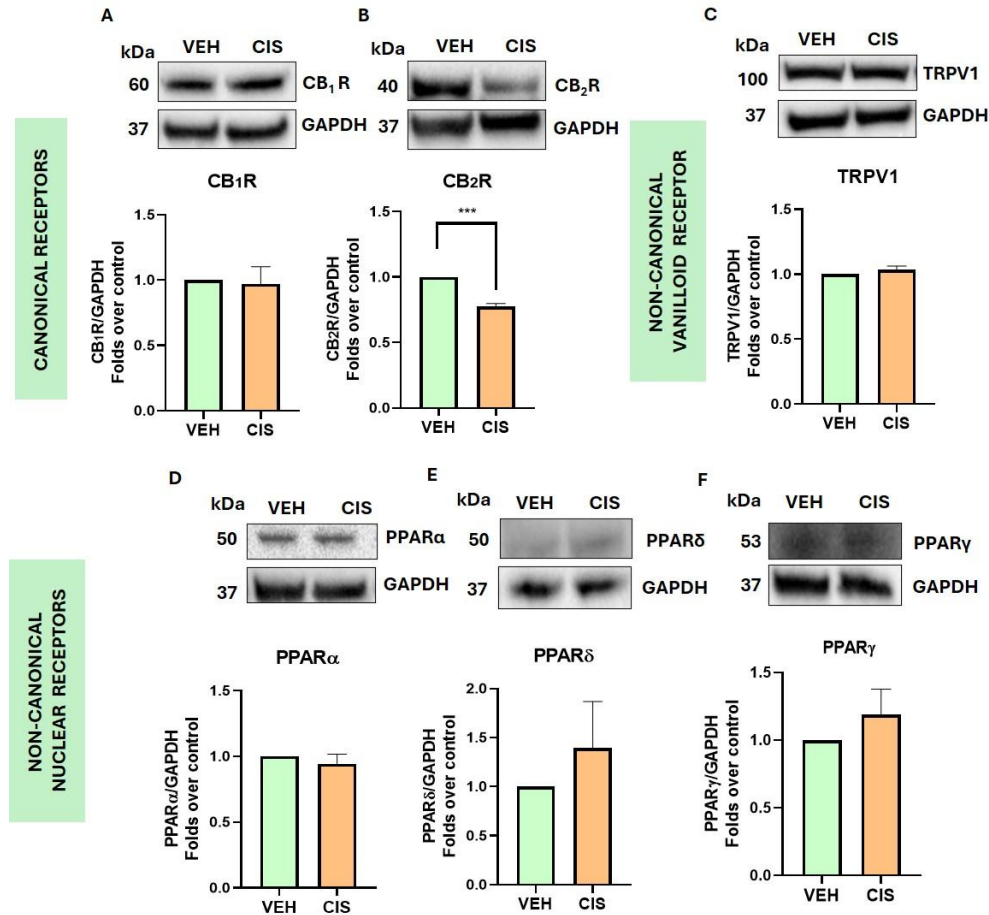


Figure 13. ECS receptors in UB/OC1 cells upon cisplatin-induced ototoxicity. Representative Western blot bands and densitometric quantification of proteins CB₁R (A), CB₂R (B), TRPV1 (C), PPAR α (D), PPAR δ (E) and PPAR γ (F) with respective GAPDH housekeeping in VEH and CIS-treated cells. Data is presented as mean $\pm$ SEM for three independent experiments. Statistical analysis was performed using two-tailed unpaired Student's *t*-test ($p^{***}<0.001$ vs VEH). Abbreviations: CB₁R: cannabinoid receptor 1; CB₂R: cannabinoid receptor 2; CIS: cisplatin; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; PPAR $\alpha/\delta/\gamma$: Peroxisome proliferator-activated nuclear receptors $\alpha/\delta/\gamma$; TRPV1: Transient receptor potential vanilloid receptor 1; VEH: Vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

Therefore, we sought to investigate the possible perturbation of CB₂R localization in addition to its reduced expression. To this end, an immunofluorescence staining of CB₂R in vehicle and cisplatin-treated cells was performed. Heterogenous expression of the receptor CB₂R was observed in this cell line as shown in Figure 14A-F. To our surprise, cell-membrane surface receptor CB₂R (as red puncta) was also observed in the nucleus of both control and cisplatin-treated cells. To

confirm this unexpected result, the localization pattern of CB₂R was further investigated by live-cell imaging using a highly potent CB₂R selective agonist fluorescent probe (SiR-8) developed recently [111]. Consistent with classical immunostaining, both intracellular and nuclear localization of CB₂R were observed as shown in the time-dependent images (Figure 15). Cisplatin treatment did not induce any marked sub-cellular localization differences as seen from Figures 14 and 15. It should be noted that the intensity/pattern of the staining for the CB₂R receptor is quite different using an anti-CB₂R antibody and SiR-8, owing to technical differences where fixation and permeabilization can alter the staining to a greater extent [123].

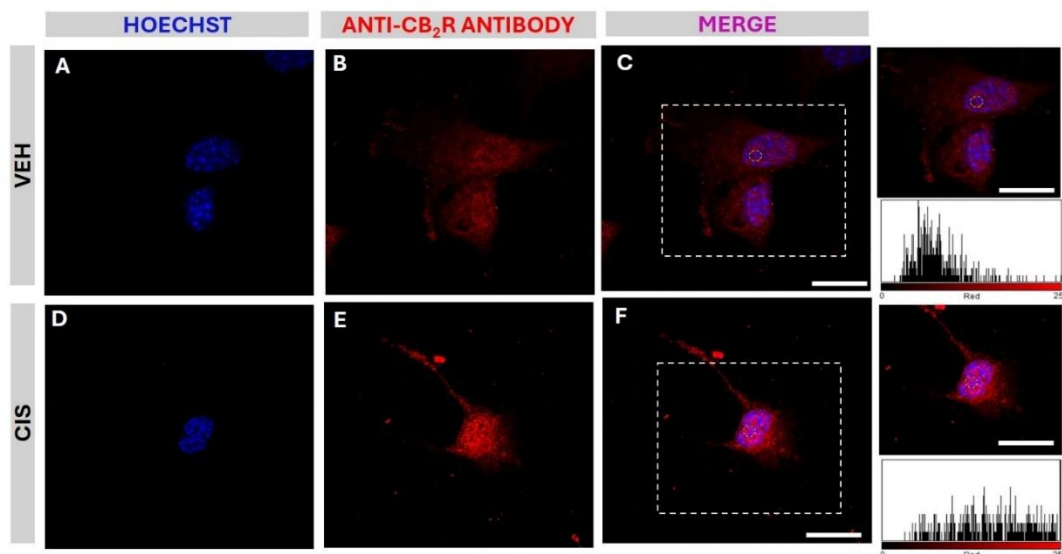


Figure 14. Sub-cellular localization pattern of CB₂R using classical immunostaining. Representative confocal images of VEH-treated cells immunostained with anti-CB₂R antibody (red) and counterstained with Hoechst nuclear dye (blue) (A, B, C). Representative confocal images of CIS- treated cells immunostained with anti-CB₂R antibody (red) and counterstained with Hoechst nuclear dye (blue); scale bar: 20µm (D, E, F). Abbreviations: CB₂R: cannabinoid receptor 2; CIS: cisplatin; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

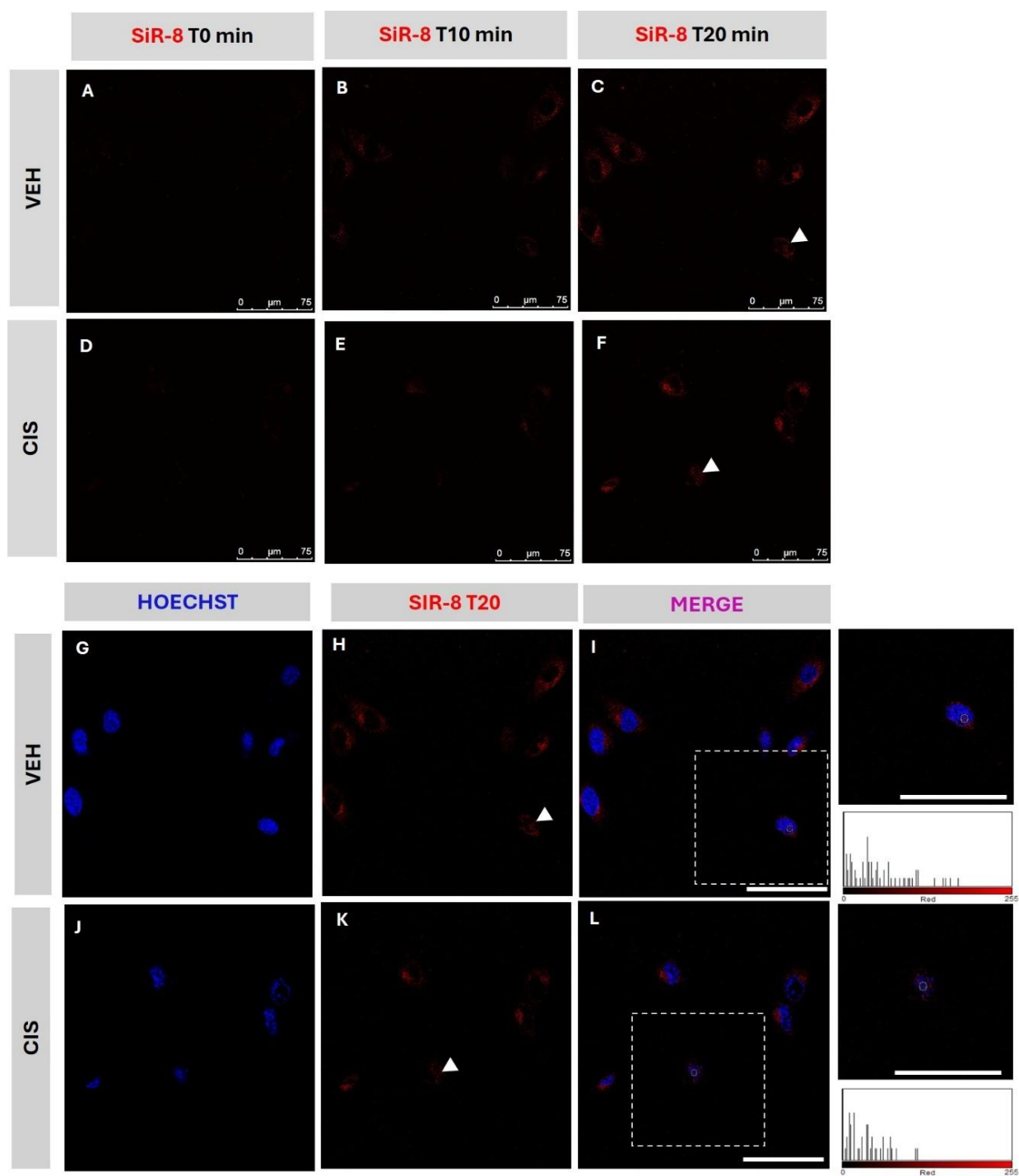


Figure 15. Sub-cellular localization pattern of CB₂R using the selective SiR-8 probe. Representative confocal microscopy images of VEH-treated cells stained with SiR-8 probe (red) at T0 min (A), T10 min (B) and T20 min (C) after incubation with SiR-8. Representative confocal microscopy images of CIS-treated cells stained with CB₂R SiR-8 probe (red) at T0 min (D), T10 min (E) and T20 min (F) after incubation with SiR-8. Scale bar: 75 μ m. Representative confocal microscopy images of VEH (G-I) and CIS-treated cells (J-L) stained with CB₂R SiR-8 probe (red)

and counterstained with Hoechst nuclear dye (blue) at T20 min after exposure to SiR-8. White dotted boxes show zoomed-in views and the corresponding fluorescence intensity histograms relative to the circled areas. Abbreviations: CB₂R: cannabinoid receptor 2; CIS: cisplatin; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

4.5 Cisplatin causes perturbation of the 2-AG metabolic pathway

After assessing the modulation of eCB receptors in our ototoxic model, possible effects of cisplatin on the biosynthetic and degradative machinery of AEA and 2-AG in UB/OC1 cells were investigated. Upon cisplatin, western blot analysis of the 2-AG metabolic pathway (Figure 16A) revealed a significant downregulation of the principal 2-AG biosynthetic enzyme DAGL β compared to vehicle (Figure 16B), while no differences were observed for DAGL α between groups (Figure 16C). Moreover, the 2-AG degradative enzyme ABHD6 (Figure 16E) was significantly reduced after treatment with cisplatin compared to vehicle, while ABHD12 remained unchanged (Figure 16F). On this basis, DAGL β and ABHD6 were also analyzed through immunofluorescence staining followed by confocal microscopy to evaluate their sub-cellular localization patterns (Figure 16D,G). No changes in their sub-cellular localization were observed upon cisplatin treatment. However, consistent with western blotting analysis, the fluorescent signal of DAGL β and ABHD6 was clearly reduced in cisplatin-treated cells compared to vehicle (Figure 16D, G).

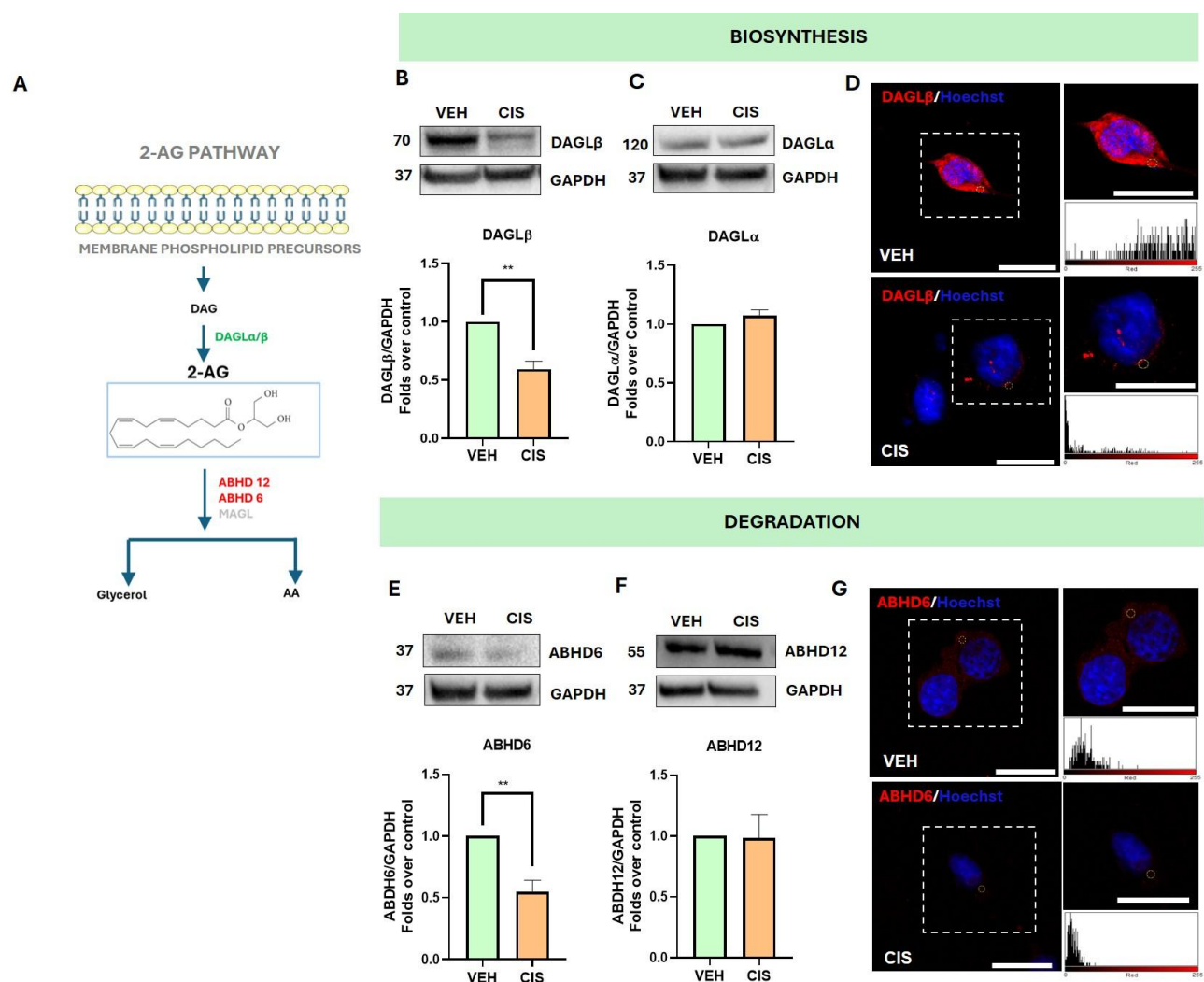


Figure 16. Effects of cisplatin on 2-AG metabolic pathway. Schematic representation of 2-AG metabolism. The biosynthetic enzymes are marked in green; the degradative enzymes are marked in red and the enzymes which were not expressed in the cell line are marked in grey (A). Representative western blots and densitometric quantification of DAGLβ (B), DAGLα (C), ABHD6 (E), ABHD12 (F), with GAPDH housekeeping in VEH and CIS-treated cells. Representative confocal microscopy images of DAGLβ (red) (D) and ABHD6 (red) (G) in VEH- and CIS-treated cells, counterstained with Hoechst nuclear dye (blue); scale bar: 25 μm. White dotted boxes show zoomed-in views and the corresponding fluorescence intensity histograms are representative of the circled areas. Statistical analysis was performed using two-tailed unpaired Student's *t*-test (***p* < 0.01 vs VEH). Data is presented as mean ± SEM for three independent experiments. Abbreviations: 2-AG: 2-arachidonoylglycerol; AA: arachidonic acid; ABHD6/12: α/β hydrolase domain-containing protein 6/12; CIS: cisplatin; DAG: diacylglycerol; DAGLα/β: diacylglycerol lipases α and β; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MAGL: monoacylglycerol lipase; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

Looking into the AEA metabolic enzymes (Figure 17A), no significant modulations were observed (Figure 17B-D). Hence, under our experimental conditions, cisplatin selectively impacted the 2-AG metabolic pathway.

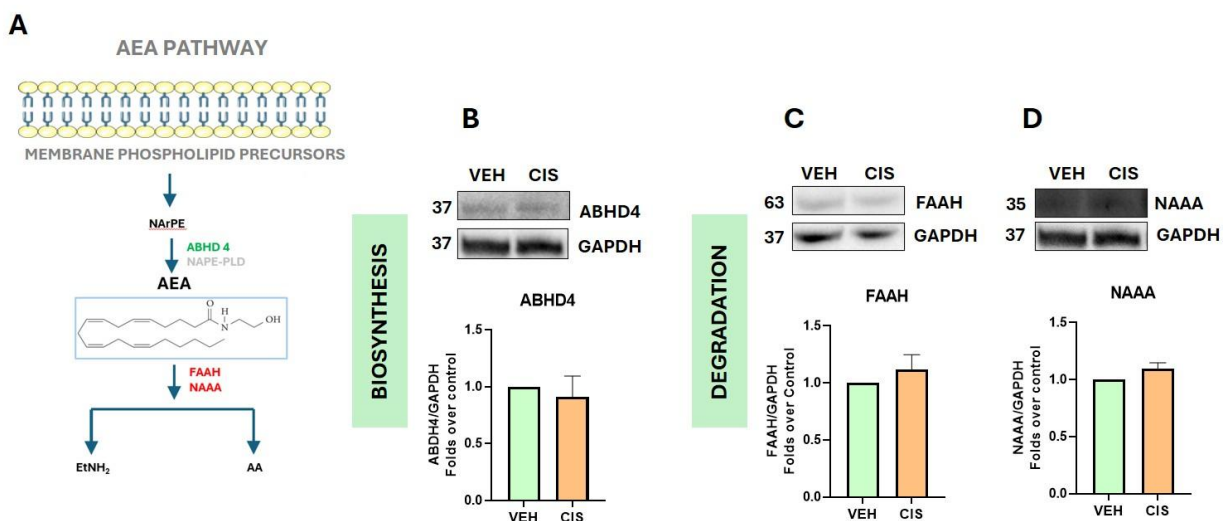


Figure 17. Effects of cisplatin on AEA metabolic pathway. Schematic representation of AEA metabolic pathway (A). The biosynthetic enzymes are marked in green; the degradative enzymes are marked in red and the enzymes which were not found to be expressed in the cell line are marked in grey. Representative western blots and densitometric quantification of ABHD4 (B), FAAH (C), NAAA (D), with GAPDH housekeeping in VEH and CIS-treated cells. Data is presented as mean $\pm$ SEM for three independent experiments. Abbreviations : AA: arachidonic acid; ABHD4: α/β hydrolase domain-containing protein 4; AEA: N-arachidonylethanolamine; CIS: cisplatin; EtNH₂: ethanolamine; FAAH: fatty acid amide hydrolase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NAAA: N-acylethanolamine acid amidase; NAPE-PLD: N-acyl-phosphatidylethanolamine-specific phospholipase D; NArPE: N-arachidonylethanolamine; VEH: vehicle. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

4.6 Cisplatin does not alter eCB lipid levels in UB/OC1 cells

Following the investigation on the eCB receptors and metabolic enzymes, we sought to quantify eCBs (AEA, 2-AG) and other eCB-like lipids (PEA, OEA, POEA, EPEA, DHEA, LEA and SEA) upon cisplatin damage. Based on the observed dysregulation in the protein levels of 2-AG metabolic enzymes upon cisplatin (Figure 16), we hypothesized that eCB levels might be altered

upon cisplatin, particularly affecting 2-AG lipid levels. However, no changes were observed in the levels of both the major eCBs (AEA and 2-AG) as seen from figure 18A. This result can likely be attributed to the reduction in protein levels of both the biosynthetic enzyme-DAGL β and degradative enzyme-ABHD6 of the 2-AG pathway, as evidenced by the ratio of DAGL β /ABHD6 (Figure 18B). Additionally, the levels of other eCB-like lipids were also found to be unaltered upon cisplatin as shown in Figure 18D. To capture overall trends in lipid distribution between control and cisplatin-treated samples which could have been overlooked by univariate *t*-tests, we also performed multivariate PCA analysis. The PCA biplot of the eCB lipids revealed partial separation (PC1-78.3% variance, PC2-21.7%) between groups indicating a trend towards increase in eCB levels after cisplatin treatment, with both AEA and 2-AG contributing to this shift. However, the modest overlap suggests subtle shift in lipid profiles rather than a clear distinction (Figure 18C). On the other hand, the PCA biplot of the eCB-like lipids revealed substantial overlap with no clear cluster separation, suggesting these lipids do not distinctly vary in treated *versus* control samples (Figure 18E).

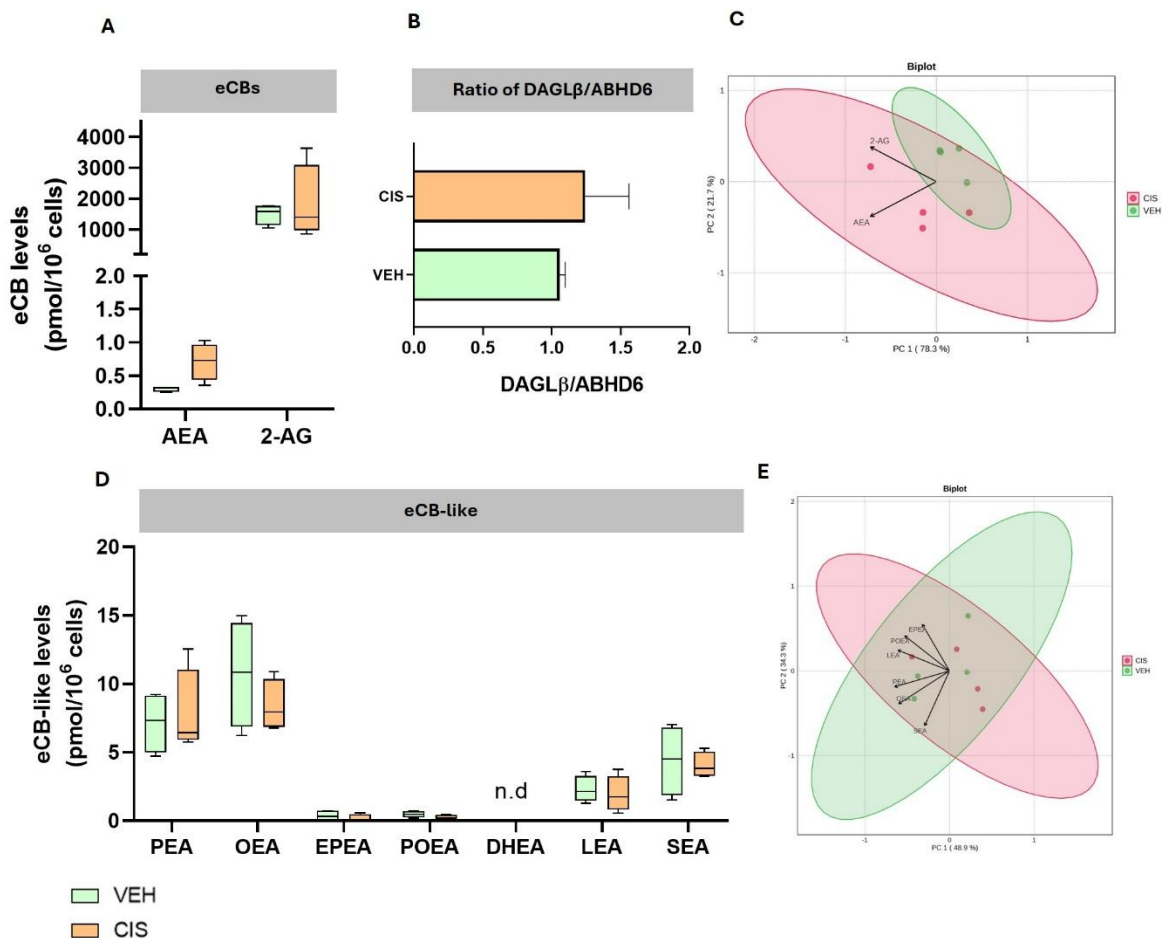


Figure 18. Quantification of eCBs and eCB-like lipids in the *in vitro* model of cisplatin-induced ototoxicity. Levels of AEA and 2-AG measured using UHPLC-MS/MS in VEH- and CIS-treated UB/OC1cells (**A**). Ratio of the protein levels of biosynthetic-to-degradative enzymes (DAGLβ/ABHD6) in VEH and CIS-treated UB/OC1 cells. Data are presented as mean ± SEM for 3 experiments (**B**). PCA biplot of eCB lipids (**C**). Levels of eCB-like compounds PEA, OEA, POEA, EPEA, DHEA, LEA and SEA, in VEH- and CIS-treated UB/OC1 cells measured using UHPLC-MS/MS. When the expression of the lipids falls below the lower limit of quantification (LOQ), the value is assumed as 0. The data is presented as a box plot with whiskers ranging from min to max values for 4 independent experiments. (**D**). PCA biplot of eCB-like lipids (**E**). Abbreviations: 2-AG: 2-arachidonoylglycerol; ABHD6: α/β hydrolase domain-containing protein; AEA: N-arachidonylethanolamine; CIS: cisplatin; DAGL β: diacylglycerol lipases β; DHEA: docosahexaenylethanolamine; EPEA: epoxyeicosatetraenyl ethanolamide; LEA: linoleoylethanolamide; OEA: N-oleoylethanolamine; PEA: N-palmitoylethanolamine; POEA: N-palmitoleoylethanolamine; SEA: N-stearoylethanolamine; VEH: vehicle Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

4.7 CB₂R antagonism inhibits cisplatin-mediated caspase-3-dependent apoptosis in UB/OC1 cells

Since in our *in vitro* model we observed the reduction of CB₂R, ABHD6 and ABHD12 protein levels upon cisplatin, we next proceeded to investigate the mechanistic implications of the reductions observed. For this purpose, we selected only CB₂R as our major target of interest due to availability of well-defined selective agonists and antagonists for the receptor. CB₂R was pharmacologically targeted using selective CB₂R antagonist SR144528 (2 μ M) in the presence of cisplatin in UB/OC1 cells. MTT assay revealed that SR144528 improved cell viability compared to cisplatin alone ($p=0.0256$) in our model. On this basis, we wondered whether this protective effect was associated with the modulation of caspase-3, which was activated by cisplatin in our model. Notably, SR144528 reduced the activation caspase-3 ($p=0.0444$), as indicated by decreased protein levels of its cleaved form compared to cisplatin alone. Collectively, these data demonstrate that blockade of CB₂R mitigates cisplatin-induced HC damage by inhibiting caspase-3-dependent apoptotic events.

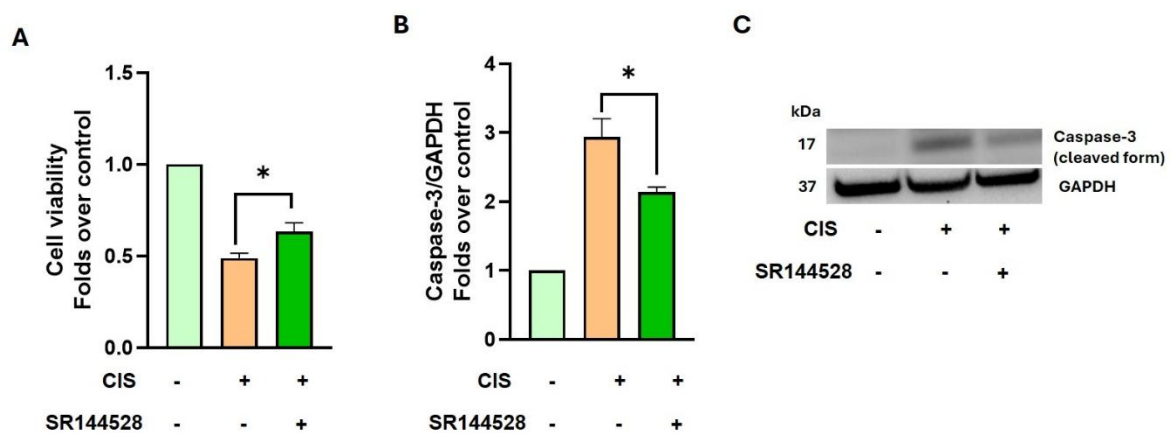


Figure 19. Pharmacological manipulation of CB₂R with the selective antagonist SR144526 in UB/OC1 cells upon cisplatin treatment. Cell viability of UB/OC1 cells upon SR144528 treatment determined by MTT assay (**A**). Statistical analysis was performed by two-tailed unpaired student's *t*-test ($*p<0.05$ vs CIS). Data is represented as mean $\pm$ SEM for six independent experiments. (**B-C**) Densitometric quantification of cleaved caspase-3 with GAPDH housekeeping (**B**), with representative western blot bands (**C**). Statistical analysis was performed by two-tailed unpaired student's *t*-test ($*p<0.05$ vs CIS). Data is presented as mean $\pm$ SEM for three independent experiments. Abbreviations: CB₂R: Cannabinoid receptor 2; CIS: Cisplatin. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

5. Results of the *in vivo* study

5.1 Development of the cisplatin-induced ototoxic mouse model

Following the analysis of the ECS *in vitro*, we extended our investigation *in vivo*. To this end, we tested different doses and routes of administration of cisplatin and furosemide (see section 3.5) in order to establish the best experimental paradigm to induce ototoxicity. The administration of furosemide in combination with ototoxic drugs is a widely used strategy to boost ototoxicity *in vivo* while reducing systemic toxicity and death [102]. This is because furosemide is a loop diuretic which disrupts the EP, enhancing uptake of cisplatin in the cochlea [124]. This allows one to use a lower cisplatin dose while still reaching ototoxic thresholds. The ototoxic damage was evaluated functionally through ABR thresholds along with amplitudes and latencies of waves I and II on days 0 and 7. Additionally, clinical signs of nephrotoxic damage such as substantial weight loss were monitored throughout the experiment.

Out of all groups analyzed (Figure 4), only group 4 characterized by the administration of cisplatin 2 mg/kg i.p., and furosemide 200 mg/kg i.p., included a subset of animals that exhibited mild ototoxic effects (>10 dB threshold shifts), which can potentially be used to study early sub-clinical molecular changes and was therefore selected for subsequent molecular analyses. Hence, only the data of group 4 are shown here.

In group 4, 42.85% (3/7 animals) showed > 10 dB differences in threshold shifts at day 7 with respect to day 0, hence this group included a total of 3 responders and 4 non-responders. Since the study aimed to include only animals demonstrating functional hearing impairment (>10 dB threshold shifts), only responders' data are shown here (Figure 20) and are used for subsequent histological analysis. The individual thresholds of the 3 responders on day 0 and day 7 are shown in figure 20A. Collective analysis of the responders' data showed a statistically significant decrease in the thresholds on day 7 compared to day 0 (Figure 20B). The representative waveforms on days 0 and 7 are shown in figure 20C. Importantly, the weights of the animals remained constant throughout the study and no systemic toxicity was noted (Figure 20D). Furthermore, no significant changes in amplitudes and latencies were observed on day 7 compared to day 0 (Figure 21). Additional exploratory analysis on the non-responders' is shown in appendix I.

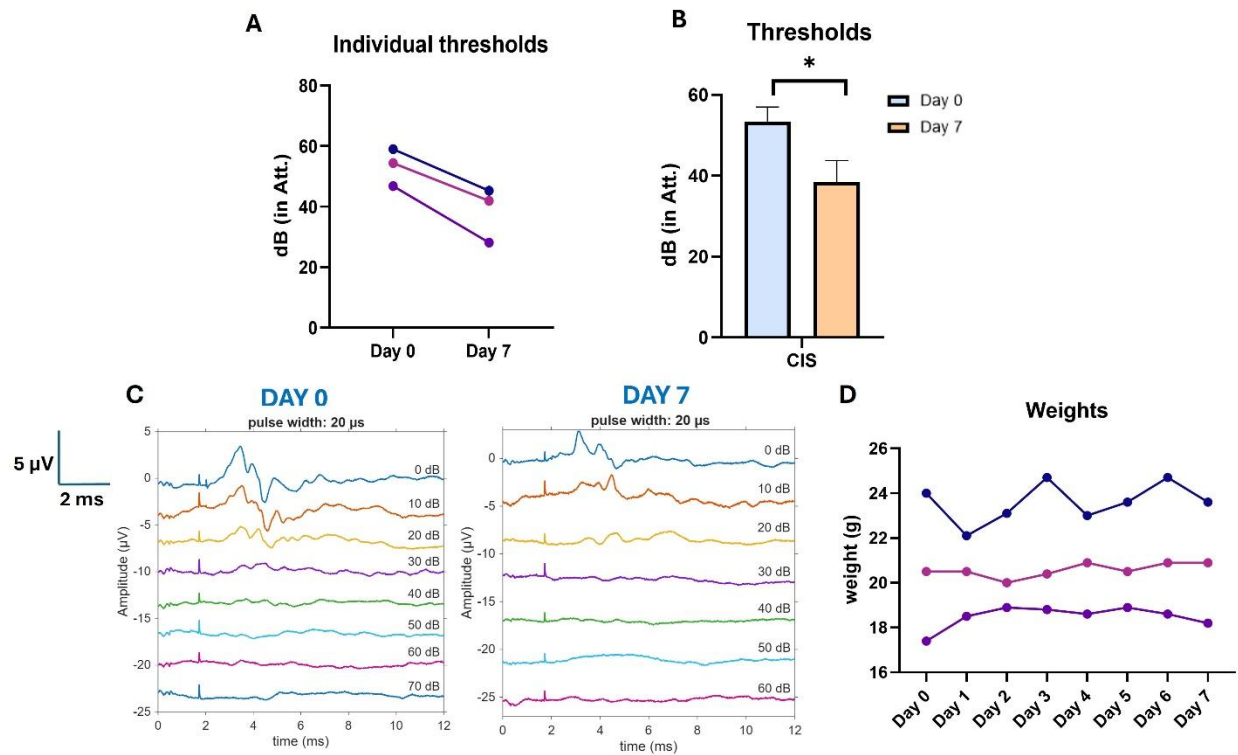


Figure 20. ABR analysis of the cisplatin-induced ototoxic model. Animals were treated with a combination of furosemide (200 mg/kg; i.p.) and cisplatin (2 mg/kg; i.p.). Individual thresholds of 3 animals (each color represents an animal) on day 0 and day 7 shown as symbols with connecting lines between days 0 and 7 (**A**). Collective thresholds of 3 animals on day 0 and day 7. Statistical analysis was performed using two-tailed paired *t*-test ($*p < 0.05$ vs Day 0) (**B**). Representative ABR waveform of the group on day 0 and on day 7 (**C**). Weights of each animal (each color represents an animal) measured across the experiments from day 0 to day 7 shown as symbols with connecting lines (**D**). Abbreviations: ABR: Auditory brainstem response; CIS: cisplatin; IP: Intraperitoneal. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

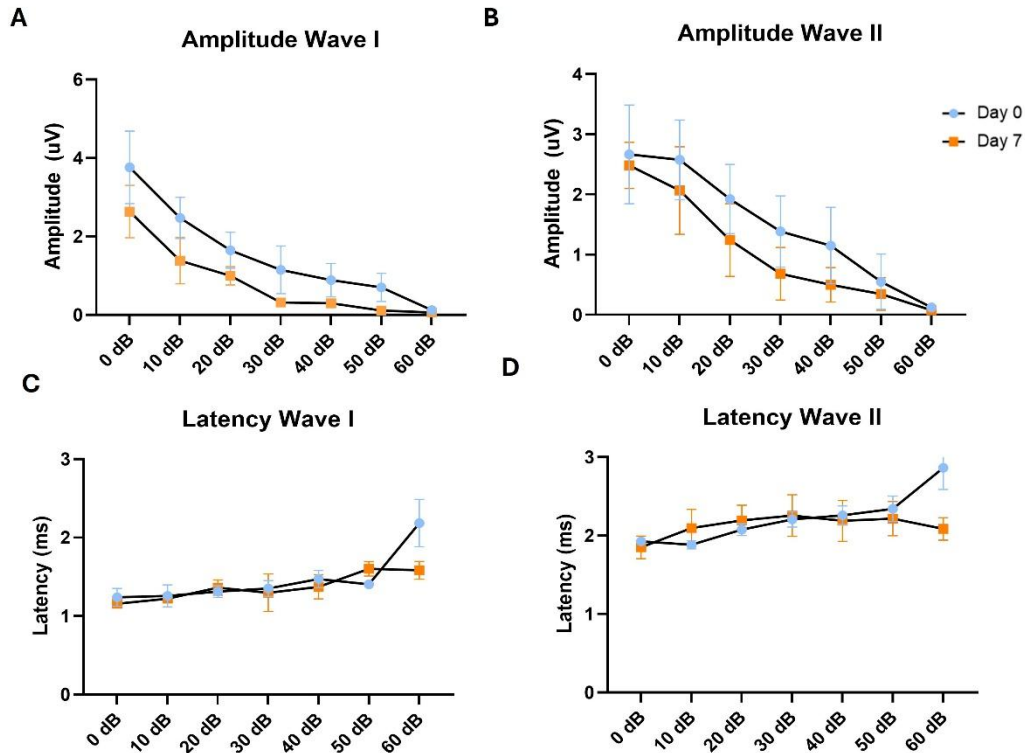


Figure 21. Amplitude and latency analysis of waves I and II in cisplatin-induced ototoxic model. Amplitudes of wave I (A) and wave II (B) measured across 0 dB (in attenuation) to 60 dB (in attenuation) for 3 animals. Latencies of wave I (C) and wave II (D) measured across 0 dB (in attenuation) to 60 dB (in attenuation) for 3 animals. Statistical analysis was performed using repeated measures two-way ANOVA (mixed effects model) with Bonferroni post hoc comparison test. Data is represented as mean $\pm$ SEM for 3 animals.

5.2 Cisplatin significantly downregulates CB₂R, DAGL β and ABHD6 in the murine OC

Building on the observed reduction in the protein levels of CB₂R, DAGL β and ABHD6 in our *in vitro* ototoxic model of HC-like cells, we next investigated the alterations of the same proteins in the OC of our selected *in vivo* model. Interestingly, consistent with our *in vitro* data, we observed a significant decrease in the expression of all the proteins analyzed (Figure 22), thereby strengthening the physiological relevance of our findings.

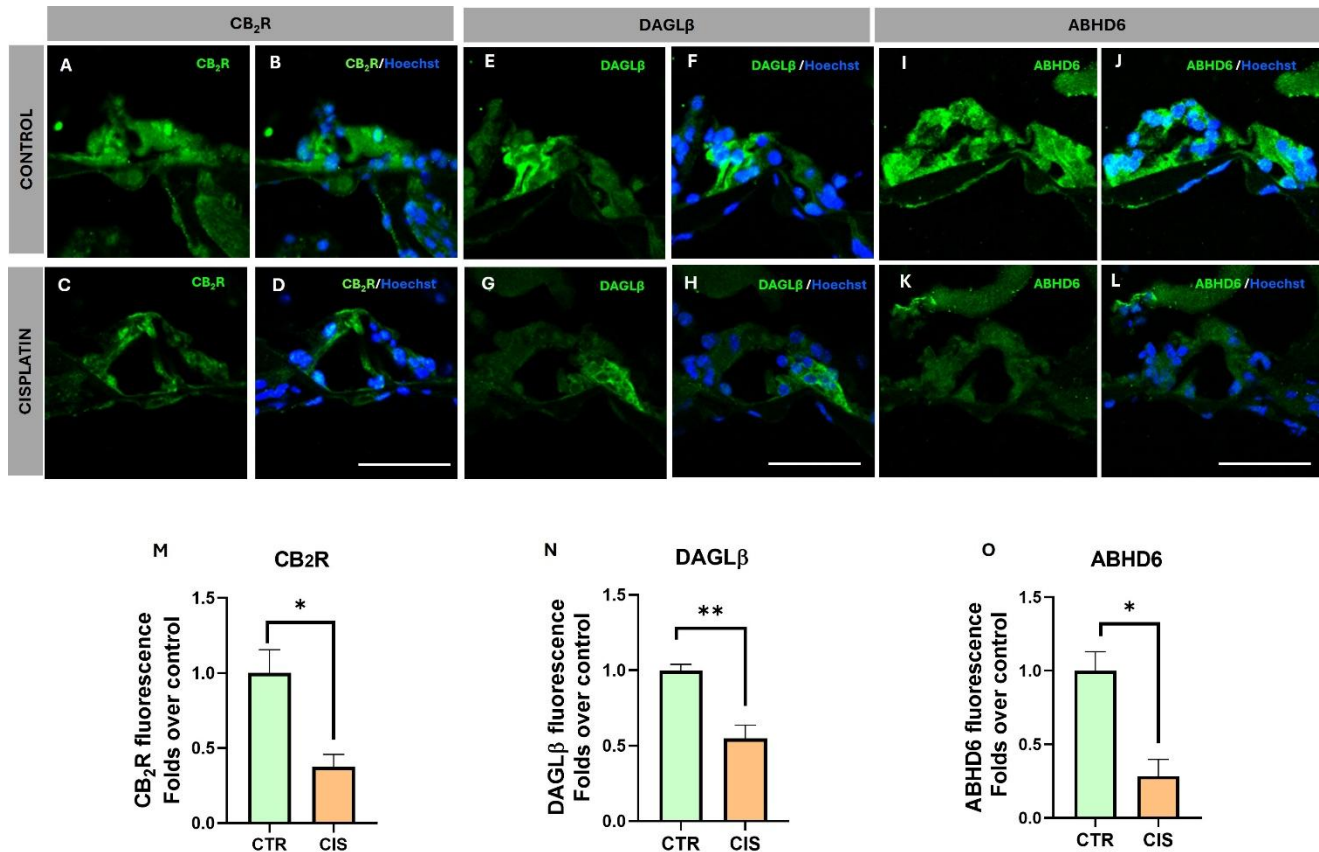


Figure 22. CB₂R, DAGL β and ABHD6 IF in the OC upon cisplatin-induced ototoxicity *in vivo*. Representative confocal microscopy images of CB₂R (green) counterstained with Hoechst nuclear dye (blue) in control (A,B) and cisplatin-treated OC (C,D). Representative confocal microscopy images of DAGL β (green) counterstained with Hoechst nuclear dye (blue) in control (E,F) and cisplatin-treated OC (G,H). Representative confocal microscopy images of ABHD6 (green) counterstained with Hoechst nuclear dye (blue) in control (I,J) and cisplatin-treated OC (K,L). Fluorescence intensity of CB₂R (M), DAGL β (N) and ABHD6 (O) in control and cisplatin-treated OC, scale bar-50 μ m. Statistical analysis is performed using two-tailed unpaired student's *t*-test (**p* < 0.05 vs CTR, ***p* < 0.01 vs CTR). Data is presented as mean $\pm$ SEM for 3 animals. Abbreviations: ABHD6: α/β hydrolase domain-containing protein; CB₂R: Cannabinoid receptor 2; CIS: cisplatin; DAGL β : Diacylglycerol lipase β ; IF: Immunofluorescence. Figure adapted from [114] under creative commons attribution 4.0 license (CC BY 4.0).

6. Discussion

Overall, this dissertation explores the ECS in cochlear HC physiology and ototoxicity through an integrated approach, employing both *in vitro* and *in vivo* models. Our findings underscore the critical involvement of the ECS in cisplatin-mediated cochlear damage, revealing novel biomolecular targets with potential therapeutic relevance. We discuss below in detail the key findings of the work.

6.1 Identification of the ECS (primary receptor targets, metabolic enzymes and endogenous lipids) in auditory HC-like UB/OC1 cells

The first step of the thesis was to perform a systematic profiling of the ECS in the UB/OC1 cell line, which serves as a suitable auditory HC-like model owing to the expression of key sensory HC markers like Myo7a [125].

Firstly, the expression of primary eCB receptors CB₁R, CB₂R, TRPV1 and PPAR $\alpha/\delta/\gamma$ in UB/OC1 cells was demonstrated at the protein level (Figure 6). This agrees with previously reported data showing the expression of the same receptors in the OC of animal models [87,88,94,97,126,127]. We then turned our attention to the metabolic components of AEA and 2-AG signalling, which have been poorly investigated until now. Indeed, only transcriptomic data from the otic vesicle or early developmental stages are available [59]. Here, we show that the classical AEA biosynthetic enzyme NAPE-PLD and the principal 2-AG degrading enzyme MAGL are not expressed in UB/OC1 cells (Figure 6). Strikingly, secondary metabolic routes of both AEA (via ABHD 4 and NAAA enzymes) and 2-AG (via ABDH 6/12 enzymes) pathways were found to predominate this cell line (Figure 6). This suggests that the eCB signalling in HCs could primarily employ alternative metabolic routes, and that the ABHD class of enzymes may play a key role in auditory function.

Then, through a targeted lipidomic approach using UHPLC-MS/MS, quantification of eCBs and eCB-like lipids in UB/OC1 cells was performed. Interestingly, both primary eCBs AEA and 2-AG were identified in the cell line, with 2-AG levels being substantially higher (~5000 folds higher) than that of AEA. Additionally, other eCB-like lipids namely PEA, OEA, LEA, SEA, POEA, EPEA were also detected; however, their levels were lower compared to 2-AG, which indeed resulted to be the most abundant eCB in the cell line (Figures 7,8). Of note, no studies have ever investigated the presence of eCBs in any auditory system, therefore our data represents the very first evidence on the presence of bioactive eCBs in auditory HC-like cells. The existence of this

extensive ECS network in UB/OC1 cell line makes it a suitable auditory HC-like model to investigate ECS functions in physiological and pathological contexts.

6.2 Cisplatin impacts selective elements of the ECS in HC-like cells and in the mouse OC

The next objective of the study was to investigate the impact of cisplatin on the ECS. Therefore, following the characterization of the ECS in UB/OC1 cells, we explored its possible perturbation in cisplatin-induced ototoxic conditions in the same cell line. To achieve this, an *in vitro* model of ototoxicity was established by treating the cells with cisplatin for 24 hours [39,99,101]. A dose-dependent cytotoxic effect of cisplatin was observed and 30 μ M concentration (corresponding to the IC₅₀ dose) was selected for subsequent investigations. Besides cytotoxicity, the suitability of our experimental paradigm was also confirmed by other key molecular events involved in cisplatin-induced ototoxicity, such as the reduction of the HC marker Myo7a, the activation of NF- κ B and downstream caspase-3-mediated apoptosis. Indeed, the expression of Myo7a is known to be affected by cisplatin due to the degeneration of HCs [128]. Furthermore, NF- κ B activation and induction of caspase-3 mediated cell death agrees with data reported elsewhere in *in vitro* and *in vivo* ototoxic models [27,122,129]. It should be noted that, in our model, cisplatin did not induce IL-1 β release, which aligns with the observed absence of caspase-1 activation. While other proteases including caspase-3 can process pro-IL-1 β [130,131], the lack of IL-1 β in our model further supports the central role played by caspase-1 in this context.

In such a model, receptor CB₂R (Figure 13B), the biosynthetic and degradative enzymes of the 2-AG pathway, DAGL β (Figure 16B) and ABHD6 (Figure 16E) respectively were found to be significantly downregulated in response to cisplatin-treated cells compared to vehicle. To evaluate whether our *in vitro* observations translate to a more physiologically relevant setting, we analyzed the same components in the murine OC. For this purpose, we first established a model of cisplatin-induced ototoxicity, which was confirmed to exhibit functional hearing impairment using click-evoked ABR measurements. Interestingly, *in vivo* analysis revealed a significant decrease in CB₂R consistent with our *in vitro* findings (Figure 22M). In contrast, a previous study in rats observed an upregulation of the CB₂R after cisplatin-induced ototoxic insult [88]. Nevertheless, the quantification of CB₂R was performed only at a gene level and additionally, the analyses resulted from whole cochlear lysates, which do not provide HC specific expression levels. Interestingly, we showed that blockade of CB₂R using SR144528 protects UB/OC1 cells from cisplatin-induced

apoptosis by inhibiting caspase-3 activation (Figure 19). This key finding provides new mechanistic insights into the role of CB₂R in cisplatin-induced ototoxicity, indicating that CB₂R antagonism may serve as a new therapeutic strategy to mitigate cisplatin-induced hearing loss. In addition to CB₂R, a significant reduction in the biosynthetic enzymes DAGL β (Figure 22N) and the degradative enzyme ABHD6 (Figure 22O) was also observed, in agreement with the *in vitro* observations. This is the first study to provide evidence for the involvement of these enzymes in cisplatin-induced ototoxicity. Of note, a similar scenario has been reported for the enzyme DAGL β in the brain and gastrointestinal system upon cisplatin treatment [132]. However, looking at the eCB lipid levels, all eCBs, including 2-AG, resulted to be unaltered in response to cisplatin *in vitro* (Figure 18). Consistently, the ratio of DAGL β /ABHD 6 protein levels resulted to be similar between vehicle and cisplatin cells (Figure 18). While protein levels provide valuable support for the data, measuring enzyme activity can offer additional information on the functional effectiveness of the enzymes. Moreover, both these enzymes control additional eCB-independent processes in the human body [133,134], suggesting that alternative ototoxic mechanisms may be mediated by cisplatin-induced perturbation of DAGL β and ABHD6. It is important to emphasize that the ECS is a highly complex signalling network with multiple intersecting elements [135–137] that were beyond the scope of this investigation and could potentially contribute to the changes observed here. Nevertheless, our study provides the first evidence of ECS signalling in auditory HC-like UB/OC1 cells and identified new ECS elements targeted by cisplatin *in vitro* and *in vivo* which may represent promising therapeutic targets to counteract cisplatin-induced ototoxicity. From a clinical standpoint in the application of platinum-based chemotherapeutics, cisplatin is the most commonly used drug due to its high effectiveness against a broad range of cancers. However, other less ototoxic structural analogs such as carboplatin/oxiplatin are also used, although they are generally considered less effective than cisplatin [138]. The mechanisms underlying these diverse toxicity profiles remain incompletely understood. In this context, further investigation of ECS may shed light into the mechanisms underlying the ototoxicity of platinum-based chemotherapeutics to identify novel targets for hearing preservation.

7. References

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Appendix I Preliminary results of the ongoing work

Appendix I contains preliminary data that were collected as an exploratory part of the study but were not sufficiently developed to be included in the main thesis. These findings are provided to document ongoing research related to the characterization of the ECS in cochlear physiology and ototoxic conditions. In this section, we presented preliminary results on the characterization of the eCB receptors' gene expression and protein localization in NH murine cochlea. We placed our focus on receptors as they are pharmacologically druggable targets with higher therapeutic relevance. To further investigate alterations of ECS receptors in ototoxic conditions, we established a mouse model of kanamycin-induced ototoxicity in addition to cisplatin model described in the thesis. Due to the inherent characteristics of the two models, limitations in tissue availability and time constraints, different phenotypic models were adopted to study ECS receptors. In this exploratory study, we report data on the responders of the kanamycin model and non-responders of the cisplatin model. Responders and non-responders of both ototoxic models will be analyzed in future work to further comprehensively dissect ECS related molecular changes in different stages of ototoxicity.

Characterization of eCB receptors in NH murine cochlea

1.1 The adult murine cochlea expresses the transcripts of principal eCB receptor targets

We investigated the presence and abundance of the eCB receptors in the adult mouse cochlea using bulk mRNA transcriptomics. Bulk transcriptomics was selected as our initial approach because it allows for a comprehensive analysis of all the ECS receptor genes using a relatively small amount of material (2 pooled cochleae). Contrastingly, protein techniques such as western blotting require pooling multiple cochleae to obtain sufficient material - due to the very low dimension of a single cochlea - and limit the analysis to a few selective targets rather than the entire gene set. To measure the abundance of the transcripts and thereby to know the expression levels of each transcript in the cochlea, transcripts per million (TPM) levels were determined. Tissues for transcriptomic analyses were found to express key markers of cochlear cell types *pou4f3* and *myo7a* (for HCs), *sox2* (for SCs), *tubb3* and *pou4f1* (for SGNs) (Figure 1.1A). The TPM values of these transcripts reflect the known relative abundance of each cell type in the cochlea. Individual TPM of the transcripts in all four animals analyzed are presented as a heatmap (Figure 1.1B). Subsequently, eCB receptor transcriptomic profiling was performed. The analysis revealed the presence of transcripts *cnr1*,

cnr2, *trpv1* and *ppara/d/g* as shown in figures 1.1C. The mean TPM $\pm$ SEM levels from 4 animals were identified to be the following: *cnr1* (6.16 ± 2.99), *cnr2* (0.18 ± 0.12), *trpv1* (5.86 ± 5.64), *ppara* (0.31 ± 0.07), *ppard* (2.04 ± 0.50) and *pparg* (1.87 ± 1.05). Among the transcripts analyzed, *cnr1* was found to be the most abundant followed by *trpv1*, *ppard*, *pparg*, *ppara* and finally *cnr2*.

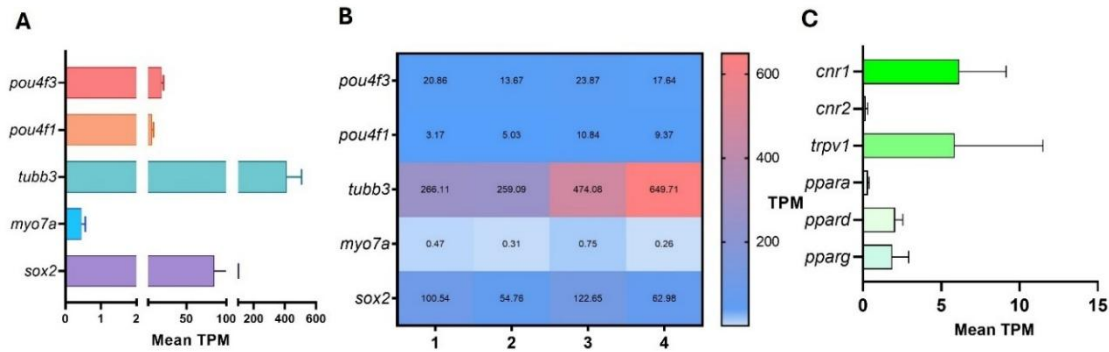


Figure 1.1. Transcriptomic analysis of major cochlear cell type markers and principal eCB receptor targets in the adult mouse cochlea. Mean TPM levels of cochlea cell type markers *pou4f3*, *myo7a*, *tubb3*, *pou4f1* and *sox 2* (A). Individual TPM levels of transcripts *pou4f3*, *myo7a*, *tubb3*, *pou4f1* and *sox2*. Data are expressed as mean $\pm$ SEM for four animals (B). Mean TPM levels of receptors *cnr1*, *cnr2*, *trpv1*, *ppara/d/g* (C). Abbreviations: TPM: transcripts per million

1.2. eCB receptors have distinct region-specific expression patterns in the adult mouse cochlea

To confirm the expression of the eCB receptors at a protein level and to investigate their spatial localization we performed immunohistochemistry assays on NH mouse cochlear cryosections.

CB_{1/2}R

Firstly, we detected the expression of CB₁R in all the examined regions of the cochlea with a characteristic membranous distribution pattern (Figure 1.2). In the OC, CB₁R was robustly expressed in IHCs, OHCs, and in SC populations as shown in Figures 1.2A-C. CB₁R labeling was also detected in the soma of SGNs (Figure 1.2D-F). Notably, in the SV, CB₁R was found to be enriched in the marginal cells with respect to intermediate and basal cell layers as evidenced by the plot profile (Figure 1.2G-J). Similarly, strong CB₂R labeling was observed in the OC, soma of SGNs and SV but with a different expression profile compared to CB₁R (Figure 1.3). In fact, within the OC, CB₂R was detected in the IHCs, OHCs and SCs with IHC showing a robust signal

compared to other cell types (Figure 1.3A-C). Moreover, unlike CB₁R, CB₂R exhibited strong signal across all the layers of SV (Figure 1.3G-I) and was found to be localized in both nucleus and cytoplasm of the cells in all the investigated cochlear regions (Figure 1.3).

TRPV1

Regarding the non-canonical vanilloid receptor TRPV1 (Figure 1.4), we observed its expression in the OC (Figure 1.4A-C), SGNs (Figure 1.4D-F) and SV (Figure 1.4G-I). TRPV1 immunostaining exhibited a punctate pattern and was predominantly localized in the cytoplasmic membranes in all assessed regions (Figure 1.4).

PPARs

Lastly, we turned our attention to PPAR family of nuclear receptors. PPAR α was detected in IHCs, OHCs and SCs in the OC (Figure 1.5A-C), in the soma of SGNs (Figure 1.5D-F) and in all SV layers (Figure 1.5G-I). Notably, within the key cell types of the OC, PPAR α expression was found to be the strongest in the IHC. Furthermore, in the SV, PPAR α showed a selective robust distribution pattern along the marginal cells as seen from the plot profile (Figure 1.5 G-J). As for PPAR δ , it exhibited widespread expression across all key cell types in the OC (Figure 1.6A-C) and SGNs (Figure 1.6 D-F). In the SV, similar to PPAR α , a strong signal was observed in the marginal cells as evidenced by the plot profile (Figure 1.6G-J). Moreover, differently from PPAR α , PPAR δ immunolabeling was most pronounced in SC populations. Regarding PPAR γ , its expression appeared weak but was detected in all the analyzed cochlear regions: OC (Figure 1.7A-C), SGNs (Figure 1.7D-F) and SV (Figure 1.7G-I).

Overall, these data provide a comprehensive analysis of key eCB receptors in the adult murine cochlea while also demonstrating for the first time the presence of PPAR δ at the gene and protein level. Importantly, spatial localization of the receptors in the OC, SGNs and SV revealed novel insights into the microanatomical and sub-cellular context of eCB signaling.

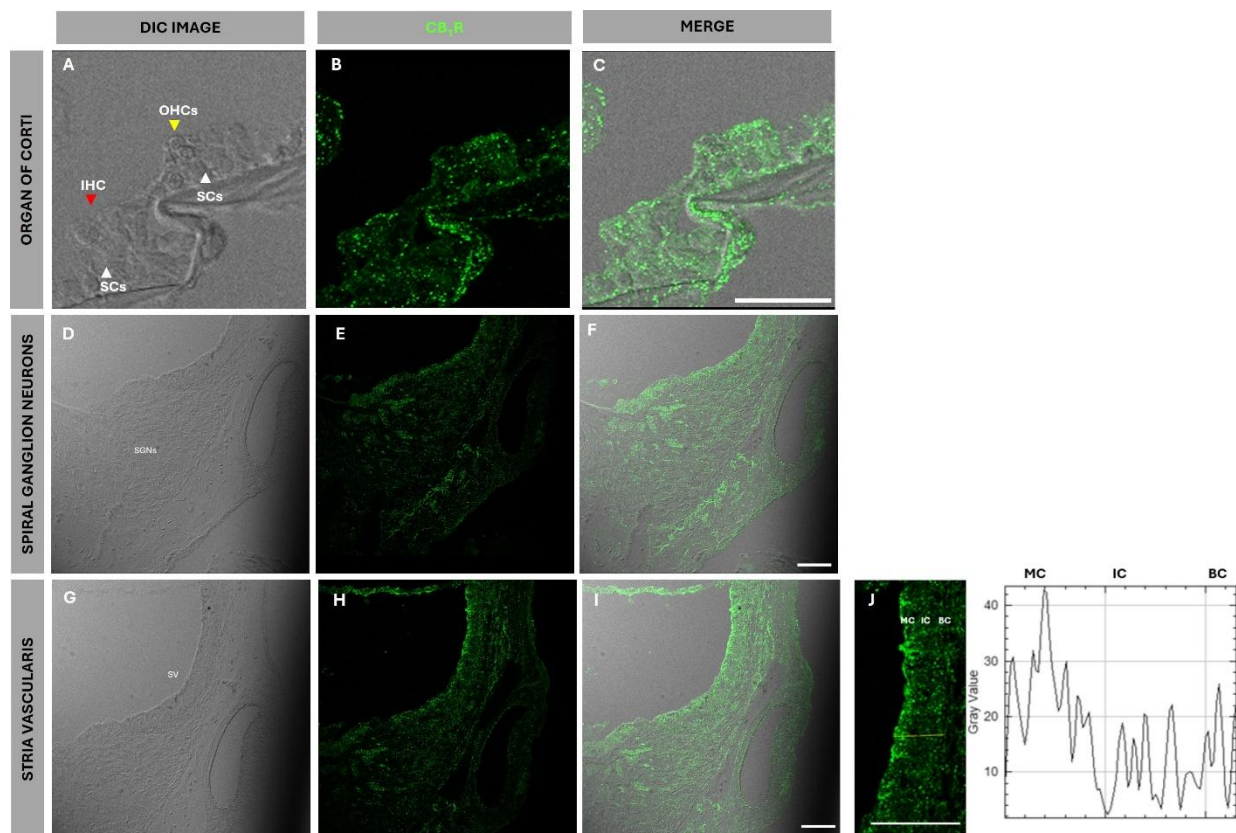


Figure 1.2. CB₁R localization in the OC, SGNs and SV of the adult murine cochlea. DIC microscopy image of the OC (A). IHC is indicated by red arrows, OHCs are indicated by yellow arrows and SCs are indicated by white arrows. Representative maximum projection confocal image showing CB₁R expression (in green) in the OC (B). Overlay of CB₁R with corresponding DIC image (C). DIC microscopy image of the SGN (D). Representative maximum projection confocal image showing CB₁R expression (in green) in the SGN (E). Overlay of CB₁R with corresponding DIC image (F). DIC microscopy image of the SV (G). Representative maximum projection confocal image showing CB₁R expression (in green) in the SV (H). Overlay of CB₁R with corresponding DIC image (I). Zoomed image showing CB₁R expression in the MC, IC and BC layers of SV and the yellow line indicates the region used to generate fluorescence intensity plot profile (J). Scale bar: 50 μ m. Abbreviations: BC; Basal cell layer; CB₁R: cannabinoid receptor 1; DIC: Differential interference contrast; IC: Intermediate cell layer; IHC: Inner hair cell; MC: Marginal cell layer; OHC: Outer hair cell; OC: organ of Corti, SC: Supporting cell; SGN: spiral ganglion neuron; SV: Stria vascularis.

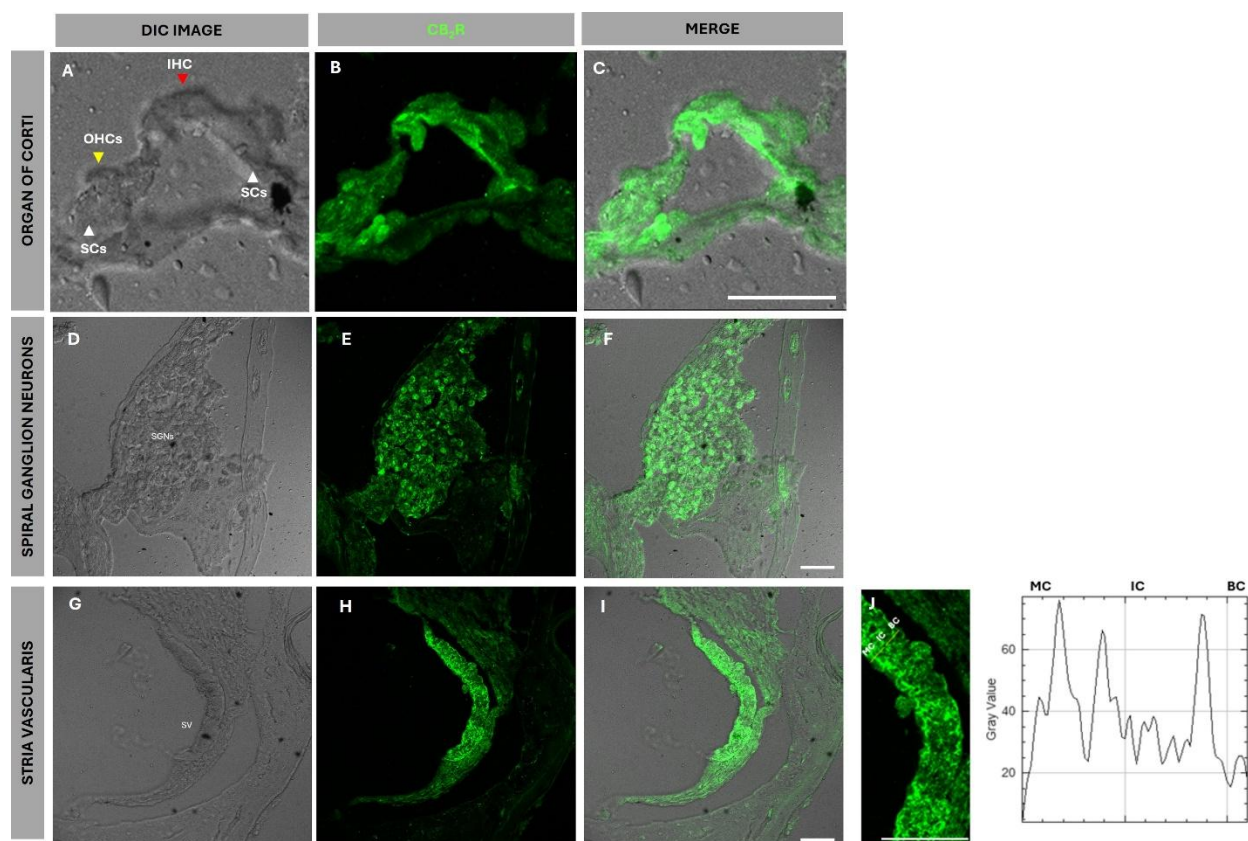


Figure 1.3. CB₂R localization in the OC, SGNs and SV of the adult murine cochlea. DIC microscopy image of the OC (A). IHC is indicated by red arrows, OHCs are indicated by yellow arrows and SCs are indicated by white arrows. Representative maximum projection confocal image showing CB₂R expression (in green) (B) in the OC. Overlay of CB₂R with corresponding DIC image (C). DIC microscopy image of the SGN (D). Representative maximum projection confocal image showing CB₂R expression (in green) in the SGN (E). Overlay of CB₂R with corresponding DIC image (F). DIC microscopy image of the SV (G). Representative maximum projection confocal image showing CB₂R expression (in green) in the SV (H). Overlay of CB₂R with corresponding DIC image (I). Zoomed image showing CB₂R expression in the MC, IC and BC layers of SV and the yellow line indicates the region used to generate fluorescence intensity plot profile (J), scale bar: 50 μ m. Abbreviations: BC: Basal cell layer; CB₂R, cannabinoid receptor 2; DIC, Differential interference contrast; IC: Intermediate cell layer; IHC, Inner hair cell; MC: Marginal cell layer; OHC: Outer hair cell; OC: organ of Corti, SC: Supporting cell; SGN: spiral ganglion neurons; SV: Stria vascularis.

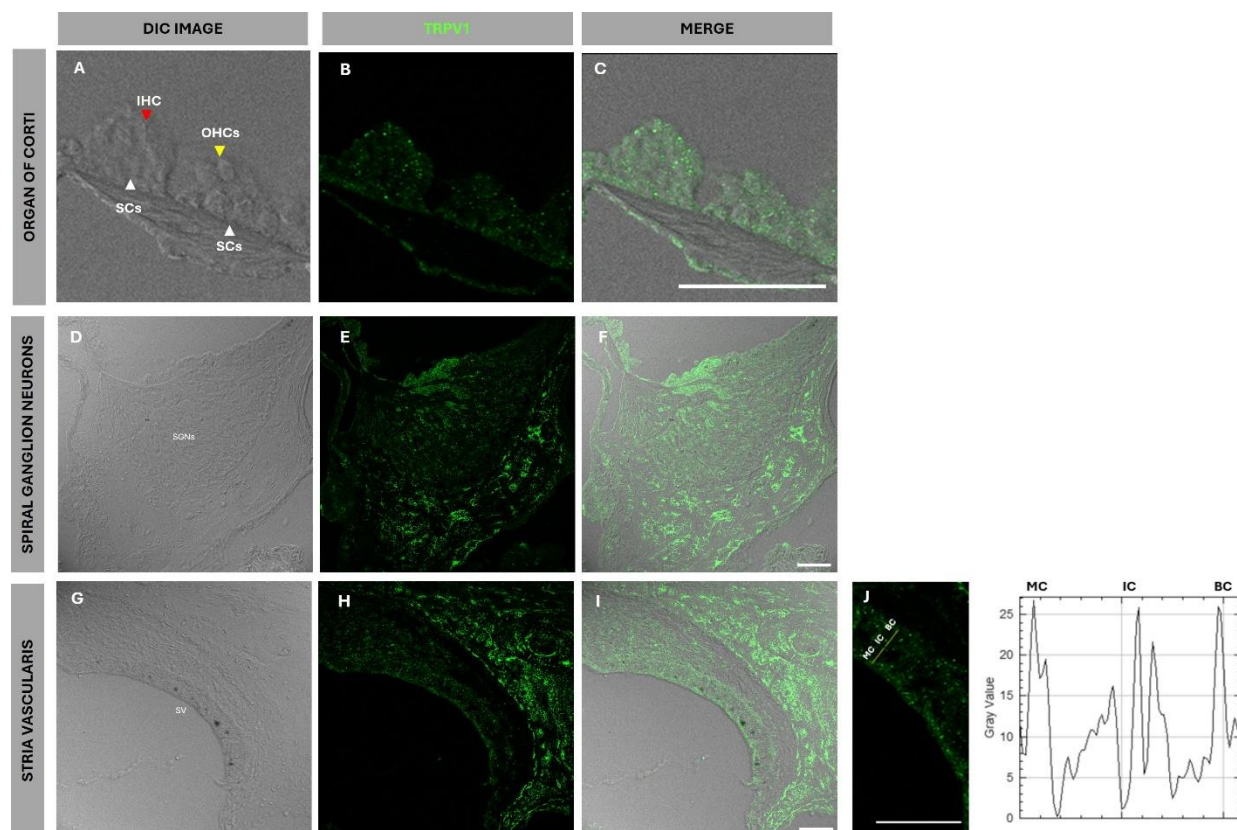


Figure 1.4. TRPV1 localization in the OC, SGNs and SV of the adult murine cochlea. DIC microscopy image of the OC (A). IHC is indicated by red arrows, OHCs are indicated by yellow arrows and SCs are indicated by white arrows. Representative maximum projection confocal image showing TRPV1 expression (in green) (B) in the OC. Overlay of TRPV1 with corresponding DIC image (C). DIC microscopy image of the SGN (D). Representative maximum projection confocal image showing TRPV1 expression (in green) (E) in the SGN. Overlay of TRPV1 with corresponding DIC image (F). DIC microscopy image of the SV (G). Representative maximum projection confocal image showing TRPV1 expression (in green) in the SV (H). Overlay of TRPV1 with corresponding DIC image (I). Zoomed image showing TRPV1 expression in the MC, IC and BC layers of SV and the yellow line indicates the region used to generate fluorescence intensity plot profile (J), Scale bar: 50 μ m. Abbreviations: BC: Basal cell layer; DIC: Differential interference contrast; IC: Intermediate cell layer; IHC: Inner hair cell; MC: Marginal cell layer; OHC, Outer hair cell; OC: organ of Corti, SC: Supporting cell; SGN: spiral ganglion neurons; SV: Stria vascularis; TRPV: Transient receptor potential vanilloid.

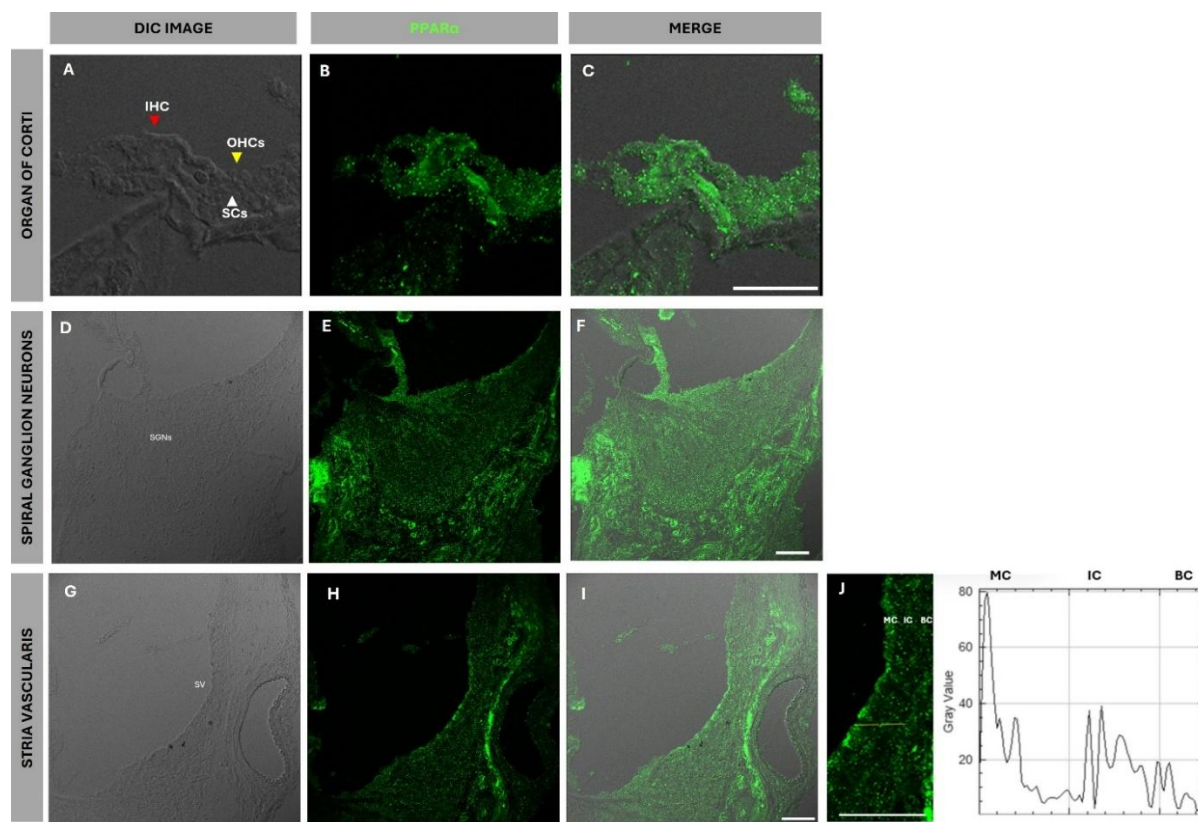


Figure 1.5. PPAR α localization in the OC, SGNs and SV of the adult murine cochlea. DIC microscopy image of the OC (**A**). IHC is indicated by red arrows, OHCs are indicated by yellow arrows and SCs are indicated by white arrows. Representative maximum projection confocal image showing PPAR α expression (in green) (**B**) in the OC. Overlay of PPAR α with corresponding DIC image (**C**). DIC microscopy image of the SGN (**D**). Representative maximum projection confocal image showing PPAR α expression (in green) in the SGN (**E**). Overlay of PPAR α with corresponding DIC image (**F**). DIC microscopy image of the SV (**G**). Representative maximum projection confocal image showing PPAR α expression (in green) in the SV (**H**). Overlay of PPAR α with corresponding DIC image (**I**). Zoomed image showing PPAR α expression in the MC, IC and BC layers of SV and the yellow line indicates the region used to generate fluorescence intensity plot profile (**J**), scale bar: 50 μ m. Abbreviations: BC: Basal cell layer; DIC: Differential interference contrast; IC: Intermediate cell layer IHC: Inner hair cell; MC: Marginal cell layer; OHC: Outer hair cell; PPAR α : peroxisome proliferating activated receptor α ; OC: organ of Corti; SC: Supporting cell; SGN: spiral ganglion neurons; SV: Stria vascularis.

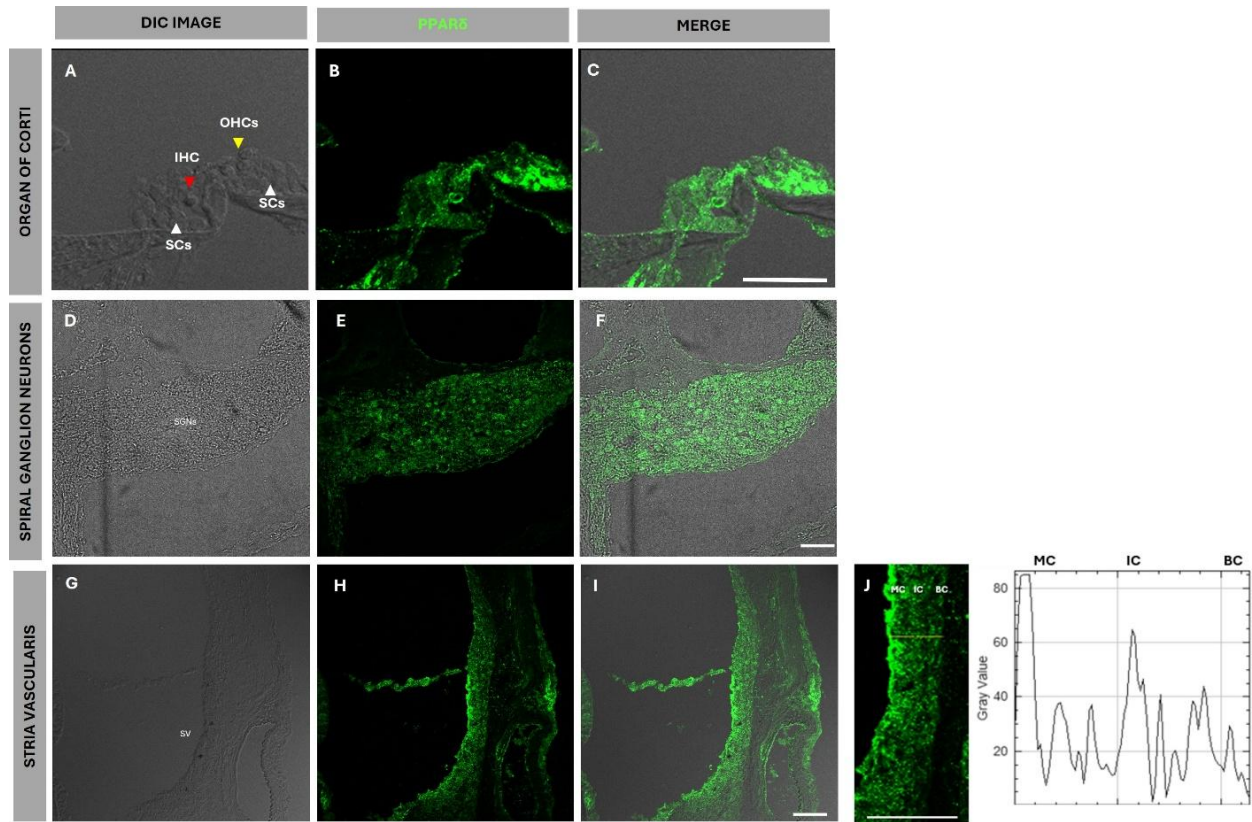


Figure 1.6. PPAR δ localization in the OC, SGNs and SV of the adult murine cochlea. DIC microscopy image of the OC (**A**). IHC is indicated by red arrows, OHCs are indicated by yellow arrows and SCs are indicated by white arrows. Representative maximum projection confocal image showing PPAR δ expression (in green) (**B**) in the OC. Overlay of PPAR δ with corresponding DIC image (**C**). DIC microscopy image of the SGN (**D**). Representative maximum projection confocal image showing PPAR δ expression (in green) (**E**) in the SGN. Overlay of PPAR δ with corresponding DIC image (**F**). DIC microscopy image of the SV (**G**). Representative maximum projection confocal image showing PPAR δ expression (in green) in the SV (**H**). Overlay of PPAR δ with corresponding DIC image (**I**). Zoomed image showing PPAR δ expression in the MC, IC and BC layers of SV and the yellow line indicates the region used to generate fluorescence intensity plot profile (**J**), scale bar: 50 μ m. Abbreviations: DIC: Differential interference contrast; IHC: Inner hair cell; OHC: Outer hair cell; PPAR δ : peroxisome proliferating activated receptor δ ; OC: organ of Corti; SC: Supporting cell; SGN: spiral ganglion neurons; SV: Stria vascularis.

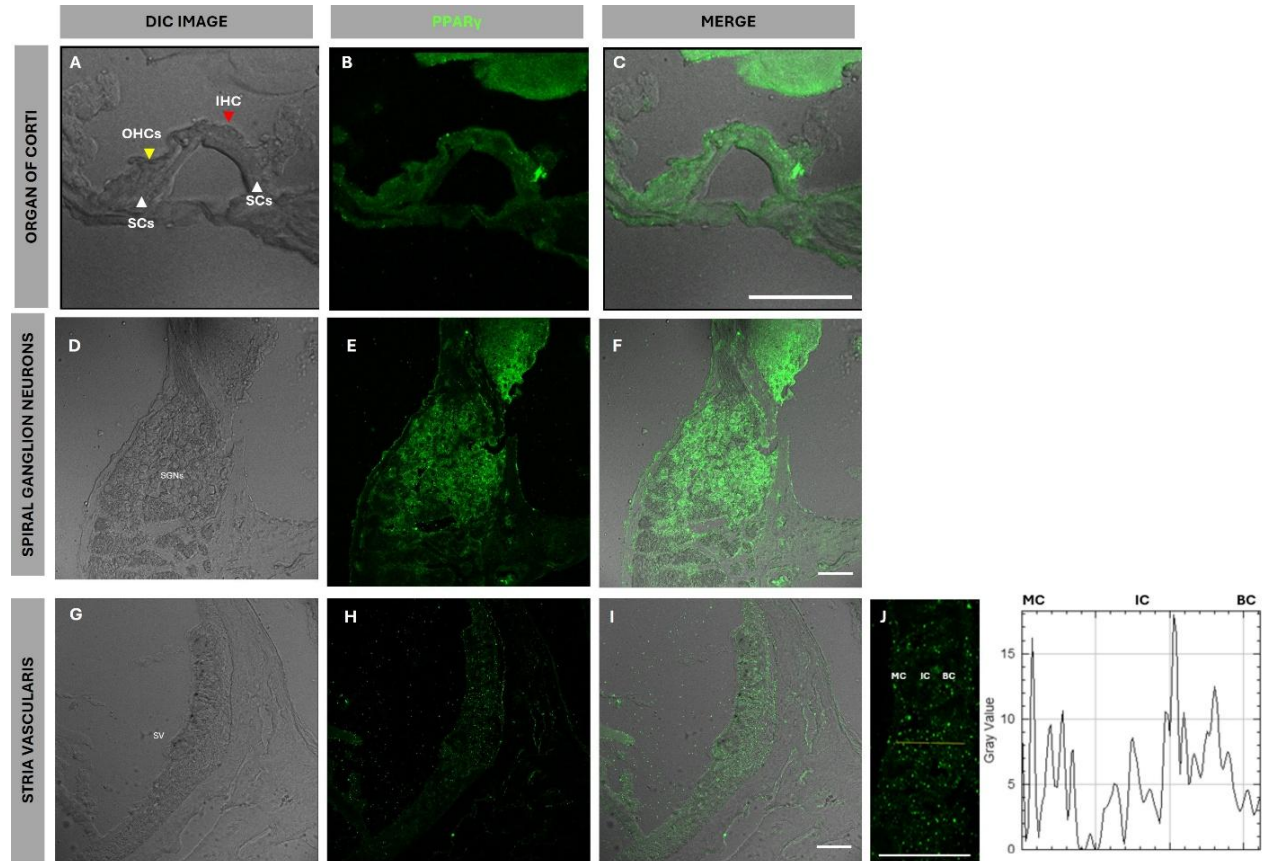


Figure 1.7. PPAR γ localization in the OC, SGNs and SV of the adult murine cochlea. DIC microscopy image of the OC (A). IHC is indicated by red arrows, OHCs are indicated by yellow arrows and SCs are indicated by white arrows. Representative maximum projection confocal image showing PPAR γ expression (in green) (B) in the OC. Overlay of PPAR γ with corresponding DIC image (C). DIC microscopy image of the SGN (D). Representative maximum projection confocal image showing PPAR γ expression (in green) in the SGN (E). Overlay of PPAR γ with corresponding DIC image (F). DIC microscopy image of the SV (G). Representative maximum projection confocal image showing PPAR γ expression (in green) in the SV (H). Overlay of PPAR γ with corresponding DIC image (I). Zoomed image showing PPAR γ expression in the MC, IC and BC layers of SV and the yellow line indicates the region used to generate fluorescence intensity plot profile (J), scale bar: 50 μ m. Abbreviations: BC: Basal cell layer; DIC: Differential interference contrast; IC: Intermediate cell layer; IHC: Inner hair cell; MC: Marginal cell layer; OHC: Outer hair cell; PPAR γ : peroxisome proliferating activated receptor γ ; OC: organ of Corti, SC: Supporting cell; SGN: spiral ganglion neurons; SV: Stria vascularis.

2. Molecular assessment of cochlear damage in non-responders of the selected cisplatin-induced ototoxic model

Since some animals in our established cisplatin model cohort (see section 5.1) did not exhibit functional impairment (non-responders), we aimed to understand if subtle early molecular changes could occur in the cochlea prior to functional damage. For this purpose, principal cochlear markers (*pou4f3*, *myo7a*), SCs (*sox2*) and SGNs (*pou4f1*, *tubb3*) were profiled through bulk-RNA sequencing in the cochleae of non-responders. Upon cisplatin we observed statistically significant decrease in the expression of *pou4f3* transcript ($p=0.0014$) indicating HC related subtle molecular changes relevant to ototoxic damage (Figure 2.1A). This finding is consistent with previous studies showing reduced POU4F3 levels at the gene and protein level upon ototoxic damage [1,2]. Mean TPM levels of each transcript for NH animals (n=4) and cisplatin-treated animals (n=3) are provided as a heatmap (Figure 2.1B).

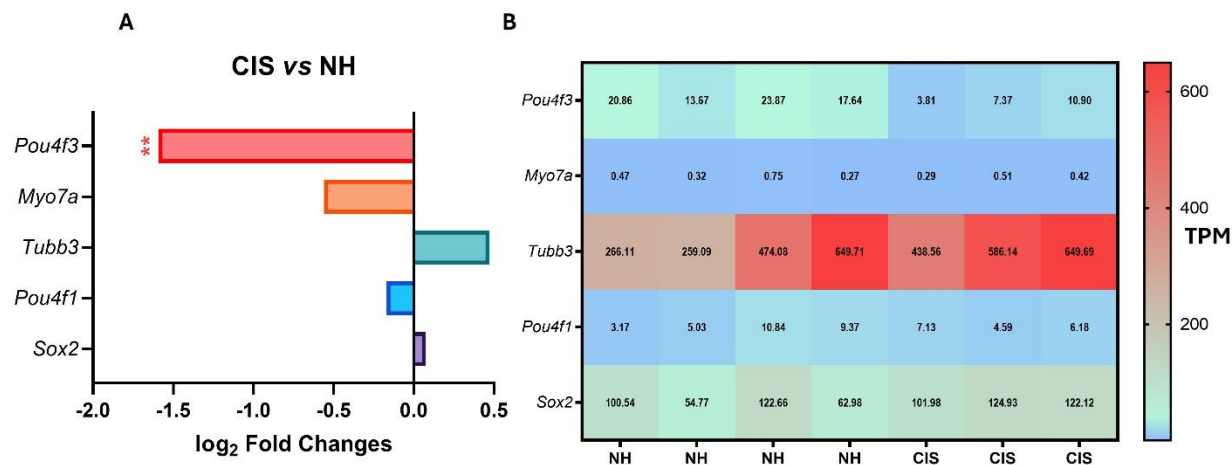


Figure 2.1. Transcriptomic analysis of key HC, SC and SGN markers upon cisplatin-induced ototoxicity. Log fold changes of genes *pou4f3*, *myo7a*, *tubb3*, *pou4f1*, and *sox2* in CIS-treated cochleae compared to untreated cochleae. Statistical analysis was performed using Benjamini–Hochberg FDR approach (** $p<0.05$ vs NH) (A). Individual TPM levels of each gene *pou4f3*, *myo7a*, *tubb3*, *pou4f1*, and *sox2* in NH and CIS-treated cochleae are represented as a heatmap (B). Abbreviations: CIS: cisplatin; HC: hair cell; NH: normal hearing, SC: Supporting cell; SGN: spiral ganglion neuron; TPM: transcripts per million.

3. Kanamycin-induced ototoxic mouse model

To evaluate the possible alterations of ECS receptors in response to ototoxicity, we employed a second model alongside cisplatin because it is a well-established and validated model developed at UMC Utrecht (our project partner) [3]. Additionally, kanamycin is also widely used for investigating general and broader molecular mechanisms underlying ototoxicity [4-8]. The ototoxic effects of kanamycin were assessed by measuring ABR threshold shifts along with amplitudes and latencies of waves I and II on day 0 and day 7. Among the 21 animals used in the study, 42.85% of animals (9/21 animals) showed ~10 dB - 55 dB of differences in thresholds between day 0 and day 7, 6 animals showed <10 dB of differences in thresholds whereas the rest did not show any changes at day 7 compared to day 0. Inter-individual variability with kanamycin has also been documented in other studies [3,8,9]. Collective analysis from the cohort showed significant differences in thresholds between day 0 and 7 ($p=0.0044$), as shown in figure 3.1A. Representative waveforms on day 0 and 7 for the cohort are shown in figure 3.1C. Importantly, animals showed no significant loss in body weight (Figure 3.1B) throughout the study, suggesting no overall systemic toxicity. Additionally, amplitudes and latencies of waves I and II were analyzed across 0-60 dB (in attenuation) intensities, to look for potential alterations in the strength and conduction of neural responses. In our model, amplitudes of wave I showed a trend toward decrease at day 7 compared to day 0 across all intensities, but statistically significant differences were observed only at 0 dB in attenuation ($p=0.0003$) (Figure 3.2A), pointing towards impaired synaptic transmission from HCs to the auditory nerve [10]. However, latencies of wave I across sound levels did not show any differences (Figure 3.2B). Regarding wave II, no statistically significant changes in both amplitudes and latencies were observed on day 7 with respect to day 0 (Figure 3.2C, D). Four responders with >20 dB differences in thresholds were chosen to perform molecular studies. To characterize the molecular damage in the cochlea, key representative markers of HCs, SCs and SGNs were profiled. No statistical significance was observed for any transcript analyzed (Figure 3.3A). This finding might suggest that the observed functional impairment may not be driven by transcriptomic changes but could involve post-transcriptomic/post-translational alterations. Alternatively, subtle cell type specific changes might be masked by the bulk tissue analysis. Individual TPM levels of each gene for untreated cochleae (n=4) and kanamycin-treated (n=4) cochleae are provided in figure 3.3B.

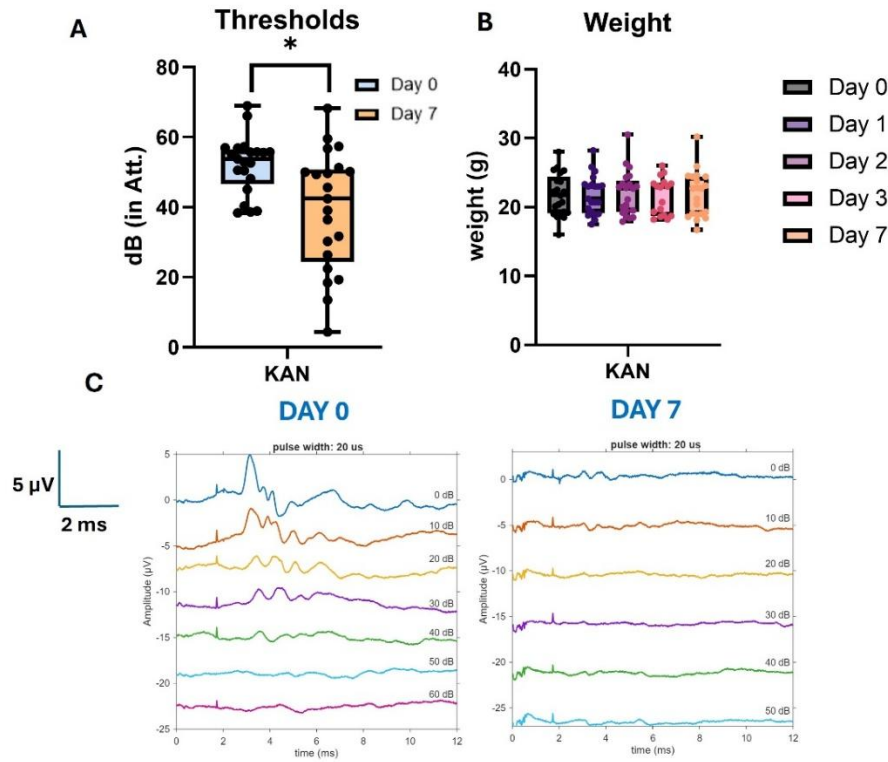


Figure 3.1. ABR analysis of kanamycin-induced ototoxic model. Collective thresholds of 21 animals on day 0 and day 7 represented as a box and whiskers plot with min and max values. Statistical analysis is performed using two-tailed paired t -test, * $p < 0.05$ (A). Weights of each animal (each color represents an animal) measured across the experiments at days 0, 1, 2, 3 and 7 shown as symbols with connecting lines (B). Representative ABR waveforms of the group on day 0 and on day 7 (C). Abbreviations: ABR: Auditory brainstem response; KAN: kanamycin.

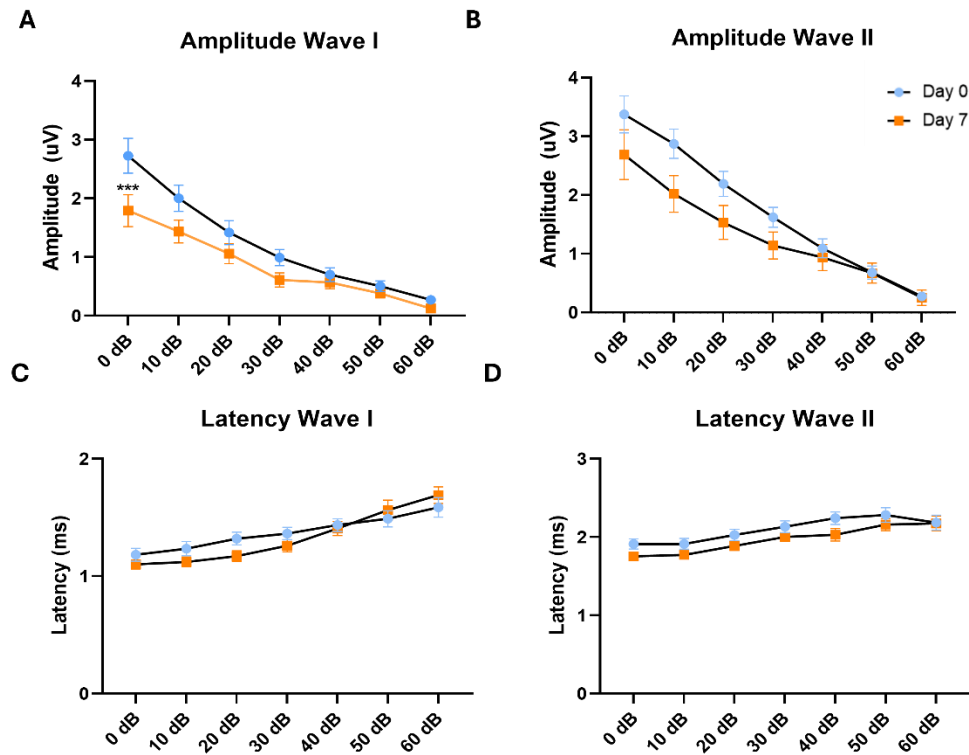


Figure 3.2. Amplitude and latency analyses of ABR waves I and II of kanamycin-induced ototoxic model. Amplitudes of wave I (**A**) and wave II (**B**) measured across 0 dB attenuation to 60 dB in attenuation for 21 animals. Latencies of wave I (**C**) and wave II (**D**) measured across 0 dB attenuation to 60 dB attenuation for 21 animals. Statistical analysis was performed using repeated measures two-way ANOVA (mixed effects model) with Bonferroni post hoc comparison test. *** $p < 0.001$, Data are represented as mean $\pm$ SEM for 21 animals.

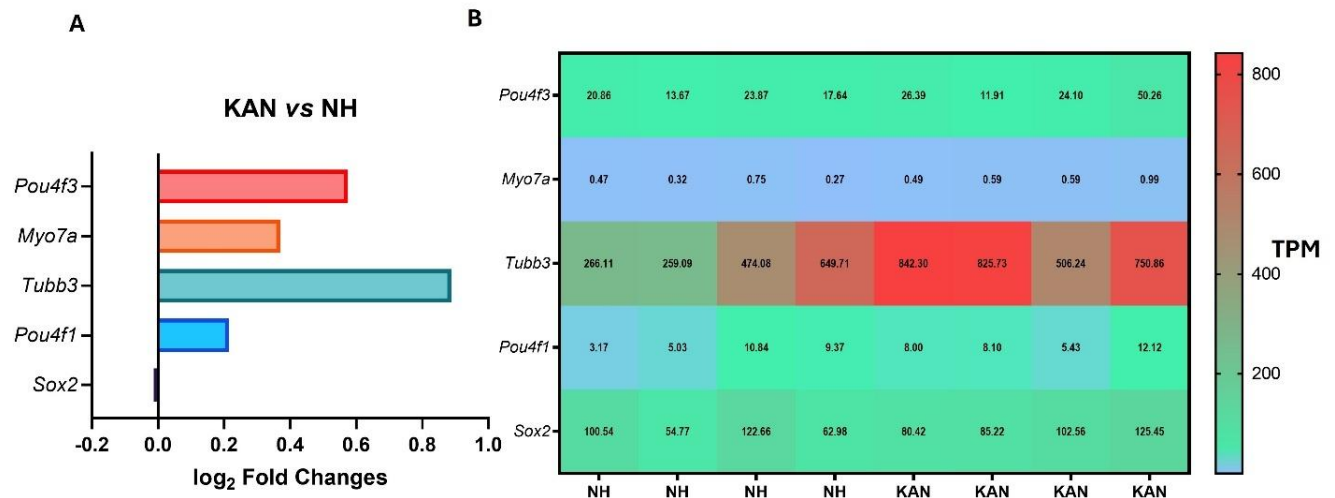


Figure 3.3. Transcriptomic analysis of key HC, SC and SGN markers upon kanamycin-induced ototoxicity. Log fold changes of genes *pou4f3*, *myo7a*, *tubb3*, *pou4f1*, and *sox2* in KAN-treated cochleae compared to NH cochleae. Statistical analysis was performed using Benjamini–Hochberg FDR approach (**A**). Individual TPM levels of each gene *pou4f3*, *myo7a*, *tubb3*, *pou4f1*, and *sox2* in NH and KAN-treated cochleae represented as a heat map (**B**). Abbreviations: FDR: False discovery rate; HC: hair cell; KAN: kanamycin; NH: normal hearing, SC: Supporting cell; SGN: spiral ganglion neuron; TPM: transcripts per million.

4. Kanamycin and cisplatin differentially regulate gene expression of eCB receptors

To investigate if ototoxic drugs impact eCB receptor gene expression, bulk transcriptomic analysis of treated cochleae were compared with those of untreated cochleae. Interestingly, kanamycin selectively upregulated *trpv1* gene expression ($p=0.0402$) in a statistically significant manner (Figure 4.1A) compared to NH cochleae. No changes in other receptor transcripts were observed upon kanamycin-induced ototoxicity. On the other hand, cisplatin did not induce any modulation of eCB receptors (Figure 4.1B). However, a trend toward decrease close to the statistical significance was observed for *pparg* ($p=0.0701$). To further highlight the divergence in transcriptional responses between the ototoxic drugs, differential gene expression analysis of the eCB receptors in cisplatin-treated cochleae *versus* that of kanamycin-treated was performed (Figure 4.1C). The analysis showed a significant downregulation of *trpv1* ($p=0.0002$) in kanamycin compared to cisplatin, indicating that *trpv1* gene modulation is a kanamycin-specific effect. This finding highlights potential drug-specific involvement of the ECS in ototoxic scenarios.

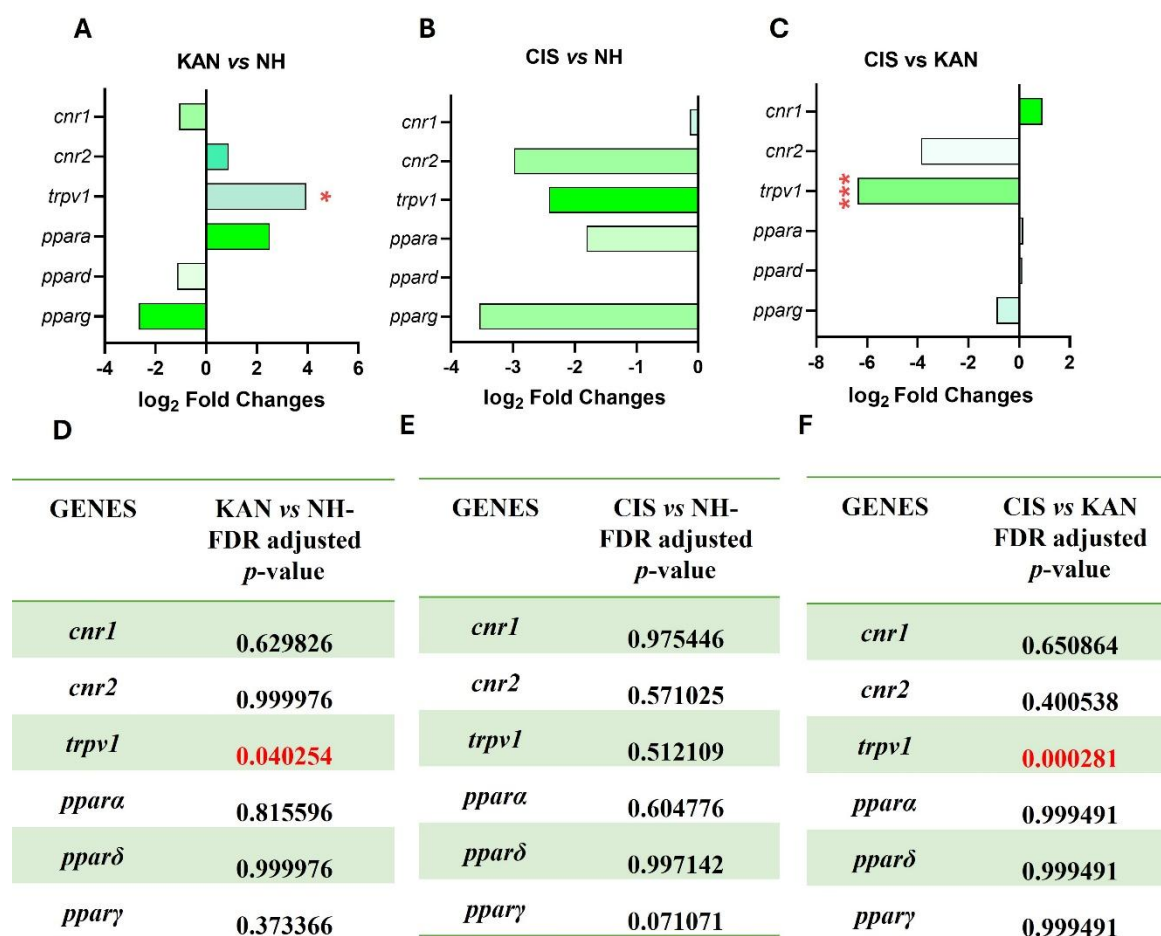


Figure 4.1. Differential gene expression analysis of eCB receptors in kanamycin and cisplatin-induced ototoxicity. Log₂ Fold changes of eCB receptor genes in KAN-treated *versus* NH animals (**A**), CIS-treated *vs* NH animals (**B**), CIS-treated *vs* KAN-treated animals (**C**). Log₂ Fold changes and their corresponding adjusted *p*-value for KAN-treated *versus* NH (**D**), CIS-treated *vs* NH (**E**) and CIS-treated *versus* KAN-treated (**F**). Statistical analysis was performed using Benjamini–Hochberg FDR approach, * *p* < 0.05 *** *p* < 0.001. Abbreviations: CIS: cisplatin; eCB: endocannabinoid; FDR: False discovery rate; KAN: kanamycin; NH: normal hearing; TPM: transcripts per million.

Overall, the abovementioned preliminary findings provided a comprehensive overview of the ECS transcriptomic signature in two experimental models of ototoxicity. Furthermore, drug-specific and possibly convergent molecular players in ototoxicity were shown, strengthening the involvement of the ECS in cochleotoxicity. Given the limited sample size, further studies with larger number of samples are planned to strengthen the statistical validity of our results.

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List of Publications (peer-reviewed)

- [1] **Palaniappan, S***, Tisi, A*, Di Meo, C., Urbano, C., Fenton, G. E., Della Valle, F., Fanti, F., Compagnone, D., Nazarè, M., Versnel, H., Aramini, A., Allegretti, M., & Maccarrone, M. (2026). Unraveling Endocannabinoid Signaling Pathways in Cisplatin-Induced Ototoxicity. *FASEB journal* 40 (4), e71568. 10.1096/fj.202502888RR
- [2] Di Meo, C., Tisi, A., Lizzi, A, R., **Palaniappan, S.**, Pulcini, F., Cinque, B., Delle Monache, S., Nazarè, M., Hsu, E., Rapino, C & Maccarrone, M. (2025) Development of a Human RPE In Vitro Model with AMD-like Features Reveals Blue Light-Induced Modulation of the Endocannabinoid System. *Biochemical and Biophysical Research Communications*, 767.10.1016/j.bbrc.2025.151896
- [3] Tisi, A., Scipioni, L., Carozza, G., Di Re, L., Cimino, G., Di Meo, C., **Palaniappan, S.**, Valle, F. D., Fanti, F., Giacobazzo, G., Compagnone, D., Maccarrone, R., Oddi, S., & Maccarrone, M. (2024). Alterations of endocannabinoid signaling and microglia reactivity in the retinas of AD-like mice precede the onset of hippocampal β -amyloid plaques. *Journal of neurochemistry*, 169. 10.1111/jnc.16256
- [4] Tisi, A*, **Palaniappan, S***, & Maccarrone, M. (2023). Advanced Omics Techniques for Understanding Cochlear Genome, Epigenome, and Transcriptome in Health and Disease. *Biomolecules*, 13(10), 1534. 10.3390/biom13101534
- [5] Cappelletti, P., Gallo, G., Marino, R., **Palaniappan, S.**, Corbo, M., Savoia, C., & Feligioni, M. (2023). From cardiovascular system to brain, the potential protective role of Mas Receptors in COVID-19 infection. *European journal of pharmacology*, 959. 10.1016/j.ejphar.2023.176061
- [6] Marino, R., Buccarello, L., Hassanzadeh, K., Akhtari, K., **Palaniappan, S.**, Corbo, M., & Feligioni, M. (2023). A novel cell-permeable peptide prevents protein SUMOylation and supports the mislocalization and aggregation of TDP43. *Neurobiology of disease*, 188. <https://doi.org/10.1016/j.nbd.2023.106342>

***Shared first authorship**

List of conferences attended

➤ Talks

[1] Systematic profiling of the endocannabinoid system and their alterations in an *in vitro* model of cisplatin induced ototoxicity – Lazio-Abruzzo-Molise-Sardegna Interregional Section of the Italian Society of Biochemistry and Molecular Biology (LAMS-SIB) meeting (July 2025), L'Aquila, Italy

➤ Poster presentations

[2] Emerging novel biomolecular targets of cisplatin -induced ototoxicity: the imbalance of the endocannabinoid system – Inner Ear Biology (IEB) meeting (September 2025), Tübingen, Germany

[3] Imbalance in endocannabinoid signalling within an *in vitro* model of cisplatin-induced ototoxicity unveils novel biochemical targets- Annual DISCAB Young researcher's day, University of L'Aquila (February 2025), Italy

[4] Alterations of endocannabinoid signalling in an organ of Corti cell line upon cisplatin induced ototoxicity- 48th FEBS meeting (July 2024), Milan, Italy

[5] Characterization of the endocannabinoid system in organ of Corti cells and its modulation upon cisplatin-induced ototoxicity-3rd Annual DISCAB Young researcher's day, University of L'Aquila (February 2024), Italy

[6] The role of bioactive lipids and resolution of inflammation in the sensorineural degeneration of the cochlea-2nd Annual DISCAB Young researcher's day, University of L'Aquila (February 2023), Italy

Tesi redatta con il cofinanziamento dell'Unione europea-*NextGeneration EU*

Piano Nazionale di Ripresa e Resilienza Missione 4, Componente 2 –

Investimento 3.3. del PNRR – “Dottorati innovativi che rispondono ai fabbisogni di innovazione delle imprese” – CUP E11I22000230001