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Extracellular Vesicles-mediated communication among brain cells

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Preface

This thesis results from the research activity conducted during my Ph.D. in Biochemistry and Molecular Biology (BiBiM 2.0) starting in 2019. The Pegaso initiative of the Tuscan Region sponsored the BiBiM 2.0 Ph.D. program, and I conducted most of my work in the Biochemistry lab of Claudia Martini, Full Professor of Biochemistry, at the Department of Pharmacy of the University of Pisa, Italy. In 2021, I spent six months in the lab of Marçal Vilar at the Molecular Basis of Neurodegeneration Unit lab, located in the Institute of Biomedicine of València, Spain.

The main topic of my Ph.D. thesis is the role of extracellular vesicles (EVs) as mediators of intercellular communication in the brain. This choice was motivated by a naïve interest in the basic biochemistry underlying these subcellular structures. Furthermore, EVs represent an excellent opportunity to build fully biological protein- or nucleic acid- based drugs in the future. This premise prompted our research group to investigate two aspects of EV function. On the one hand, I set-up and validated one protocol for extracting EVs from two human microglial cell lines. I performed a biophysical characterization on these EVs and investigated their biological effect both on microglia itself and on a line of glioblastoma cells. We started analyzing the effect on EVs from untreated microglia, and we carried on by analyzing EVs from inflamed microglia. Next, I investigated one possible role of neurotrophins as a molecular system by which EVs could gain specificity of action. This was motivated by my previous studies on neurotrophins and their receptors, performed during my master's degree, and by the possibility of joining the lab of Marcel Vilar, an internationally recognized expert on neurotrophins. The results of these studies have been organized into three chapters, and one appendix.

In Chapter 1, I will introduce EVs, presenting a brief historical context and how they are classified. I will focus on their function in cell-to-cell communication and their implication in neuroinflammation, which will be a significant part of this thesis. I will also talk about the present knowledge of their production and secretion from the parental cells and their uptake by the recipient cells.

In Chapter 2, I am going to give an introduction concerning microglia and the role of microglia-derived EVs in the communication among brain cells. After that, I will present my experimental work in characterizing the biophysical properties of EVs extracted from two microglia cell lines (C20 and HMC3), investigating their biological effect and ability to propagate inflammation to neighboring cells.

Chapter 3 will focus on a possible mechanism to increase EVs' targeted delivery. The first part will introduce the neurotrophin system, and the second part will integrate all the experimental work I performed on the neurotrophins and their receptors, with a particular focus on the investigation of the NGF-mediated p75-TrkA interaction as a molecular mediator of EVs uptake by recipient cells.

In Chapter 4, I will draw the main conclusions from my experimental work and delineate how this could be prosecuted in the following years.

Finally, appendix I will describe the work I've done during my period abroad in the Institute of Biomedicine of Valencia. This work didn't involve extracellular vesicles but was dedicated to a better understanding of the neurotrophin system.

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Abbreviation List

5-HT	Serotonin
AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
AuNPs	Gold Nanoparticles
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CHOL	Cholesterol
CNS	Central Nervous System
DLS	Dynamic Light Scattering
ECD	Extracellular Domain
EnR	Endoplasmic Reticulum
ESCRT	Endosomal Sorting Complex Required For Transport
ESE	Early Sorting Endosome
EVs	Extracellular Vesicles
GBM	Glioblastoma Multiforme
GSCs	Glioma Stem Cells
hnRNPA2B1	Heterogeneous Nuclear Ribonucleoprotein A2B1
ICD	Intracellular Domain
IL-1 β	Interleukin-1 β
IL4	Interleukin-4
IL-6	Interleukin-6
ILVs	Intraluminal Vesicles
iPSCs	Induced Pluripotent Stem Cells
ISEV	International Society Of Extracellular Vesicles
LEVS	Large Extracellular Vesicles
LPS	Lypopolysaccharide
LSEs	Late Sorting” Endosomes
MS	Multiple Sclerosis
MSCs	Mesenchymal Stem Cells
MVBs	Multivesicular Bodies
NGF	Nerve Growth Factor
NK	Natural Killer
NT	Untreated
NTs	Neurotrophins
PD	Parkinson's Disease

PS	Phosphatidylserine
RIP	Regulated Intramembrane Proteolysis
ROS	Reactive Oxygen Species
SAPs	Single And Aggregated Proteins
SGCs	Superior Cervical Ganglion
SEVs	Small Extracellular Vesicles
SM	Sphingomyelin
SNARE	Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptors
TACE	Tnf α -Converting Enzyme
TAMs	Tumor-Associated Microglia/Macrophages
TEM	Transmission Electron Microscopy
TEMs	Tetraspanin-Enriched Microdomains
TGF- β	Transforming Growth Factor B
TKD	Tyrosine-Kinase Domain
TNF- α	Tumor Necrosis Factor A
TrkA	Tropomyosin-Related Kinase A

1 Extracellular Vesicles: what they are and what they do

1.1 General knowledge of Extracellular Vesicles (EVs)

The first observations of EVs date back to the mid-1900s; they were conducted in various tissues differently but without fully understanding the importance and significance of this form of cellular communication. The real discovery came in the 80s. Ultrastructural studies conducted during the differentiation of sheep red blood cells showed that multivesicular bodies released vesicles after their fusion with the membrane cells; “exosome” was the name given to these vesicles, but until 1989 they were classified as cellular debris (Johnstone et al., 1987). More than a decade later, Zitvogel et al. observed that B lymphocytes and dendritic cells transfected with Epstein Barr virus could secrete EVs with antigen-presenting membrane and were able to induce a T-lymphocyte

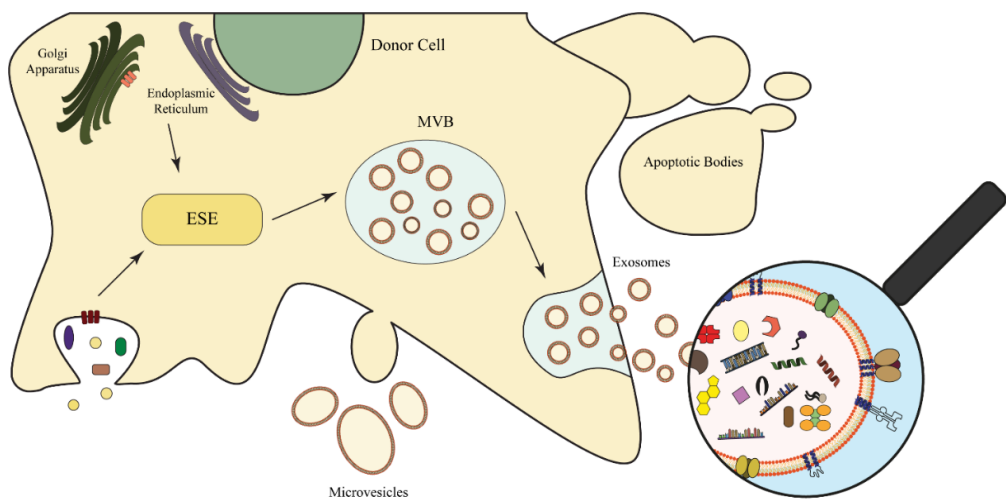


Figure 1-1 Schematics of all type of Extracellular Vesicles, each of them have very different diameters, biogenesis and secretion mechanisms. Extracellular vesicles are produced through a variety of different cellular processes: apoptotic bodies (>1000 nm in diameter) derive from cell disassembly, microvesicles (>200 nm in diameter) are secreted by budding of plasma membrane, and exosomes (<200 nm in diameter) are intraluminal vesicles derived from multivesicular bodies (MVB).

response (Zitvogel et al., 1998). After discovering that EVs could also carry RNA and miRNA, they were considered carriers of potential

immunomodulatory effects and anti-tumor immunity (Raposo et al., 1996; Valadi et al., 2007; Zitvogel et al., 1998).

At first, we thought EVs' only function was to dispose of cellular waste. In reality, cells can exploit this mechanism for communication. Our current knowledge is that most cell types secrete EVs, which can play a vital role in cell-to-cell communication under physiological and pathological conditions (Huang et al., 2022; Kluszczynska and Czyz, 2023). They exchange genetic information and all kinds of biomolecules (Sung and Weaver, 2017; Yang et al., 2017) and play an essential role in supporting many physiological processes, such as cell growth, development, differentiation, and apoptosis (Men et al., 2019).

EVs can contain macromolecules specific to the cell of origin, such as lipids, proteins, and nucleic acids, thus determining both the protection of information and communication with target cells. However, they can also contain different molecules, such as mRNA and miRNAs, capable of modifying or altering the gene expression profile of target cells (Aili et al., 2021; Gurunathan et al., 2021; Kalluri and LeBleu, 2020; Xu et al., 2018).

EVs also play a role in several pathological conditions. Indeed, altered EVs-mediated intercellular communication appears to be involved in some neurodegenerative diseases. In cancer cells, EVs are released not only in a significantly altered manner, but they can also alter the function of healthy cells, such as lymphocytes, by reducing their anti-tumor immune response (Kalluri and LeBleu, 2020). Recent studies state, with increasing clarity, that secretion of abnormal and dysfunctional EVs significantly affects the genesis, development, and therapy of human malignancies (Boriachek et al., 2018; Steinbichler et al., 2017).

Finally, the presence of EVs in different biological fluids (plasma, cerebrospinal fluid, urine, saliva) is essential for their possible use as biomarkers or as vectors for drugs in gene therapy.

Despite the tremendous increase in scientific interest in EVs, the knowledge of these nanometer-sized particles is still limited, and understanding the complex molecular mechanisms guiding EVs' biogenesis, role, and physiological relevance is an urgent need (Heidarzadeh et al., 2021). As a result, developing biotechnological products based on EVs is still far from meeting the clinical standard.

1.2 EVs classification

Cells can release various subtypes of membrane structures, EVs being one of them. The content, size, and composition of the membrane of EVs are very heterogeneous and variable and depend on the state of the parental cell and the microenvironment conditions. This heterogeneity has led to a relatively disordered scientific definition of vesicles. Currently, the International Society of Extracellular Vesicles (ISEV) defines the classification of EVs, intending to unify the nomenclature and methodology for isolating EVs. The most accredited way of defining them is by clear and measurable characteristics such as size (Small EVs < 100 nm or < 200 nm and medium/large EVs > 200 nm), density (low, medium, high), biochemical composition (CD63+ /CD81+ /Annexin...), experimental conditions (hypoxic, normoxic) and cells of origin (Cocucci and Meldolesi, 2015). According to recent guidelines, the MISEV 2018 recommends using “**extracellular vesicles**” as “*a generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus.*”

However, the classification of EVs is constantly evolving, so today, two main categories of vesicles are defined, ectosomes and exosomes (Mathieu et al.,

2019). Ectosomes are vesicles generated by budding directed outward from the plasma membrane, while exosomes originate from the endosomal pathway (Lötvall et al., 2014; Mathieu et al., 2019).

1.3 Exosome biogenesis

The biogenesis of exosomes begins with the formation by endocytosis of vesicles from specialized regions of the plasma membrane (**Figure 1-2**). Firstly, the plasma membrane invaginates, forming a cup that includes the cell surface and soluble proteins associated with the extracellular environment. This cup will become an early sorting endosome (ESE), which in some cases

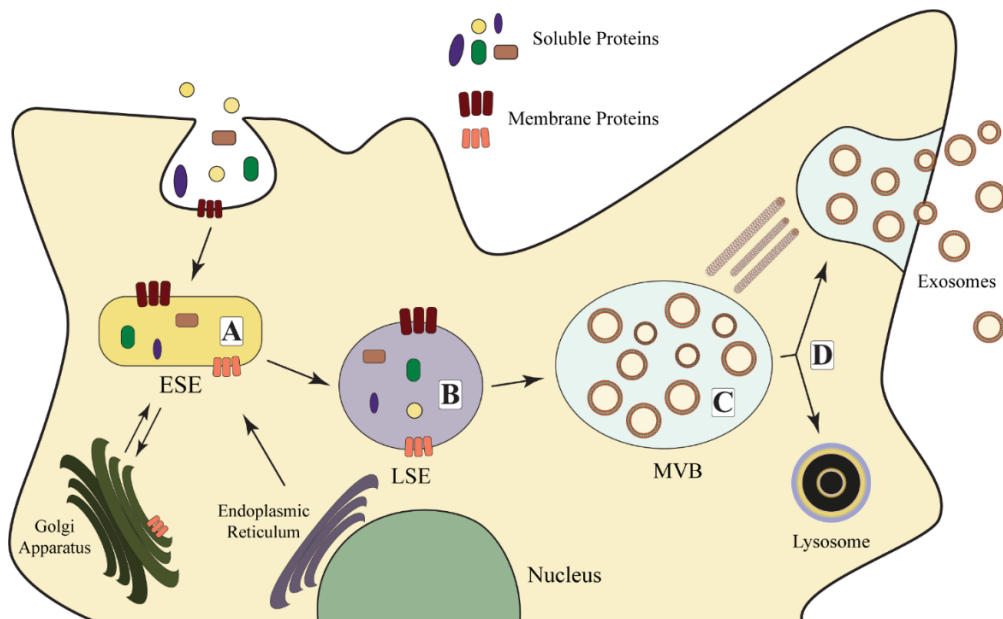


Figure 1-2 Schematics of exosome biogenesis-related portion of the endosomal pathway. The formation of an Early Sorting Endosome (ESE) (A) starts with the invagination of the outer membrane, later a Late Sorting Endosome (LSE) (B) is formed after the sorting of molecules inside the ESE. LSE can evolve in a Multivesicular Body (MVB) (C) upon the formation of Intraluminal Vesicles (ILVs) which in turn can (D) fuse with the lysosome for content degradation or fuse with the plasma membrane to secrete exosomes.

may fuse directly with a preexisting ESE or receive material for formation and content from the Golgi apparatus and endoplasmic reticulum (**Figure 1-2, A**). It is a sorting center for proteins, lipids, and nucleic acids. ESEs can mature into “late sorting” endosomes (LSEs) by acidification of the environment

within them (**Figure 1-2, B**). These two organelles can be distinguished not only by their content but also by their shape and location: in fact, the ESE possesses a more tubular shape and is located near the outer edge of the cell, while the LSE is located much closer to the nucleus and has a spherical shape.

Within the LSE, smaller vesicles can form, called intraluminal vesicles (ILVs). During this process, proteins, nucleic acids, and lipids are sorted into these small vesicles. Late endosomes containing many intraluminal vesicles are called Multivesicular Bodies (MVBs) (**Figure 1-2, C**). MVBs can then follow two routes: fuse with the lysosome and degrade all its content or fuse with the cell membrane releasing ILVs as exosomes into the extracellular space (**Figure 1-2, D**) (Kalluri and LeBleu, 2020; Keller et al., 2006).

The formation of ILVs requires two distinct processes: the first requires the presence of proteins called tetraspanins, while the second requires the presence of the ESCRT (endosomal sorting complex required for transport) complex.

Tetraspanins are part of a large family of proteins that organize the membrane into microdomains by forming aggregates with different membrane and cytosolic proteins. These microdomains are known as TEMs (tetraspanin-enriched microdomains), and exosomes are rich in such domains.

The two tetraspanins that seem to play a critical role in exosome formation are the CD9 and CD63 proteins. Indeed, they can induce membrane curvature, which is necessary for the formation of ILVs, and in addition, their connection with the cytoskeleton influences the process of vesicle fission (Andreu and Yáñez-Mó, 2014).

The other key point in forming ILVs is the recruitment of the ESCRT. This machinery consists of about 20 proteins that assemble to form four complexes together with other associated protein components, such as Vsp4, VTA1, and

Alix; these proteins collaborate to facilitate the formation of multivesicular bodies, budding of vesicles and sorting of proteins within vesicles (Frankel and Audhya, 2018; Juan and Fürthauer, 2018; Zhang et al., 2019).

However, although the ESCRT pathway is the primary process for exosome biogenesis, some studies have shown a formation process that does not involve ESCRT. Indeed, the inactivation of ESCRT does not inhibit the formation of MVBs (Stuffers et al., 2009).

This alternate process appears to involve lipid rafts (lipid rafts, microdomains of the plasma membrane rich in membrane proteins, sphingomyelin, and cholesterol). Such microdomains are rich in sphingomyelinases, enzymes that can hydrolyze sphingomyelin, leading to the formation of ceramides which, due to their cone-shaped structure, can cause inward curvature of the exosomal membrane, thus promoting microdomain invagination and the formation of ILVs (Zhang et al., 2019).

Regarding nucleic acid sorting, the mechanisms underlying this process are still not very clear. Nevertheless, their sequence, cellular abundance, and competition with targets could influence the sorting of nucleic acids, particularly miRNAs.

Analyses conducted on exosomal miRNAs have identified common sequences, called EXO-motifs, that may facilitate nucleic acid binding to RNA binding proteins, such as annexin A2, hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2B1), SYNCRIP or Y-box protein 1 (Li et al., 2018).

1.4 Exosome secretion

As previously mentioned, MVBs can be directed to lysosomes or reach the plasma membrane, with which they fuse, releasing exosomes into the

extracellular environment. However, the molecular mechanisms that regulate this balance are not yet well understood.

Recently, Villarroya-Beltri et al. observed that induction of ISGylation (a ubiquitin-like post-translational modification) could impair exosome secretion by promoting the fusion of MVBs with the lysosome, thus directing the vesicles toward degradation. This process may be a regulatory signal for the fate of MVBs (Villarroya-Beltri et al., 2017, 2016).

The release of exosomes into the extracellular environment requires a series of steps: transport, docking to the membrane, and fusion with the membrane.

The transport of MVBs to the plasma membrane requires interaction with actin and microtubule molecules. Studies have shown the importance of cortactin, a cytoplasmic actin-binding protein. This protein is essential in transporting and docking MVBs to the plasma membrane: inhibiting its expression leads to a decrease in exosome secretion, while its overexpression causes the opposite effect (Muriel et al., 2016).

Rab GTPases are also involved in regulating many steps in MVBs trafficking, including vesicle budding, vesicle transport, and membrane fusion, although their precise mechanism of action is not yet known.

Membrane fusion is the last step to release exosomes into the external environment. Among the proteins involved in membrane fusion are SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins, tethering factors, Rabs, and Ras GTPases. SNAREs proteins facilitate the fusion of vesicles with their target membrane, lysosomes or other organelles, or the plasma membrane (Hessvik and Llorente, 2018).

Finally, it is essential to note that, as with many other cellular secretion types, intracellular calcium can regulate the exosome secretion process (Savina et al., 2003).

1.5 Exosomes uptake by recipient cells

Exosomes are used by cells for the elimination of waste products in order to maintain cellular homeostasis, but they are also vehicles used for intercellular communication under physiological and pathological conditions. For example, exosomes from cancer cells can promote angiogenesis, modulate the immune system, and remodel the surrounding parenchymal tissue (all processes that promote tumor progression) (Hessvik and Llorente, 2018). In the immune system, on the other hand, exosomes play a significant role in immunoregulation: antigen presentation, activation, and suppression of the immune system (Zhang et al., 2019).

All these functions, along with many others, can be exerted by exosomes through two different mechanisms: direct receptor-ligand stimulation or internalization of exosomes, which can occur through endocytosis or membrane fusion (Yáñez-Mó et al., 2015).

Regarding internalization by endocytosis, there are several mechanisms, among which we find clathrin-mediated or caveolin-mediated endocytosis, lipid raft-mediated, macropinocytosis, and phagocytosis. The choice of one process over another depends on the type of cell and its state. For example, both clathrin-dependent mechanism and phagocytosis have been observed in neurons, while in microglia, the process of macropinocytosis is preferred, endothelial cells prefer caveolin-mediated endocytosis, and finally, cancer cells prefer the process mediated by lipid rafts.

The other uptake mechanism occurs through the direct fusion of the vesicle membrane with the plasma membrane of the recipient cell. This process occurs primarily in cancer cells, requiring acidic pH conditions, which we find in this type of cell (Abels and Breakefield, 2016).

The exosome cargo is released into the target cells once internalized. The contained molecules can then influence the recipient cell's function through three mechanisms: ligand-receptor interaction, transfer of activated receptors to the target cell, and epigenetic reprogramming through functional proteins, lipids, and RNA (Zhang et al., 2019).

1.6 EVs Functions

Intercellular communication is an essential process for the survival of a multicellular organism. In the past, most researchers thought that cells could only communicate with each other in two ways: through direct cell-cell contact (due to gap junctions and surface protein interactions) or the secretion of specific soluble molecules, such as hormones, cytokines, and growth factors. In recent decades, however, another method of communication has been proposed, which occurs through the release of EVs (Raposo and Stoorvogel, 2013).

Questions surrounding the function of exosomes are focused mainly on understanding the fate of their constituents and the phenotypic and molecular alterations they induce in recipient cells. Exosome uptake and secretion pathways may intersect, resulting in a mixed population of exosomes produced in both the cell and the environment over time.

The distinct mechanisms and pathways associated with exosome uptake and the presumed specificity of exosomes for specific cell types further complicate the function of exosomes in intercellular communication (Mathieu et al.,

2019). For example, in human pancreatic cancer cells, oncogenic signals induced by the expression of mutated KRAS promote exosome uptake via macropinocytosis into human pancreatic cancer cells (Kamerkar et al., 2017). Human melanoma cells uptake exosomal cargo through their fusion with the plasma membrane (Parolini et al., 2009), and neurosecretory PC12 cells (derived from rat adrenal medullary tumor) rely more readily on clathrin-dependent endocytosis for uptake (Tian et al., 2014). The “turnover rate” for internalized exosomal cargo may vary depending on the cargo’s nature and the recipient cell’s metabolic state that regulates the uptake of extracellular molecules and vesicles. It is unknown whether a different mode of uptake of exosomes by recipient cells results in distinct localization, degradation, or functional outcomes of exosomal constituents (Kalluri and LeBleu, 2020). EVs enter numerous processes where intense cellular communication is required (*Table 1-1*).

Table 1-1 Main processes which involve intense intercellular communication mediated by EVs.

Involvement	Literature
Human reproduction, pregnancy, and embryonic development require precise, finely tuned, and dynamic intercellular communication.	(Capra and Lange-Consiglio, 2020; Smith and Russell, 2022)
Sperm maturation.	(Sullivan, 2015)
Presence in amniotic fluid, blood, and breast milk.	(Sullivan, 2015)
Immune responses.	(Robbins and Morelli, 2014)
Metabolic diseases and cardiovascular activity. EVs transfer metabolites and facilitate intercellular communication	(Deng et al., 2009)

through the exchange of exosomal miRNAs between pancreatic β -cells, adipose tissue, skeletal muscle, and liver in both animal and human macrophage stimulation and insulin resistance.	
In neoplasms, tumor growth and metastasis, paraneoplastic syndromes, and resistance to therapy.	(Kalluri and LeBleu, 2020)
Physiological condition in the brain. Neurons: synaptic transmission, retrograde signaling, and neurodevelopmental processes. Glia differentiation, neurite development, and implication in neuroinflammation. Pathological condition in the brain. Spreading and clearance of toxic aggregates and AD lesions. Inflammation and degradation of extracellular matrix.	(Brenna et al., 2021; Busatto et al., 2021; Gassama and Favereaux, 2021)

The role of exosomes in cancer progression is likely to be dynamic and specific depending on the type of cancer. Exosomes can (a) induce or promote neoplasia; (b) modulate cancer progression and metastasis by reciprocal exchange of exosomes from the stroma to tumor cells; (c) help in remodeling the angiogenic and extracellular matrix of the tumor microenvironment, which is vital for tumor growth and metastatic dissemination; and (d) promote resistance to various chemotherapeutic agents and antibodies (Kalluri and LeBleu, 2020).

Thus, it is clear that EVs are involved in many intercellular signals by performing autocrine and paracrine functions and controlling processes such as development, proliferation, migration, and pathogenetic mechanisms.

1.7 EVs implication in neuroinflammation

Three types of cells populate the central nervous system (CNS): neurons, astrocytes, and microglia. The primary brain cells are neurons linked together to form a dense network; they enable us to interact with our surroundings, analyze stimuli, and initiate our actions. Astrocytes help the neurons, ensuring their proper support and functioning, while microglia, representing the brain's immune component, defend other cells from external aggression that could undermine their functionality (Van den Broek et al., 2020). This complex cellular architecture uses EVs to maintain cell-cell communication and cellular homeostasis (Li and Barres, 2018). The first study on particles in the CNS was conducted in *Drosophila* about 20 years ago (Greco et al., 2001). From there on, research on the role of EVs in the brain has been increasing. It is clear that the particles are directly involved in numerous physiological processes specific to the CNS, such as in the modulation of neuronal development and membrane protein exchange within brain cells (Sierra et al., 2016). Vesicles are also involved in many pathological processes, so they have been proposed as markers for diseases such as traumatic brain injury (TBI) and stroke, Alzheimer's disease, and generally for some types of cancer and CNS disorders (Wolf et al., 2017).

In neurodegenerative diseases, the intersection of exosomal biogenesis and secretory vesicle regulation in neuronal cells has offered new insights into the putative connection between exosomes and neurodegenerative disease pathogenesis. Exosomes can promote or limit the aggregation of unfolded and

abnormally folded proteins in the brain or participate in deleting misfolded proteins, thereby exerting detoxifying and neuroprotective functions. Otherwise, exosomes may participate in the propagation and aggregation of misfolded proteins, effectively promoting the “infectivity” of protein aggregates and contributing to disease progression (Leng and Edison, 2021; Li et al., 2019; Shaimardanova et al., 2020; Sproviero et al., 2018). Such opposite functions of exosomes may not be mutually exclusive (Budnik et al., 2016).

Neuroinflammation is a significant contributing cause of all neurodegenerative diseases and has become an important therapeutic target demonstrating to reduce disease severity and improve patient outcomes in a majority of cases. Many recent studies confirmed the specific interplay between neurodegenerative disease and neuroinflammation (Amor et al., 2010; Ransohoff, 2016; Schain and Kreisl, 2017). The role of EVs in neuroinflammation can be both beneficial and detrimental, but at the moment, they are mostly known for their capability of spreading inflammatory signals in the environment. The main actors in this process are microglial cells: resident macrophages of the brain and the first line of defense and response against every sort of stimulus (Trotta et al., 2018; Vandendriessche et al., 2020).

Once microglia cells are activated by the inflammatory stimulus, they secrete ATP-containing EVs. Those EVs signal danger for neighboring cells (mainly microglia and astrocytes) which also get activated to secrete more inflammatory stimuli and further disseminate inflammation (Ceccarelli et al., 2021a; Hwang et al., 2021; Paolicelli et al., 2019; Sanwlani and Gangoda, 2021). The transport of inflammatory materials from the peripheral to the brain can induce neuroinflammation as EVs move across the blood-brain barrier

(BBB); therefore, inflammation from the periphery can cause neuroinflammation.

EVs detrimental role does not end by worsening inflammation; they are also crucial in the progression of several neurodegenerative diseases, namely Alzheimer's Disease, TBI, Parkinson's Disease, and Multiple Sclerosis. The common denominator of all these diseases is chronic neuroinflammation, which EVs can sustain, and which is the leading cause of loss of cognitive functions (Delpech et al., 2019; Groot and Lee, 2020; Hwang et al., 2021). The spreading of aggregated or misfolded proteins ($\alpha\beta$, phosphorylated Tau, α -Syn) causes the primary disruptive effects; as the cells try to get rid of these aggregates through EVs, they only worsen it by causing the formation of more aggregates in the extracellular space. Several miRNAs implicated in neural disorders, notably a part of EVs cargo, also support the already described protein-mediated effect (Delpech et al., 2019).

1.8 Therapeutic uses of EVs

In recent years, a vast number of studies on EVs have made it possible to understand their structure and many of their functions. Extracellular vesicle-mediated information exchange, including exosomes, is a newly identified form of intercellular communication that is critical in the development and physiology of brain systems. These vesicles, contain information as signaling proteins and both coding and regulatory RNAs, and can be picked up by target cells, permitting multilayer information transfer. Recent research shows that they play an important role in physiological processes such as neuron regeneration, synapse function, and behavior (Gassama and Favereaux, 2021; Rajendran et al., 2014). These vesicles also play a role in the spread of toxic amyloid proteins in neurodegenerative conditions such as prion diseases, Alzheimer's and Parkinson's diseases, inducing neuroinflammation through

information exchange between neurons and glia, and aiding tumor progression in the brain through subversion of normal cells (Rajendran et al., 2014).

The structural features of EVs, compared with their parental cells, have many advantages: small size, good stability, high selectivity for target and size distribution, diverse content within them, low immunogenicity, good tissue penetration ability (such as BBB and blood vessels) and biocompatibility (Shi et al., 2020). These vesicles can be created to carry therapeutic molecules since they can be taken up by cells and released their luminal contents. They have the potential to be biomarkers in the diagnosis of brain disorders because their composition reflects the cell of origin (Gassama and Favereaux, 2021; Rajendran et al., 2014).

The scientific community is becoming more persuaded by the results acquired, along with the numerous clinical and preclinical trials, that EVs can serve as prospective targets and therapeutic tools in a number of areas of interest in addition to being a source of biomarkers. These include cancer, cardiovascular disease, diabetes, and age-related diseases (Pancholi et al., 2022).

To date, the most promising therapeutic strategies involve a different role of EVs: (a) EVs as a therapeutic target; (b) EVs as a vehicle for drug delivery; (c) EVs as a therapeutic agent (Sun et al., 2020).

1.8.1 EVs as a therapeutic target

Different strategies that view EVs as a therapeutic target aim to inhibit different functions: biogenesis, release, uptake, altered composition, and removal of EVs (Sun et al., 2020).

The mechanism of EVs biogenesis is widely known, but the involvement of different proteins in EVs biogenesis and release depending on the cell type and its activation state remains fully understood.

In cancer cells, for example, there is increased biogenesis of EVs (Raposo and Stoorvogel, 2013). Different involved proteins can be identified as targets depending on the state of the cells, and when inhibited, they will result in less EV creation and release.

The use of drugs can also lead to the inhibition of proteins involved in the biogenesis of EVs, consequently reducing their production (Catalano and O'Driscoll, 2020). In addition, *in vivo* and *in vitro* studies have shown that drugs capable of inhibiting calcium channels can impair intracellular calcium release (Savina et al., 2003), resulting in reduced release of EVs into the blood and culture medium (Chalmin et al., 2010).

Reducing EVs uptake by recipient cells can be another goal. Only a few identified EV uptake methods are endocytosis, phagocytosis, and receptor-mediated micropinocytosis (Mulcahy et al., 2014).

The uptake of EVs by cancer cells is a significant contributor to chemoresistance. The use of EV uptake inhibitors has demonstrated a reduction in their uptake by chemosensitive cells, thus maintaining their response to chemotherapy.

Another strategy to reduce the oncogenic function of EVs in this context involves their capture and removal through specific antibodies to block ligand-receptor interaction (Kogure et al., 2020).

Despite continued research on the use of EVs as therapeutic targets, there are limitations to these strategies. Currently, the test conditions of most studies on inhibitors of EVs' biogenesis, release, and uptake are *in vitro*, and preclinical and clinical investigations are unknown. Most of these inhibitors lack specificity and efficacy because of multiple targets and pathways involved in the biology of EVs. Therefore, using EVs as therapeutic targets requires a

better understanding and analysis of the pathways involved (Pancholi et al., 2022).

1.8.2 EVs as drug vehicles

The properties that characterize EVs (small size, biocompatibility, high tissue penetration capacity, cargo transfer to the recipient cell, and adhesive molecules on its surface) make them an efficient vehicle for drug delivery (de Jong et al., 2020).

As is well known, EVs can transfer miRNAs to recipient cells. Taking advantage of the latter property, many miRNA inhibitors and miRNA sponges packaged in exosomes have emerged as novel therapeutic approaches (Monfared et al., 2019).

Given these characteristics, EVs-mediated drug or gene delivery is considered the optimal therapeutic strategy against cancer. EVs derived from mesenchymal cells, tumor cells, and immune cells can be used as nanocarriers for chemotherapeutic drug delivery to treat various cancers. The main limitations of this approach are the reduced scalability and lower loading efficiency of anticancer drugs into EVs. Recent studies have demonstrated the possibility of large-scale production of functional exosomes capable of encapsulating mRNAs through cellular nano-poration (Yang et al., 2020). In addition, several techniques can increase the loading efficiency of exosomes, improve drug delivery and cancer cell recognition, and, in particular, increase the release of glioma-derived EVs capable of causing innate immune activation (Sheybani et al., 2020)

Other limitations related to the use of EVs as a vehicle include high heterogeneity, reduced endogenous yield, reproducibility, and biodistribution, lack of methods to isolate a homogeneous population, the presence of cost-effective technologies, off-site accumulation of EVs, decreased efficiency, and

lack of complete understanding of the aspects involving EVs. To get over these restrictions, designed EVs must be developed; however, since there is currently no agreed-upon standard for classifying them, it is suggested that they be referred to as artificial EVs for systematic classification. (García-Manrique et al., 2018).

Artificial EVs are either directly or indirectly engineered and are classified into: (a) fully synthetic EVs; (b) semi-synthetic EVs.

- (a) Synthetic EVs or mimetic EVs: these are synthetic analogs that can mimic the properties of natural EVs. This type of EV can be produced by two techniques: top-down and bottom-up. The former uses the larger substrate and reduces it to smaller units for the formation of nanovesicles, while the latter technique uses the smaller individual fragments that self-assemble into higher-order structures with a modular composition. The possible advantage of designing this type of EV over the production of semi-synthetic EVs lies in the increased scalability of EVs to be used in clinical trials and the high sterility and uniformity in size. One of the subcategories of this type of EV is hybrid mimetic EVs that allow the fusion of EVs with synthetic liposomes to increase stability and decrease immunogenicity for better therapeutic use (García-Manrique et al., 2018).
- (b) Semi-synthetic EVs: formed from natural cells' starting material as a result of alterations done before or after the cells were isolated to improve their intrinsic qualities, such as functional cargo transfer. This form of EV is created directly, including alteration after isolation, or indirectly, through genetic engineering procedures using donor cells. (Ramasubramanian et al., 2019).

EVs as nanocarriers are manipulated for cargo loading either endogenously or exogenously. The first methodology involves reprogramming the parental cells to overexpress the protein or nucleic acid of interest that will then be packed into the EVs and delivered to the targeted recipient cell. Exogenous stimulation of cells by various factors is an alternative method to redesign parental cells and increase their EV yield, reproducibility, and therapeutic potency (Man et al., 2020). In addition, there is a classification of methods to modify the isolation of EVs into active and passive methods:

- Active methods: these are both physical and chemical methods. Among the former, we find electroporation and sonication, which allow the transfer of the drug into the EVs. In chemical methods, on the other hand, we find saponin-assisted loading, or transfection reagents, which allow the therapeutic agent to penetrate the membrane of the EVs (de Jong et al., 2020);
- Passive methods include co-incubating isolated EVs with the target drug (García-Manrique et al., 2018).

Among active methods, there is one particularly interesting: RNA-binding proteins (RBPs)-mediated RNA loading on EV. This method employs RBPs to recruit endogenous RNA and to induce preferential loading of them in EVs (Dellar et al., 2022; Fabbiano et al., 2020). While some of the major packaging mechanisms for these RNAs have started to become apparent, it appears that a number of different elements work together to promote loading. With a better understanding of these aspects, customized payloads for therapeutic applications can be created. Furthermore, as the RNA species found in EVs are probably as diverse as EVs themselves, it is crucial to identify which EV subpopulations contain which RNAs and how this relates to EV biogenesis in order to comprehend the physiological activities of EVs.

All of these methods allow the modification of EVs on their surface, but despite these advances in their engineering, the use of these methods remains a challenge in the field of therapy and regenerative medicine, as controlled reaction conditions such as pH and temperature are required to avoid membrane rupture of EVs (Man et al., 2020). Ye et al. investigated an example of the application of these EVs on murine glioblastoma multiforme. Both BEE and glioma cells overexpress the low-density lipoprotein receptor on their surface. EVs were loaded with the anti-tumor agent methotrexate and conjugated with a peptide that targets the overexpressed lipoprotein receptor. Glioblastoma-bearing mice treated with these modified EVs showed a more extended median survival period than control groups (including methotrexate alone and EVs loaded with methotrexate but no coating), and no injury or tissue damage was observed (Ye et al., 2018).

1.8.3 EVs as therapeutic agents

EVs have innate therapeutic potential determined by the presence of bioactive cargoes within them. Therefore, given their extensive involvement in normal physiological and pathophysiological processes, they are considered a possible therapeutic agent in regenerative medicine and tissue repair (EL Andaloussi et al., 2013).

Most studies have focused on EVs derived from mesenchymal stem cells (MSCs). MSCs are multipotent stem cells that can differentiate into various cell types (mainly bone, cartilage, muscle, marrow, ligament, adipose tissue, and connective tissue) (Dimarino et al., 2013). Given these characteristics, MSCs have been studied predominantly in clinical applications to repair damaged tissues. In addition to these regenerative properties, MSCs exhibit immunosuppressive and neuroprotective capabilities, but the mechanisms underlying these characteristics remain largely unknown (Ohno et al., 2016).

The complexity of MSCs, in terms of their origin and cell culture conditions, makes their use difficult.

Recent work has shown that the administration of EVs derived from MSCs has a similar effect on the transplantation of the cells (Kordelas et al., 2014). Preclinical studies have shown their therapeutic ability on various tissues, such as in disease treatment and CNS regenerative medicine (Ruppert et al., 2018).

MSC-derived EVs carry out their therapeutic activity through several processes, including cargo transfer and activation of multiple signaling pathways by surface contact with the recipient cell. This interaction may result in altered immunological responses, diminished inflammation, decreased apoptosis, reduced oxidative stress, stimulated wound healing, and promoted angiogenesis, among other effects. Together, these systems lessen the negative consequences of the disease, encourage healing, and restore function (van Koppen et al., 2012).

Despite the rich literature, given the complexity of MSCs, using EVs from MSCs as a therapeutic agent remains quite tricky.

Numerous pieces of research have concentrated on various cell types that possess stem-like characteristics and may have immunomodulatory effects that could be utilized in therapeutic applications. In preclinical models, EVs derived from other regenerative and immunomodulatory cell sources (amniotic epithelial cells, endothelial progenitor cells, embryonic stem cells, induced pluripotent stem cells, cardiac-derived cells, and dendritic cells) mediate the therapeutic effects, wound healing, pulmonary fibrosis, vascular repair, myocardial infarction, and vaccination (Wiklander et al., 2019).

In addition to stem cells, EVs derived from differentiated cell types, such as immune cells, are a potential therapeutic agent for immunomodulation. The

initial approach of EVs-based therapies using immunostimulatory properties to generate an anti-tumor effect has shown efficacy in preclinical studies (André et al., 2004). For example, exosomes derived from natural killer (NK) cells target GBM cells and inhibit their growth by localizing precisely to the tumor site as these EVs contain TNF- α , granzyme-B, FasL, and perforin (Zhu et al., 2017).

In recent years, researchers' attention has focused on CNS immune cells. In particular, microglia cells can suppress GBM invasion in a time-dependent manner in a 3D-spheroid glioma model (Murgoci et al., 2018).

Subsequent studies have shown that the immunomodulatory effects of EVs depend on the parental cells' maturation state and microenvironment. Indeed, cells exposed to stress-induced conditions (oxidative stress, acidic conditions, serum deprivation, hypoxia, ultraviolet light, irradiation, or cell-stimulating substances) generate variable numbers of EVs with a different composition and function than EVs isolated from cells under standard culture conditions (Wiklander et al., 2019).

In addition to the induction of immune response by bioactive membrane constituents, luminal cargoes of miRNAs can presumably mediate biological functions through gene regulation of the target cell. Although the transfer of small RNAs by EVs has been observed in several systems involving immune cells, the exact role of EVs in this transfer remains unclear (Ohno et al., 2016).

The application of EVs as therapeutic agents or DDS has become an active research area. However, many obstacles still need to be overcome to successfully and safely use EVs as therapeutic agents in a routine clinical setting. Clinical studies in phase I indicate that acute severe side effects do not occur. However, these studies are limited to one particular biological action, immunomodulation, lacking long-term experience (Ohno et al., 2016).

2 EVs as mediators of microglia function

Microglia consists of a family of macrophages that constitute the resident immune cells of the CNS and ensure a rapid and adequate immune response (Colonna and Butovsky, 2017). Since its first identification in 1919, microglia have been studied chiefly concerning pathological processes. Immediately, the ability of this phagocyte type to respond rapidly to cellular injury was understood. Later, factors secreted by microglia were attributed to various physiological roles, for example, in providing trophic support to neurons and eliminating myelin debris (Sierra et al., 2016; Wolf et al., 2017). Although the field of research on microglia has grown tremendously in recent years, there is still much controversy about their function, status, and activity, particularly regarding their role in inflammatory CNS diseases (Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS)). One of the most debated topics is probably the definition of the activation state of microglia from a "*resting*" phenotype. Historically, microglia phenotypes have been related to their morphological changes. Branched and amoeboid morphologies have often been referred to as "*resting*" and "*activated/reactive*" microglia, respectively (Felsky et al., 2019).

2.1 What microglia is

Neuroglia is the collection of non-neuronal cells in the brain; two populations identify it: macroglia and microglia. The former consists of astrocytes, oligodendrocytes, and NG-2 glia (also known as oligodendrocyte precursors) and differs from microglia in cell size and origin (Verkhatsky et al., 2019). Microglia constitute twenty percent of the total glia population in the CNS (Gehrmann et al., 1995). Although considered a constituent of the CNS, microglia are not derived from the ectoderm but have an ontogeny of their own: they appear to originate from myeloid progenitors in the yolk sac that migrate

into the CNS around the second trimester (Kofler and Wiley, 2011). After infiltration into the CNS, microglia cells assume a branched morphology (they deviate from the amoeboid morphology, presenting a cellular soma from which smaller processes branch off). Microglial cells that exhibit this morphology are said to be resting, and they remain there until stimulated by pathogens or other stimuli (such as trauma, neurodegenerative disease, or any other event that manages to alter CNS homeostasis); they thus become active and migrate to areas involved in the damage. However, microglia cells in the resting state are not inactive but have periods of high motility interspersed with periods when they remain static. That is why the term '*resting*' is not appropriate for describing homeostatic microglia. During periods of motility, the cells extend and retract their many processes, using these branches as sensors capable of picking up changes in the microenvironment (Wake et al., 2011).

Microglial cells are therefore referred to as the macrophages of the CNS, as they constitute this system's first and foremost defense. Indeed, they are responsible for eliminating pathogenic microorganisms, dead cells, excess synapses, protein aggregates, and other soluble particulate matter capable of damaging the CNS. Moreover, as they are a primary source of pro-inflammatory cytokines, microglia cells are critical mediators in neuroinflammatory processes and can induce or modulate a broad spectrum of cellular responses (Colonna and Butovsky, 2017).

2.2 What microglia does

Microglia performs numerous tasks in the CNS, primarily those related to immune response and maintenance of homeostasis. In addition to its protective work in disease states, microglia performs other functions closely related to CNS development. First and foremost, the task of microglia is the "scanning" of the surrounding environment to pick up any changes that might threaten the

CNS. The cells can use their mobile processes to monitor the environment in their homeostatic state. In response to these extracellular materials (viruses, pathogenic microorganisms, proteins, lipids), microglial cells undergo morphological changes that lead to their activation, resulting in phagocytosis of exogenous material and induction of inflammatory processes (Kettenmann et al., 2011).

Microglial cells release various neurotrophic factors that can promote the survival and differentiation of specific neurons. The neurotrophic factors released are BDNF, IGF-1, NGF, hepatocyte growth, and epidermal growth factor.

Microglia also can control neurogenesis: during primary development, the surplus of defective neurons is eliminated precisely through the intervention of microglial cells. First, they induce programmed cell death by releasing intrinsic factors, such as NGF and TNF, but also ROS (reactive oxygen species); then they have the task of eliminating cellular debris by phagocytosis (Gogoleva et al., 2019; Nayak et al., 2014).

Another function of microglia is to perform the so-called synaptic pruning. This process involves the elimination, by phagocytosis, of accessory synapses formed during the early stages of CNS development. In fact, during development, synapses are produced in significant excess than that remain once the CNS reaches maturity, and the elimination of them is necessary to ensure efficient neuronal transmission (an uncontrolled pruning could lead to the appearance of pathologies such as schizophrenia and autism) (Paolicelli et al., 2019).

Currently, the literature describes three distinct microglia states: M0, M1, and M2. The M0 phenotype refers to homeostatic microglia in the CNS during normal function (Lee et al., 2014; Wolf et al., 2017). The M1 phenotype is

associated with the secretion of pro-inflammatory chemokines and cytokines such as TNF α , IL-6, and IL-1 β . It is, therefore, involved in inflammatory processes typical of neurotoxicity and during the development of neurodegenerative diseases (Wolf et al., 2017). In contrast, the M2 phenotype can secrete IL-10 and TGF- β , which promote microglia anti-inflammatory and regenerative activity (Wolf et al., 2017). According to this paradigm, each subtype of microglia would thus exhibit intrinsic properties and perform unique functions essential for maintaining CNS homeostasis. Nevertheless, this strict division into M1 and M2 states is not always valid: M2 can also promote detrimental effects on the CNS as M1 have beneficial effects (Colonna and Butovsky, 2017; Paolicelli et al., 2019). Therefore, it is nowadays more accepted to speak of a "transition between activated states" to overcome the limits of the rigidity of a morphological and functional classification.

2.3 Modeling microglia *In vitro*

The functions of microglia have been studied using many immortalized cell lines derived from mice and rats, in primary cultures from fresh tissues of mice, rats, and recently also from leeches (Arab et al., 2019). As for humans, the

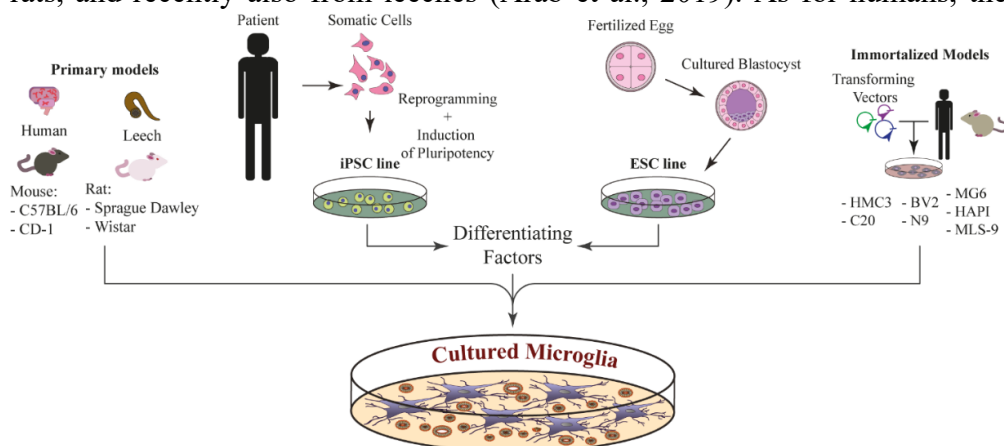


Figure 2-1 Scheme of the four main types of sources (primary cells, iPSC lines, ESC lines, and immortalized cell lines) that can be used to obtain microglia cell cultures, adapted from Ceccarelli et al., 2021b.

research work is more complex. Studies on primary cultures derived from postmortem retrievals are still scarce, with very discordant data. Currently, however, the availability of immortalized human cell lines is increasing (Ceccarelli et al., 2021b) (*Figure 2-1*):

1) HMC3 line is a microglia line obtained by immortalizing macrophages derived from the human embryonic brain and validated for several markers. It is one of the most widely used, especially for drug studies such as cocaine (Dello Russo et al., 2018; Kumar et al., 2020);

2) C20 line, a microglia line obtained from adult human brain tissue transformed with lentiviral vectors expressing either SV40 T antigen or a combination of SVR40 T antigen and hTERT. The resulting line expresses key microglial surface markers (TGF β R, P2RY12, and CD11b). Furthermore, C20 does not appear to respond like murine microglial lines to stimuli such as LPS (Garcia-Mesa et al., 2017);

3) Induced pluripotent stem cells (iPSCs) differentiated into microglia. Differentiating iPSCs into various cell types has been a widespread practice in recent years, but only recently protocols for generating human microglia have been reported (Abud et al., 2017; Douvaras et al., 2017; Hasselmann and Blurton-Jones, 2020). An increasingly attractive possibility is to obtain microglial cells from patient-derived stem cells. This technique has clear advantages in the number of cells obtained, which by replicating, reduces the ethical and practical inconvenience of harvesting human tissue. At present, however, it remains a study model yet to be fully elaborated.

2.4 Microglia-derived EVs

Microglia cells release, under normal conditions, EVs of different sizes: Small EVs (30-250nm) and Large EVs (150 -800 nm). Cell-derived membrane

vesicles carry both membrane-associated material and cytosol components such as mRNAs, miRNAs, proteins, lipids, and pro-peptides and thus can vary in size and composition (Ceccarelli et al., 2021b). Over the past decade, several studies have shown that the composition of the exosome varies according to the phenotype of the microglia cell releasing it, which allows for the modulation of communication between cells (Timmerman et al., 2018). Microglial-derived vesicles thus act as a means of propagating proteins. At the same time, other studies have indicated that they positively impact the clearance of protein aggregates and the regulation of neuronal viability and, not less notably, are significant players in cell-cell communication (Arab et al., 2019). Microglia cells can produce EVs without external stimuli; it is possible to enhance this production through particular stimuli that can be either pro-inflammatory or pro-regenerative. Depending on the stimulus applied in the culture environment, EVs with different structural and functional characteristics are obtained, resulting in different effects on the recipient cell (Bernimoulin et al., 2009; Gosselin et al., 2014; Turola et al., 2012). Depending on the microenvironment, we will have a widely varying molecular composition of the vesicles produced.

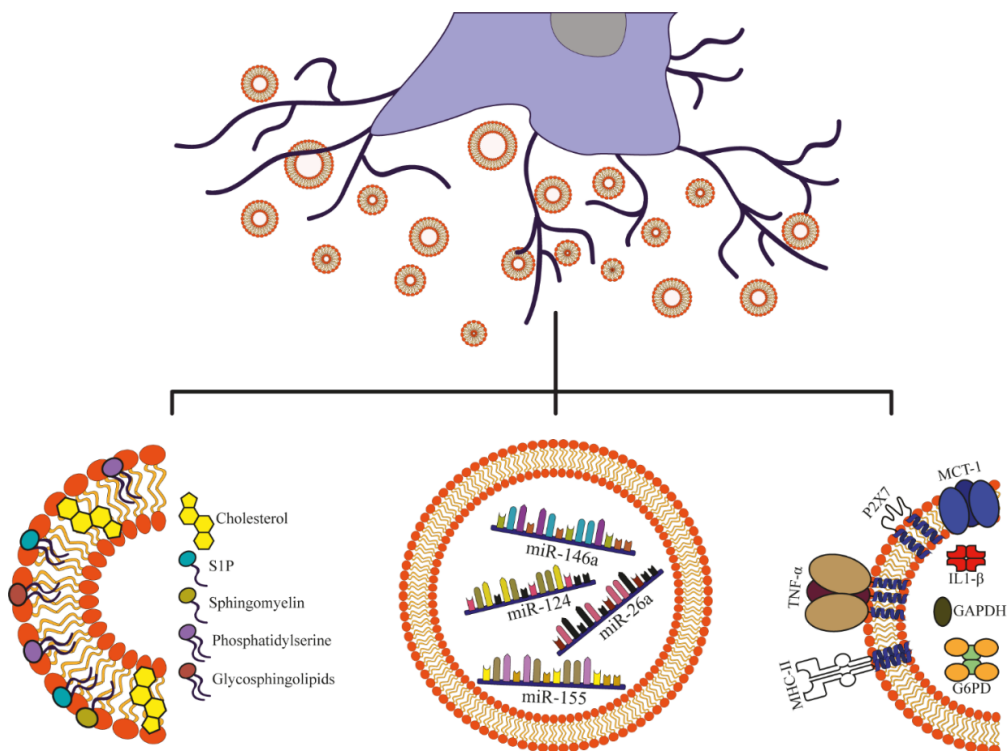


Figure 2-2 Main components of microglia-derived EVs. Molecules are grouped in Lipids (left), miRNA (center) and proteins (right). The most representative member of each category has been reported, adapted from Ceccarelli et al., 2021a.

Briefly, microglia exosomes cargo consists of (Ceccarelli et al., 2021a) (**Figure 2-2**):

- Proteins: metabolic enzymes (e.g., glyceraldehyde-3-phosphate dehydrogenase, GAPDH, pyruvate kinase), cytokines (IL-1 β , IL-6, TNF- α), microglia-specific membrane receptors (CD13 and MDC-1);
- lipids: cholesterol (CHOL), sphingomyelin (SM), glycosphingolipids, and phosphatidylserine (PS) are the most common in the membranes of microglia exosomes;
- miRNA cargo can vary a lot in composition and abundance; most miRNAs can vary in composition and amount according to donor cell status, causing a wide range of effects in recipient cells. There are about six of them which are

best-known and mostly omnipresent: miR-1860, miR-1705, miR-2284y-6, miR-146a, miR-858, and miR-7718. The cell packs them into the exosome to protect them from degradation.

2.5 Inflamed microglia & EVs

It is possible to increase the release of EVs through particular stimuli in the microenvironment, which, as already mentioned, can be either pro-regenerative or pro-inflammatory and result in different effects of the EVs on the recipient cells (Ceccarelli et al., 2021a; Salvi et al., 2017) (**Figure 2-3**).

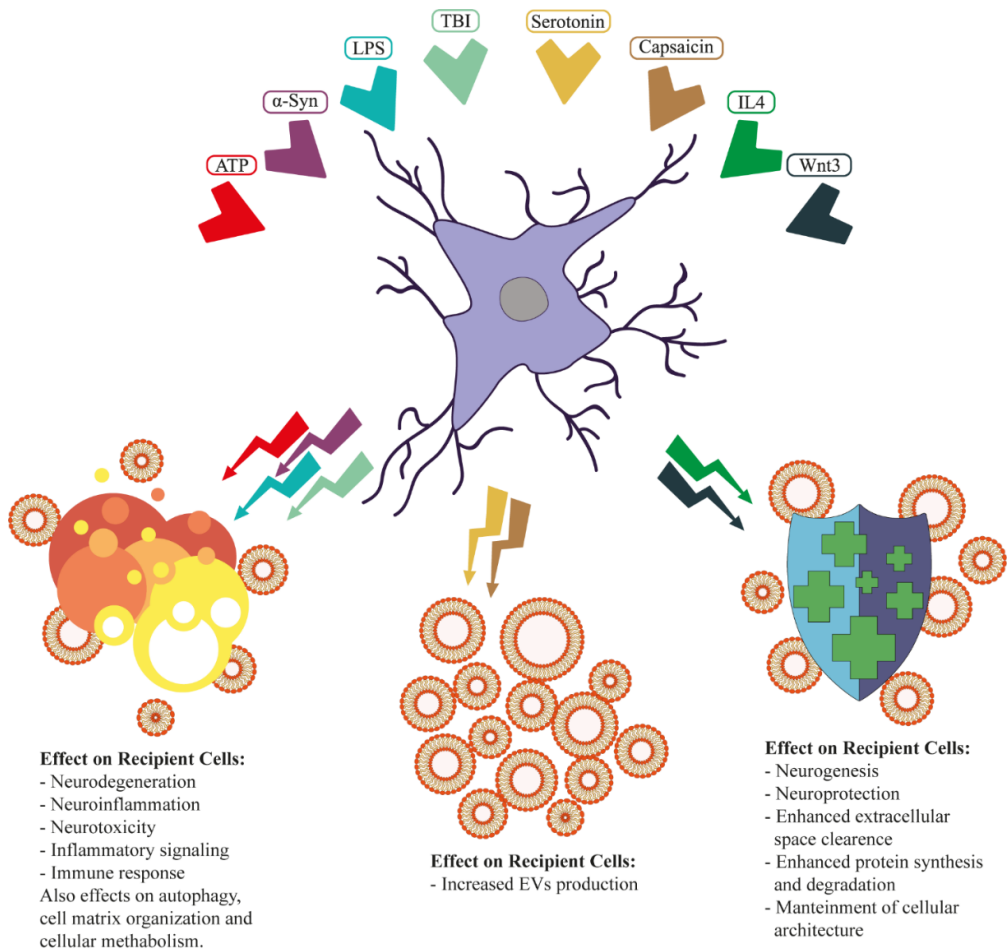


Figure 2-3 Schematic representation of the stimuli used to promote EV production by microglial cells. Stimuli are color-coded; arrows at the bottom in the same color-code indicate the main biological effect raised by each stimulated EVs in the CNS, adapted from Ceccarelli et al., 2021a.

Factors such as Interleukin-4 (a pro-regenerative cytokine) or Wnt3 (WNT family morphogenetic protein) act as stimuli for the production of EVs with pro-regenerative function: they inhibit inflammation and cellular senescence, promote neurogenesis and neuroprotection, elevate protein synthesis and

protein degradation, and maintain proper cellular architecture (Ceccarelli et al., 2021a).

On the other hand, factors such as ATP, α -synuclein, Serotonin, Capsaicin, and LPS act as stimuli for the production of EVs with pro-inflammatory effects on the recipient cell: they result in neurodegeneration, neuroinflammation, neurotoxicity, elevate the immune response, and result in inflammation.

ATP is one of the most applied stimuli. It results in a change in the content of EVs. They result enriched in proteins for cell adhesion, autophagy pathway, and enzymes for cell metabolism; they also acquire complement proteins and promote the production of fatty acids necessary for microglia activity (Drago et al., 2017; Stevens et al., 2007; Takenouchi et al., 2015).

α -synuclein stimulatory action results in the production of vesicles with a high level of TNF- α that can increase apoptosis and propagate inflammation to neighboring cells (Chang et al., 2013).

Serotonin (5-HT) causes increased intracellular Ca^{2+} levels that appear to increase the ability of MVBs to fuse with the plasma membrane leading to increased secretion of EVs (Glebov et al., 2015).

Capsaicin results in increased secretion of vesicles (Marrone et al., 2017).

Lipopolysaccharide (LPS) is a widespread method applied for *in vitro* inflammation of microglia cells to obtain EVs, mainly from murine cell lines. This stimulation results in inflamed microglia cells capable of producing EVs containing miR-155 and IL-1 β . The latter can induce an additional activation signal and propagate the inflammatory response by increasing the expression of IL-1 β , TNF- α , IL-6, NOS2 (nitric oxide synthase 2), and miR-155 (Kumar et al., 2017).

In addition, an essential effect of EVs secreted following stimulation with LPS is an anti-tumor action on glioma cells. The molecular mechanism is likely due to the miRNA content in the EVs produced (Murgoci et al., 2018).

2.6 Isolation and Characterization of Microglial EVs

Even though the process of stimulating EV production by microglia is well defined, the process of isolation of vesicles is critical. Again, there is the need to follow guidelines developed by MISEV 2018 (Théry et al., 2018b).

There are various methods of isolating EVs of microglial origin in the literature based on their different biophysical and biochemical properties, including size, bulk density, shape, charge, or antigen exposure (Murgoci et al., 2018). Among those most widely used are: differential centrifugations, Biot-Annexin-V + Streptavidin Beads, exosome isolation kits, and density gradient ultracentrifugation (Ceccarelli et al., 2021b). Each of these methods has pros and cons. Indeed, each protocol should be adapted, considering the function EVs initially have and the type of experiment needed. Currently, no universal protocol is available for isolating EVs from various tissues. In this thesis, we made use of the differential ultracentrifugation approach, which has the advantage of being an easy-to-use method and requiring little technical expertise and no sample pre-preparation (Murgoci et al., 2018).

It is necessary to check the degree of purity of the isolated EVs using various methods that include: morphological methods (transmission electron microscopy), size fractionation (dynamic light scattering), identification of different vesicular markers (Western Blot), and verification of the absence of protein contamination (CONAN assay) (Maiolo et al., 2015). Using omics sciences, one can, finally, identify the contents of EVs (Ceccarelli et al., 2021b) (*Figure 2-4*).

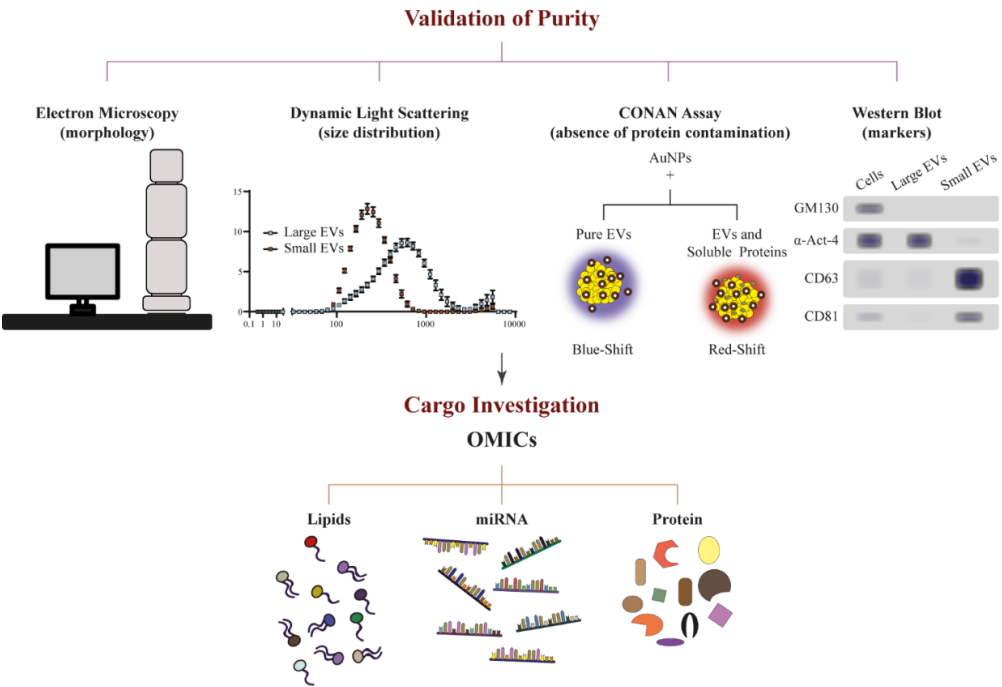


Figure 2-4 Scheme of the four main types of EV extraction and isolation methods used to gather microglia-derived EVs, adapted from Ceccarelli et al., 2021b.

2.7 Glioblastoma: an unwanted neighbor

Exactly like all others, cancer cells also release vesicles into the extracellular space, which after passing through the extracellular space, interact with neighboring cells to which they release their contents. Gliomas and primary brain tumors use various stimuli, including EVs, for their development. Glioblastoma multiforme (GBM) is one of the most lethal gliomas, accounting for 70-75% of all gliomas, with a median overall survival of only 14-17 months. The GBM microenvironment consists of non-neoplastic cells, stromal cells, resident (microglia) and infiltrating (macrophages) immune system cells, glial cells, and glioma stem cells (GSCs). Resident microglia and infiltrated macrophages comprise tumor-associated microglia/macrophages (TAMs), constituting about 30-40% of the tumor mass. Communication between tumor cells and TAMs occurs, among other means, through EVs (Pancholi et al., 2022). *In vitro* studies have shown that a single GBM cell can secrete up to 10,000 EVs in about 48h suggesting the significant role of vesicles in tumor-stromal cell communication and in directing fundamental mechanisms for GBM development (Skog et al., 2008).

Using EVs, the GBM modulates and changes the surrounding tissue in its favor. The vesicles transmit some information in tumor cells to adjacent healthy cells, helping to change their functionality and behavior (Yekula et al., 2020). Extracellular GBM vesicles, using their contents that they spill into neighboring cells, modulate:

- Tumor growth: by altering the DNA of recipient cells
- Invasiveness: through EVs secreted by the tumor that changes the phenotype of healthy cells in favor of migratory activity
- Angiogenesis: through pro-angiogenic factors (e.g.:IL-8, IL6, TGF- β)

- Chemotherapy resistance: through factors that reduce sensitivity to radiogenic therapies
- Immunosuppression: through changing the phenotype of monocytes, macrophages, and microglia in favor of tumor development

2.8 Glioblastoma & Microglia

The relationship between microglia and glioblastoma development is one hotly studied topic and the subject of scientific debate. As discussed above, EVs produced by GBM carry immunomodulatory molecules (miRNAs and ncRNAs) that, when functionally transferred to TAM, reprogram them to support glioblastoma development (Pancholi et al., 2022).

Recent studies using intravital microscopy imaging *in vivo* have amply demonstrated this close relationship between EVs release from the tumor and EVs uptake by microglia, suggesting an EVs-mediated interaction locally and systemically by the GBM.

Furthermore, 5-day exposure of microglia cells to GBM-derived EVs can change the inflamed state of microglia residing in the tumor area. Under these conditions, microglia switch to an M2 state, anti-inflammatory and regenerative. Thus, there is a secretion of many pro-tumorigenic cytokines (CXCL10, CXCL1, CCL2, CCL5, IL-6, and TIMP1) and a reduction of anti-tumor cytokines (IL-16, IL-23, IL-27) (Spellicy & Stice, 2021). Thus, microglia wholly deactivates its defense action in favor of pro-tumor activity.

Most of the studies currently in the literature focus on the effect of tumor-derived EVs on resident cells in the brain, including microglia. It is well established that GBM-derived EVs can induce tumor-supporting phenotype both on astrocytes and microglia (Catalano et al., 2022; Hallal et al., 2019; Li et al., 2022). There are reports that indicate a tumor-suppressing effect of

microglia-derived EVs on GBS (Serpe et al., 2021); the work that I am going to describe will build on that to increase the knowledge of the effect of microglia-derived EVs in brain communication.

2.9 A comparison between two human microglia-derived EVs

This paragraph describes the work that I have done to compare the effect mediated by EVs of two human microglia cell lines on a line of glioblastoma. The first step was to extract and characterize microglia-derived EVs from microglia in untreated conditions; once our samples were confirmed to be pure and characterized, we started to administer them to glioblastoma cells. Then we decided to go further and change the state of parental cells administering pro-inflammatory stimuli to assess the influence of pro-inflammatory EVs on parental cells.

In the characterization phase, some experiments were done in collaboration:

- DLS was performed by Aldo Moscardini at NEST Laboratory, Scuola Normale Superiore, Pisa
- TEM was performed by Valentina Cappello at the Center for Nanotechnology Innovation, IIT, Pontedera, Pisa
- miRNA analysis was performed by Milena Rizzo at IFC-CNR, Pisa
- CONAN assay was performed by Miriam Romano at the Department of Molecular and Translational Medicine, Brescia

All the other described experiments were done by the candidate at the Department of Pharmacy, Pisa.

2.9.1 Human microglia EV preparation and biochemical analysis

This work started with the set-up and optimization of a protocol for extracting

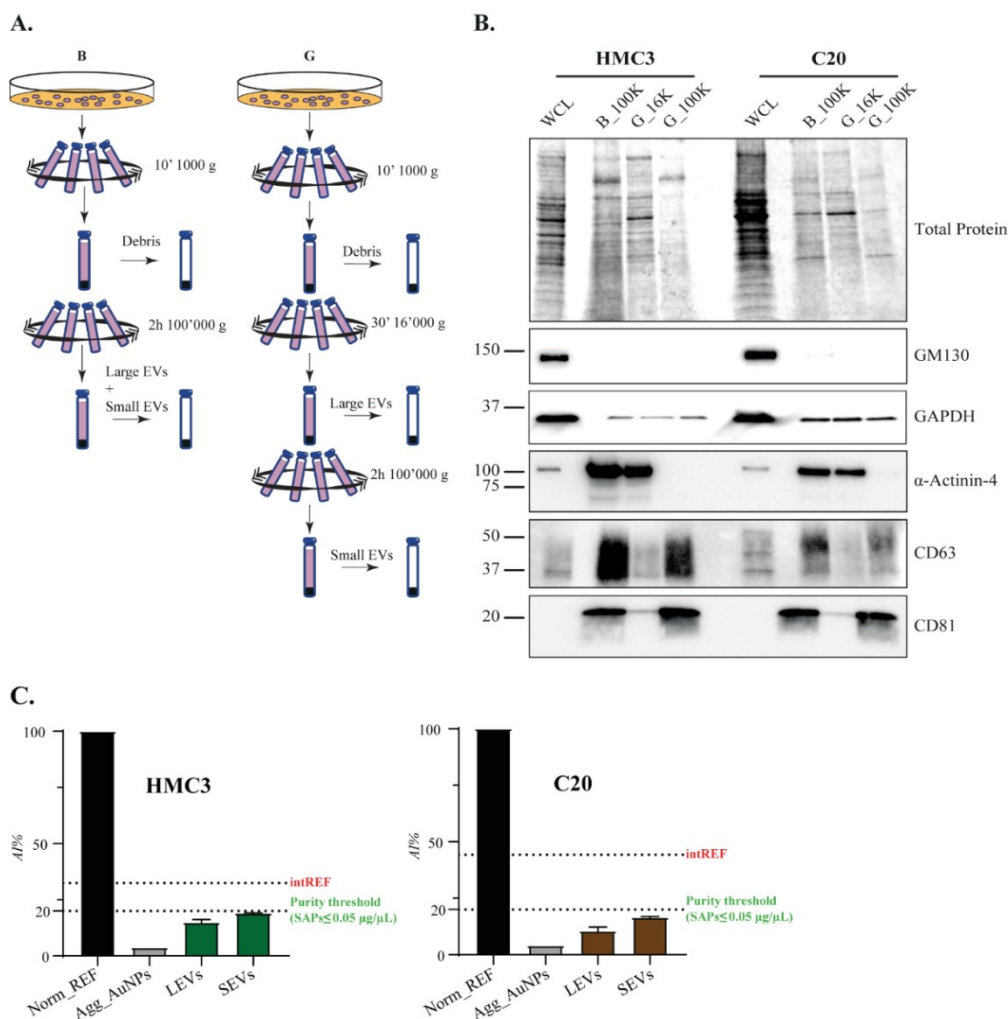


Figure 2-5 Panel A: Overview of the two protocols used in the extraction of EVs. Panel B: EVs were gathered from HMC3 and C20 cells using B and G, lysed, and the proteins separated by SDS-page. EVs gathered from one 150 mm Petri dish were loaded in each lane, together with 1 µg (α-Actinin-4 blot) or 30 µg (for all other blots) of the whole-cell lysate (WCL) of each cell line. Panel C: CONAN assay-mediated purity assessment; EVs preparations gathered from one 150 mm Petri dish were analyzed, AI (Aggregation Index) values of the EV preparations were normalized by the Aggregation Index of a solution of AuNPs in HPLC water (Norm_REF). A solution of HPLC water + AuNPs + PBS was used as negative control (intREF). See the main text for details.

EVs from the supernatant of human microglia HMC3 and C20 cells using two different isolation techniques. In the first one (named B, as Bad, **Figure 2-5**), supernatants underwent a single ultracentrifugation step at 100,000 xg

(B_100K) for two hours to extract the EVs. In the other one (named G, as Good, in **Figure 2-5**), EVs were recovered in two consecutive steps: 30' at 16,000 xg (G_16K) and 2h at 100,000 xg (G_100K). After G_16K and G_100K steps of centrifugation, LEVs and SEVs were obtained, respectively (Théry et al., 2018b).

The two cell lines collected EVs underwent Western Blot analysis comparing the two different procedures. The positive EV markers α -Actinin-4, CD81, and CD63, are only found in EV pellets, as opposed to the negative EV marker GM130, which is found in intracellular vesicles derived from the Golgi in whole-cell lysates. Since cytosolic protein was recovered in all EV fractions, GAPDH was looked into and found in both large and small EVs of the two different types of microglia. Actinin-4 demonstrated enrichment in the LEVs pellet compared to the SEVs pellet from both cell lines, indicating that this marker could be used to distinguish these two populations of EVs (Kowal et al., 2016; Théry et al., 2018b).

As anticipated, the B protocol allows the isolation of a mixed population of EVs, SEVs + LEVs. It is possible to isolate all EVs generated by cells without distinguishing between the two EV populations. In contrast, the LEVs and SEVs generated from each cell line may be satisfactorily separated using G.

To prove that the aforementioned protein analysis only applies to the EV content, the purity of EV preparations was examined using the CONAN assay. This assay combines the plasmonics of gold spherical nanoparticles with the competitive ability of citrated gold nanoparticles to cluster on EV membranes rather than being covered by a SAPs corona. Proteins in a solution can obstruct the interaction of lipid membranes and AuNPs (Montis et al., 2020). The localized peak of the surface plasmon resonance of AuNPs shifts to red in the presence of minute amounts of SAPs (0.05 $\mu\text{g}/\mu\text{L}$), which changes the

nanoplasmonics of the material. The Aggregation Index, which is defined as the ratio between the absorbance intensity at the LSPR peak of the AuNPs and the sum of the absorbance at 650 nm and 850 nm, can be used by UV-Vis spectroscopy to quantify the redshift, which is directly related to the purity grade of the EV preparation (Maiolo et al., 2015; Zendrini et al., 2019). The relative AI values for all the formulations were lower than the purity threshold (20%), indicating that the SAP concentration in each sample is less than 0.05 $\mu\text{g}/\mu\text{L}$. This outcome demonstrated that G could isolate pure EVs from human microglia cells and that all the proteins found in the EV samples are EV-related.

2.9.2 Characterization of human microglia EVs biophysical properties

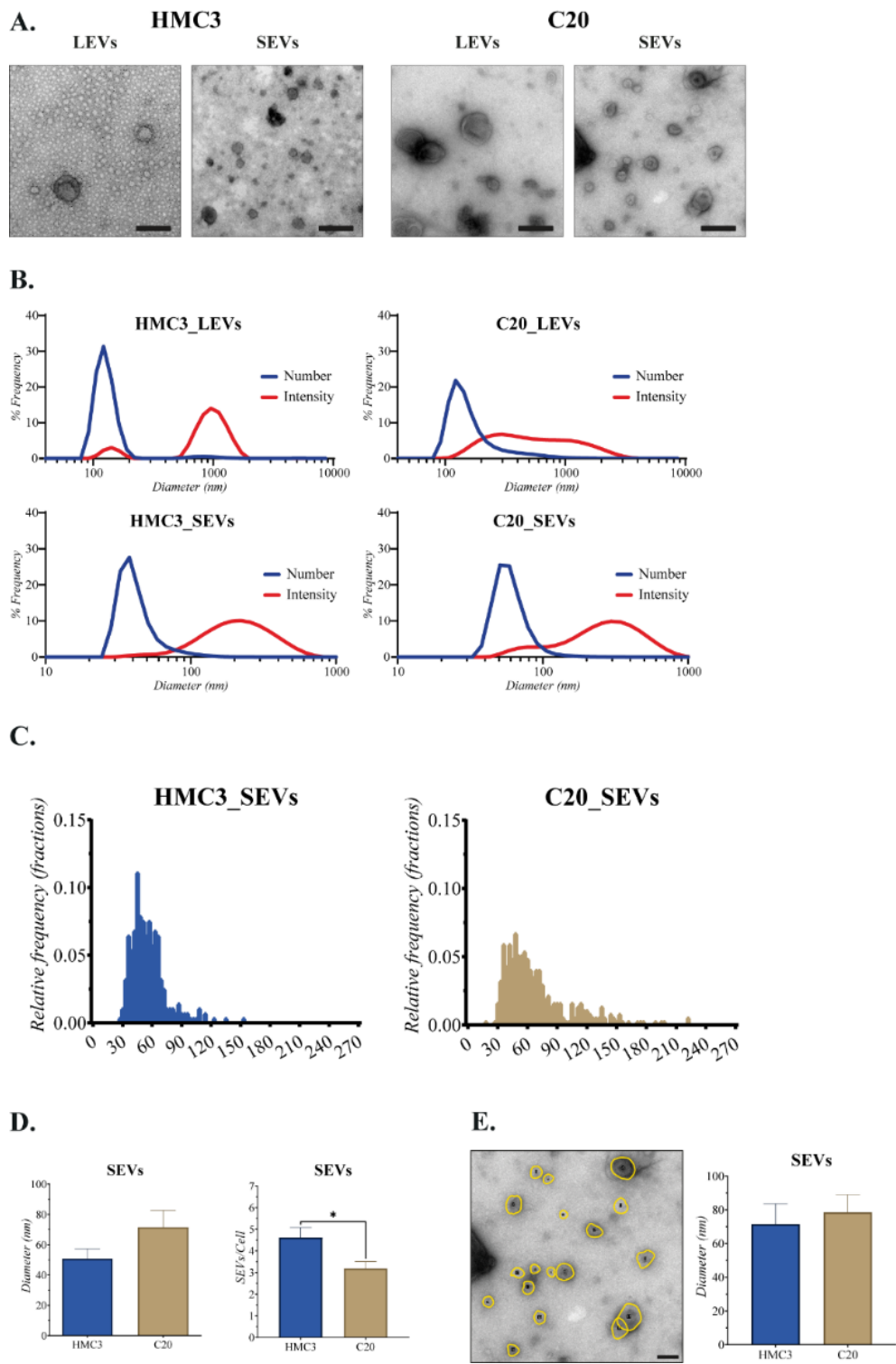


Figure 2-6 Biophysical characterization of EVs extracted from C20 and HMC3. Panel A: EVs pellets derived from two 150 mm Petri Dishes were suspended in 100 μ L PBS with ions and analyzed by TEM microscopy. TEM acquisitions were made with 25,000 $\times$ zoom; bar indicates 100 nm. Panel B: DLS analysis; EVs pellets derived from two 150 mm Petri Dishes were suspended in 100 μ L PBS with ions and analyzed, representative curves of the number and intensity values are shown for each sample gathered with G. Panel C: Relative frequencies distribution of the SEVs pellet gathered with G of each cell line. EVs pellets derived from two 150 mm Petri Dishes were suspended in 100 μ L PBS with ions and analyzed by TEM microscopy, frequency distributions were evaluated on TEM images with ImageJ Software. Panel D: DLS quantification of the SEVs pellet gathered with G of each cell line; the number of EVs/ μ L was normalized by the number of cells from which EVs are derived to calculate the number of EVs secreted per of cells. The data represent the mean values $\pm$ SEM of at least two independent experiments. The significance of the differences was determined by t-test, $*P \leq 0.05$ vs HMC3. Panel E: Schematics of quantification analysis of TEM images, perimeters (yellow lines) were measured and used to calculate the diameter of each visible EVs, images were acquired at 25'000x zoom, scale bar indicates 100 nm. The data represent the mean values $\pm$ SEM of at least two independent experiments. The significance of the differences was determined by t-Test, no significant difference between both cell line was measured.

The morphology of SEVs produced by HMC3 and C20 was next examined using Transmission Electron Microscopy (TEM). A panel of example TEM images is shown in Panel A, **Figure 2-6**.

Every EVs extraction leads to round nanoparticles with the traditional cup-shaped morphology, according to TEM examination (Kalluri and LeBleu, 2020; Pascucci and Scattini, 2021; Ramirez et al., 2018; Théry et al., 2018b). According to this approach's expected separation of LEVs from SEVs, the particle diameter in the LEVs photos of both cell lines is noticeably bigger than that in the SEVs sample (**Panel A, Figure 2-6**).

The evaluation of the LEVs and SEVs samples was performed using dynamic light scattering (Panel B. **Figure 2-6**). We calculated the particle intensity-weighted and number-weighted size distributions as %Ia and %Na, respectively. The distributions are noticeably different in our EV samples. This distinction can be explained by the fact that %Ia, but not %Na, approximates a proportionality to the sixth power of size, which results in increased sensitivity compared to the large EVs in %Ia distributions. Therefore, even if larger EVs are only present in traces, %Ia distributions enable us to appreciate their presence. This implies that LEVs and SEVs in microglia are both diverse populations of EVs with a wide range of potential sizes. Ideally, %Ia and %Na

would be nearly identical in a sample of a single EV population with a small polydispersity index. The mean diameter $\pm$ SEM was 50.77 ± 20.76 nm for HMC3 and 71.48 ± 43.25 nm for C20 cells.

C20 SEVs appear to be larger and more polydisperse than HMC3 ones, even though this difference is not statistically significant. This is further supported by the wider %Ia curve of the C20 SEVs sample compared to the HMC3 sample shown in **Panel B, Figure 2-6**. In contrast, %Na is simple particle count distribution and permitted detection of a prevalent peak, whose value was used to derive the average diameter of SEVs samples. Furthermore, these data fit well with the quantification of distribution and average diameter of SEVs analyzed from TEM images (**Panel C, Figure 2-6**).

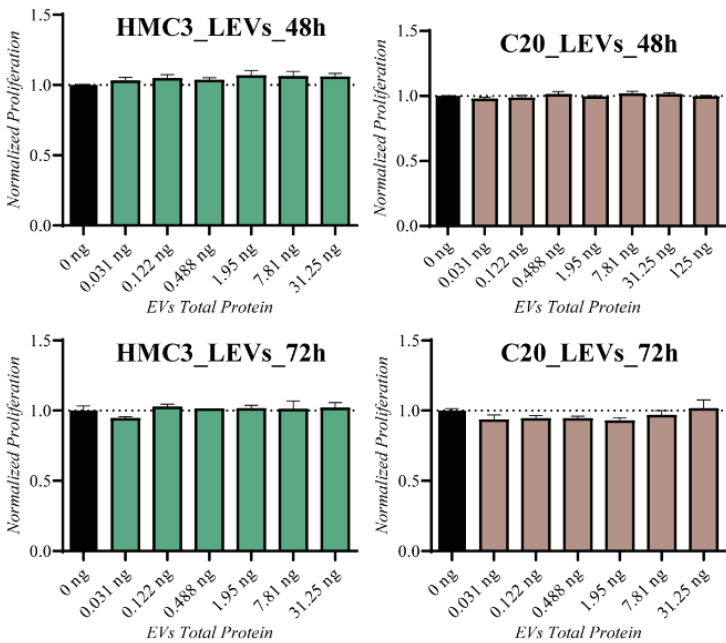
Our findings suggest that small EVs produced from human microglia are essentially the same size as those recovered from the two popular murine microglia models, BV2 and N9, which range in size from 100 to 160 nm for N9 cells and 30-180 nm for BV2 (Cunha et al., 2016; Grimaldi et al., 2019; Vaz et al., 2019; Yang et al., 2018). Using DLS curves, it was possible to calculate the number of particles in each sample, normalized to the number of generating cells (**Panel D, Figure 3-2**). According to this data, HMC3 cells emit more SEVs (4.62 ± 0.47) than C20 cells (3.19 ± 0.31) per cultured cell. Additionally, these findings highly support the quantification of distribution, and the average diameter of SEVs examined from TEM images (**Panel E, Figure 2-6**); the mean diameter of C20-derived SEVs was lower than HMC3-derived SEVs even when measured from TEM images: 71.52 ± 12.01 nm HMC3 compared to 78.4 ± 10.50 nm C20, mean $\pm$ SEM.

According to the culture conditions and extraction procedures, the number of EVs produced per cell may vary (Hurwitz et al., 2016). LEVs samples had a mixed population of EVs with widely varying sizes, which made it difficult to

precisely measure the mean diameter and the mean number of particles secreted, as was done for the SEVs samples, even though the purity of the LEVs, and SEVs was comparable.

2.9.3 SEVs generated from C20, but not HMC3 can inhibit U87MG growth

A.



B.

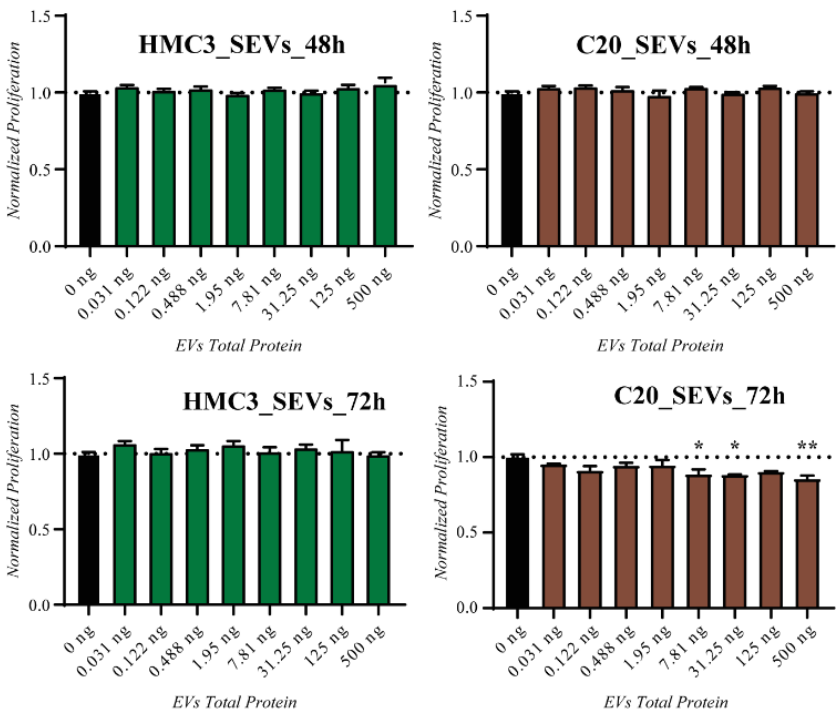


Figure 2-7 Investigation of the biological effect of SEVs on proliferation of U87MG cells. *Panel A:* Proliferation assay of U87MG cells after administration of increasing concentration of LEVs extracted from HMC3 and C20 using the G protocol. The administration was carried on for 48h and 72h. *Panel B:* Proliferation assay of U87MG cells after administration of increasing concentration of SEVs from untreated HMC3 and C20. The administration was carried on for 48h and 72h. The data were expressed as the percentage of expression versus control set to 1 and represent the mean values $\pm$ SEM of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, ** $P < 0.01$ vs control.

After evaluating the quality of human microglia EVs, the impact of LEVs and SEVs from the two cell lines on the proliferation potential of U87MG cells was investigated.

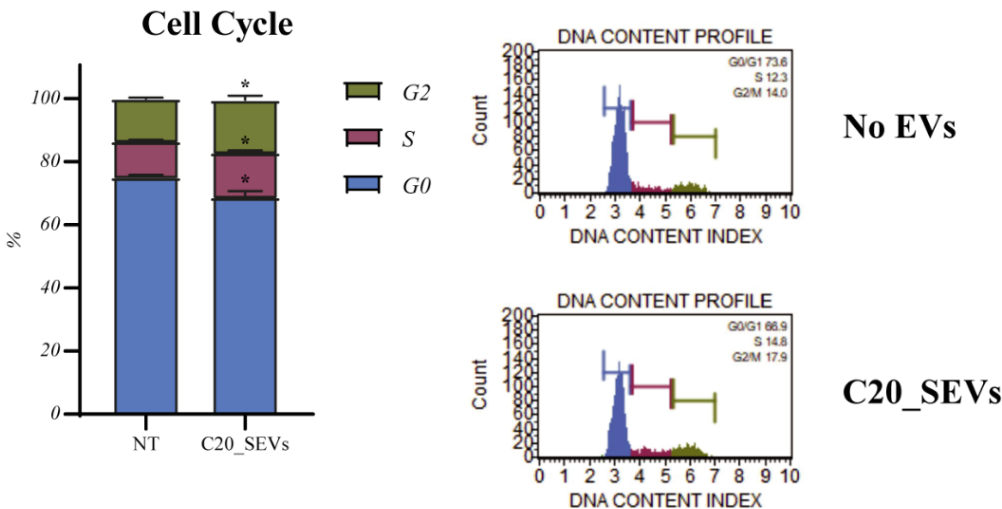
Increasing EV concentrations were given, and an MTS test was used to quantify the proliferation of the treated cells. The acquired results demonstrated that neither 48h after treatment nor 72h after treatment, HMC3- and C20-derived LEVs interfered with the ability of glioblastoma cells to proliferate at any of the tested dosages (**Panel A, Figure 2-7**). Similarly, after 48 hours, HMC3- and C20-derived SEVs could not influence the cell growth rate (**Panel B, Figure 2-7**).

C20-derived SEVs could alter cell proliferation after 72h of treatment (**Panel B, Figure 2-7**). This resulted in a significant dosage-dependent decrease in U87 proliferation, with the highest dose tested causing an average drop of about 15%. After subjecting U87MG cells to the C20-derived SEV challenge, a cell cycle study was carried out to rule out whether the observed decrease is caused by a potential obstruction of the cell cycle progression driven by SEVs or by a direct influence on tumor cell viability (**Panel A, Figure 2-8**). The cells in the S and G2/M phases accumulated little but significantly after the SEV treatment.

Then, on the same SEV-treated C20 cells, immunofluorescence was used to determine the presence of cleaved-caspase3 as an apoptotic marker, as seen in **Panel B, Figure 2-8**.

No discernible induction of apoptotic events was seen, as shown. Together, these findings imply that the ability of C20-derived SEVs to slow down the

A.



B.

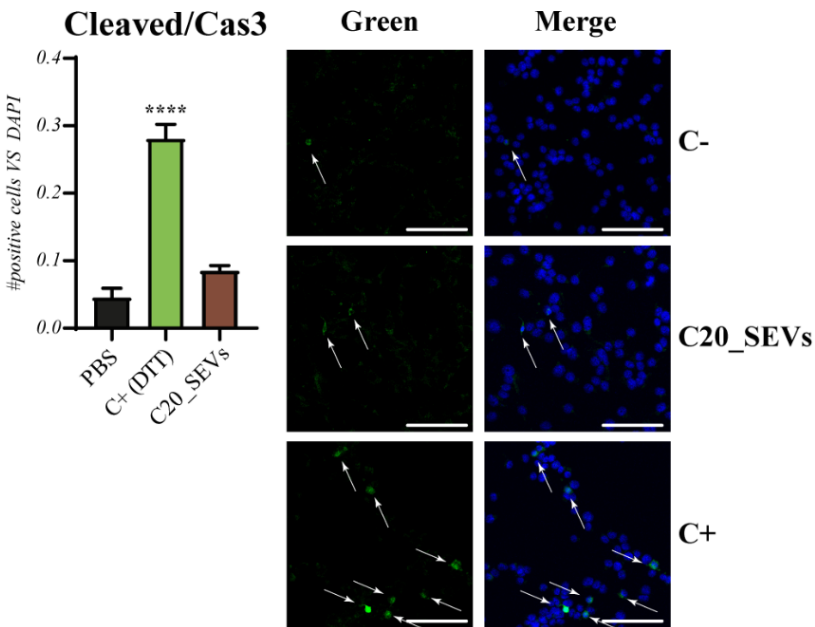


Figure 2-8 Cell Cycle and Apoptosis Analysis. Panel A: cell cycle analysis of U87MG cells treated with SEVs pellet of C20 gathered with G protocol. The significance of the differences was determined by t-test: * $P \leq 0.05$, vs the respective phase of the control. Data were collected from two independent experiments. Panel B: quantification of Cleaved/Cas3 positive U87MG cells treated with PBS (C-), SEVs pellet of C20 gathered with G, and 20mM DTT (C+). Data were quantified by counting the cell number ($n = 1929$, $n = 2183$, $n = 1694$ for C-, SEV and C+, respectively) in different analyzed fields ($n = 7$, $n = 12$, $n = 12$ for C-, SEV and C+, respectively) from two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: **** $P \leq 0.0001$ vs the negative control.

tumor cell cycle, rather than viability, is what causes the reduction in cell proliferation they cause.

2.9.4 HMC3- and C20- have distinct miRNA signatures and polarization states

Small non-coding RNA molecules, called microRNAs, play a key role in controlling the growth of gliomas, and exosomes are one of their primary sources for distribution to tumor cells. In order to understand the disparate effects on U87MG proliferation, we measured the abundance of eight distinct

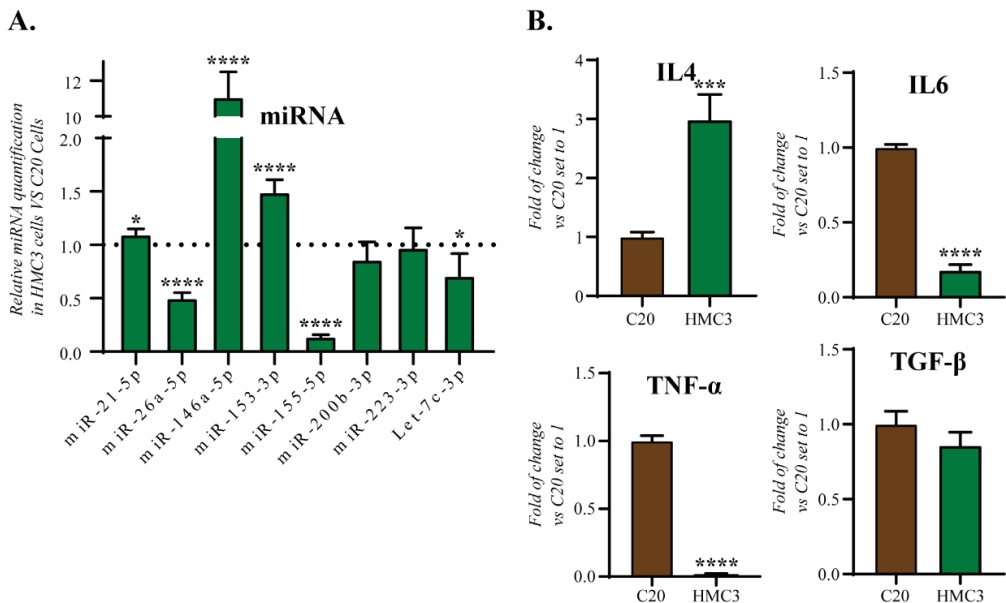


Figure 2-9: SEVs' miRNA signature and cells' mRNA expression of C20 cells compared to HMC3 cells. Panel A: Analysis of miRNA content was performed on the SEVs from both cell lines, gathered with G; the results were normalized on the value of the expression in C20-derived SEVs, set to 1. Panel B: Real Time PCR quantification of mRNA expression in C20 and HMC3 parental cells after o/n starvation. The data were expressed as the relative expression vs control set to 1 and represent the mean values $\pm$ SEM of at least two independent experiments performed in duplicate. The significance of the differences was determined by t-test: * $P \leq 0.05$, **** $P < 0.0001$ vs the respective expression in C20, set to 1.

miRNAs in SEVs produced from HMC3 and C20 (**Panel A, Figure 2-9**). EVs

from various microglia models have already been discovered to include miR-146a-5p, miR-155-5p, and miR-223-3p. Among the most often studied miRNA in neuroinflammation and tumor growth in the brain are miR-21-5p, miR-26a-5p, miR-153-3p, miR-200b-3p, and let-7c-3p. Unexpectedly, the miRNA content of the two cell lines' EVs varied.

Let-7c-3p and miR-26a-5p both displayed reduced abundance in HMC3-derived EVs, with corresponding fold decreases from C20 of 0.71 ± 0.21 and 0.50 ± 0.05 . MiR-21-5p and miR-153-3p, on the other hand, are more prevalent in HMC3 than in C20 SEVs, with fold increases of 1.10 ± 0.06 and 1.50 ± 0.12 , respectively. Interestingly, miR-146a-5p and miR-155-5p showed a tremendous difference between the two samples in HMC3 versus C20-derived samples, with an increase of 11.09 ± 1.40 for miR-146a-5p and a drop to 0.14 ± 0.02 for miR-155-5p. These findings make it appear improbable that the increased miR-155-5p in C20 SEVs, which is known to have onco-miRNA characteristics in the context of glioblastoma (Thomas et al., 2020), is connected to the decreased proliferation caused by C20 SEVs.

Thus, a qPCR study was carried out on starved cells, and two markers of the microglia M1 state (IL-6 and TNF- α) and two markers of the M2 state (IL4 and TGF- β) were chosen (**Panel B, Figure 2-9**). The results showed that IL4 gene expression significantly increased in the HMC3 while TNF- α and IL-6 gene expression significantly decreased. In the starving conditions required for the EVs collection, these data support a distinct polarization state of donor cells HMC3 (closer to M2) and C20 (closer to M1). The observed decrease in U87MG proliferation may be explained by the fact that the M1 state of TAMs, but not the M2 state, has been shown to be harmful to tumor growth (Sørensen et al., 2018). Last but not least, it is also possible that these differences are related to the various developmental stages of the cells from which the HMC3

and C20 cell lines were derived, given the varied production of miRNA during brain development.

All these findings prompted us to implement our cell model with a neuroinflammation condition to mimic the brain environment during pathological conditions.

2.10 EVs from inflamed microglia as a crucial inter-cellular way of communication

Neuroinflammation is a common condition in most, if not all, neurodegenerative diseases. This prompted us to extend our investigation on microglia-derived EVs not only to cells in untreated conditions but also after an inflammatory stimulus. We decided to go deeper and investigate the effect of inflammation on the production, cargo, and biological effect of microglia-derived EVs. We settled on using only the C20 cell line after the obtained results in the biological effect on the glioblastoma cell line (**Figure 2-8**), and we defined an inflammation protocol based on the administration of IL-1 β 20 ng/mL for 4h or 24h. Indeed, as will be shown thereafter, the two times deliver similar but not the identical inflammatory signatures.

2.10.1 Optimization of the inflammatory stimulus and EV production characterization

The first phase of the experiments focused on the production and characterization of EVs obtained through different conditions and times of inflammation of the C20 line. Initially, we used two different inflammatory stimuli, namely IL-1 β and LPS, which are widely used in the literature to boost microglia inflammatory phenotype (Ceccarelli et al., 2021a). After 4h stimulation, cells were then placed under starvation conditions for one overnight, and the EVs were collected from the culture medium.

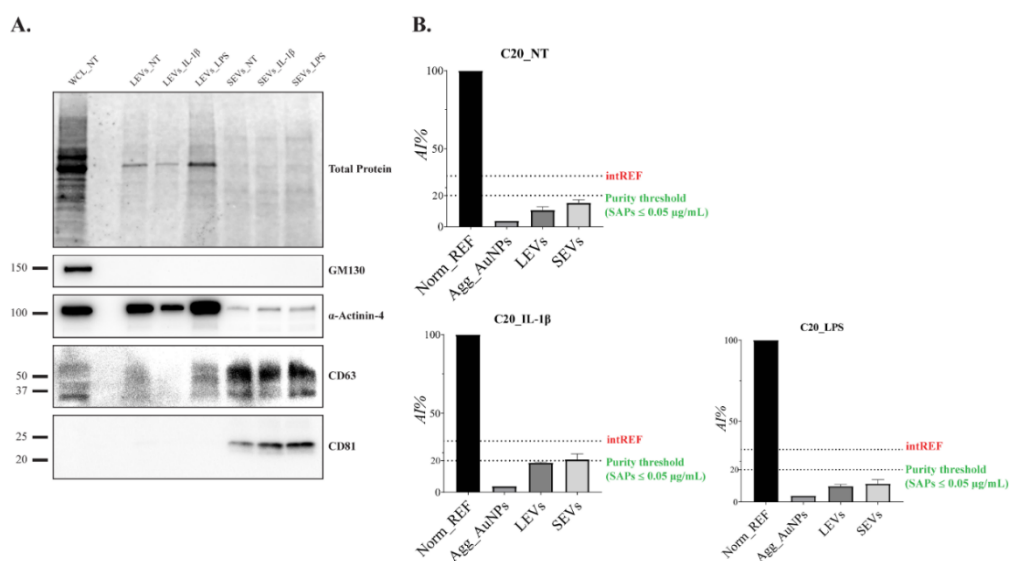


Figure 2-10: Purity assessment and EVs markers of inflamed microglia-derive EVs. Panel A: EVs were gathered from C20 cells using G protocol, lysed, and the proteins separated by SDS-page. EVs gathered from one 150 mm Petri dish were loaded in each lane, together with the whole-cell lysate (WCL) of the corresponding parental cells. Panel B: CONAN assay-mediated purity assessment; mean AI (Aggregation Index) values of the EV preparations were normalized by the Aggregation Index of a solution of AuNPs in HPLC water (Norm_REF). A solution of HPLC water + AuNPs + PBS was used as negative control (intREF). See the main text for details.

Vesicles were selected by sequential ultracentrifugation, obtaining different pellets of LEVs and SEVs, respectively, at 16,000 xg and 100,000 xg. We preliminarily performed a characterization and purity assessment of the EVs prepared, which resembles the one reported in section 2.9.1.

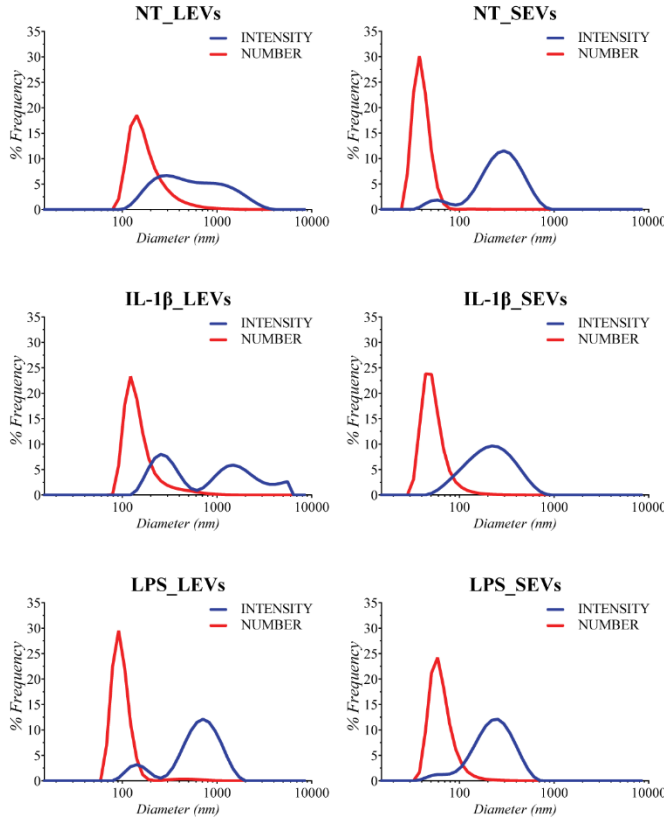
We first analyzed the expression of EVs marker in EVs extracted from C20 cells treated with IL-1 β and LPS. In particular, CD81 (positive EVs marker) show a slight increase in SEVs coming from inflamed C20.

Furthermore, the CONAN assay was performed on the same EVs preparations, confirming that they were pure with no contamination by soluble protein. This was true even after the inflammatory stimulus was administered to parental cells. Indeed, the AI value for all our SEVs samples was always under 20%.

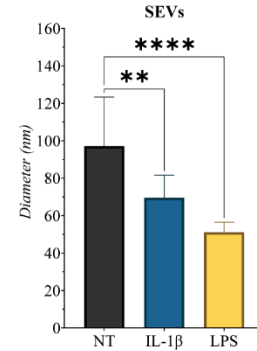
DLS analysis was also performed for the aforementioned EV samples (**Figure 2-11**). In all samples from inflamed microglia, the DLS curves of %Na showed a peak shifted more towards the smaller diameter of EVs, confirming the reduction of the mean diameter. The graphs were used to estimate the average diameter and number of EVs produced in the different conditions. We detected a net increase in the production of SEVs in both inflammatory conditions, around two-fold compared to untreated cells. Untreated cells showed production of 3.19 ± 1.13 SEVs/cell while both inflammatory conditions showed a significant increase, being 5.96 ± 2.21 SEVs/cells for IL-1 β treatment and 6.51 ± 1.87 SEVs/cell for the LPS one. This result is in line with the WB analysis showing a slightly enhanced CD81 expression in SEVs from inflamed cells.

Moreover, we found that inflammation also causes a significant reduction of the SEVs mean diameter: 69.47 ± 12.09 nm for IL-1 β and 51.10 ± 5.35 nm for

A.



B.



C.

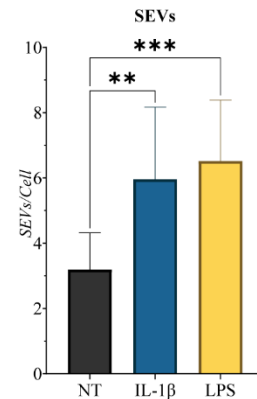


Figure 2-11: Biophysical characterization of EVs from inflamed microglia. Panel A: DLS analysis; EVs pellets derived from two 150 mm Petri Dishes were suspended in 100 μ L PBS with ions and analyzed, representative curves of the number and intensity values are shown for each sample gathered with G. Panel B,C: DLS quantification of the SEVs diameter and number gathered with G of each cell line; the number of EVs/uL was normalized by the number of cells from which SEVs are derived to calculate the number of SEVs secreted per cultured cell. The data represent the mean values $\pm$ SEM of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ vs NT.

LPS, compared to 97.11 ± 26.25 nm for NT. These findings correlate with the literature; indeed, it is known that changes in the state of microglia parental cells correlate with the secretion of different populations of EVs (Grimaldi et al., 2019; Yang et al., 2018), particularly inflammation.

Overall, these experiments validated the protocol of EV production from microglia during an inflammatory stimulus. We next sought to investigate

whether EV produced upon IL-1 β and LPS stimuli effectively carry an inflammatory signal. We first performed a gene expression analysis on the parental C20 cells to assess the relative levels of two pro- and two anti-inflammatory markers after the inflammatory stimuli used before EVs extraction. For each stimulus, the gene expression analysis was performed either after 4h or 24h of treatment (**Figure 2-12**).

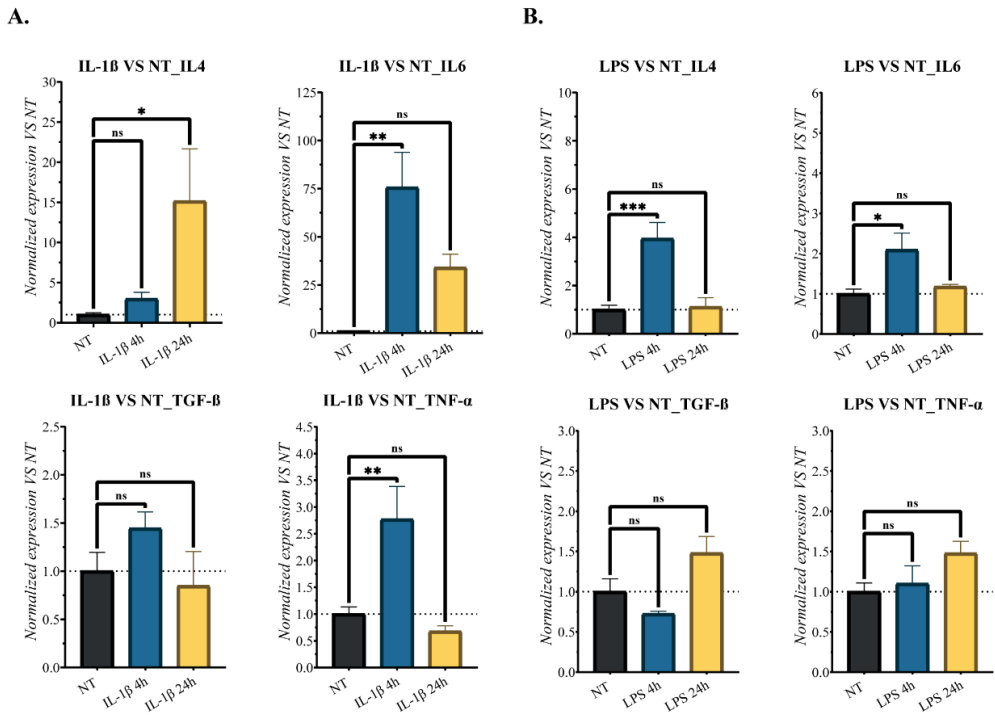


Figure 2-12: mRNA Expression in C20 parental cells after inflammation treatment. Panel A: RT-PCR quantification of mRNA expression in C20 inflamed with IL-1 β 20 ng/mL for 4 or 24h. Panel B: RT-PCR quantification of mRNA expression in C20 inflamed with LPS 10 ng/mL for 4 or 24h. The data were expressed as the relative expression vs control set to 1 and represent the mean values $\pm$ SD of at least three independent experiments performed in duplicate. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs the respective expression in untreated C20, set to 1.

The rationale for this practical choice is that the application of a 4h incubation is probably correlated with the release of particles at an early stage of inflammation, while at 24h, the cell could activate compensatory mechanisms and likely change/modulate the inflammatory output (Da Pozzo et al., 2019; Davis et al., 2018; De Felice et al., 2023; Germelli et al., 2021).

Our analysis showed that, after 4h of inflammation with IL-1 β , there is a net, significant increase in M1 markers, 75.7 $\pm$ 40.8-fold for IL6 and 2.8 $\pm$ 1.4-fold for TNF- α , while M2 markers (IL4 and TGF- β) did not show any significant differences compared to untreated cells.

On the contrary, after 24h or the same treatment, there is a reduction in the M1 markers and a high increase in IL4 expression, 15.6 $\pm$ 14.57-fold, in agreement with the hypothesis of some compensatory mechanism raised to reestablish an M1/M2 gene expression homeostasis after a prolonged inflammatory stimulus.

In cells stimulated with LPS, we only detected a comparable increase of IL6 and IL4 genes after 4h; this was almost completely recovered after 24h.

Overall, these data suggest that LPS is less capable of inflaming human microglia compared to IL-1 β , in agreement with previously published reports (Da Pozzo et al., 2019; Davis et al., 2018; Garcia-Mesa et al., 2017).

Furthermore, they suggest that the stimulus duration also affects the inflammatory status effectively achieved, with a 4h duration being apparently an optimal time to boost inflammatory phenotype with IL-1 β but not with LPS. To rule out if these changes found in the parental cells were maintained in the EVs collected in the cell medium, we analyzed at this time point of inflammatory stimulus the miRNA cargo of the EVs in a similar way to what was reported in Section 2.9.4.

We detected no significant changes in the presence of any miRNA in both inflammatory conditions, with the exception of miR-155-5p, one of the main inflammatory miRNA known, which showed a significant increase, 1.97 ± 0.51 -fold, only after stimulation of IL-1 β for 4h on the parental cells (**Figure 2-13**).

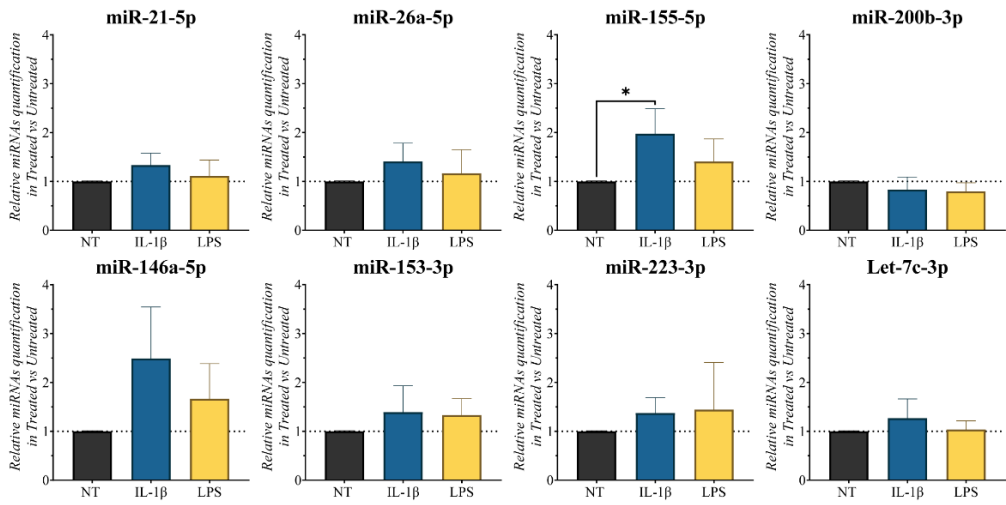


Figure 2-13: miRNA signature of inflamed microglia-derived SEVs. Analysis of miRNA content was performed on the SEVs from C20 inflamed with 20 ng/mL of IL-1 β , 10 ng/mL of LPS or untreated. SEVs pellets from inflamed C20 were gathered with G, resuspended, and lysed in Qiazol buffer, then total RNA was extracted, quantified, reverse-transcribed, and analyzed by qRT-PCR. The data were expressed as the relative expression vs control (NT) set to 1 and represent the mean values $\pm$ SEM of at least two independent experiments performed in duplicate. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, vs the respective expression in untreated

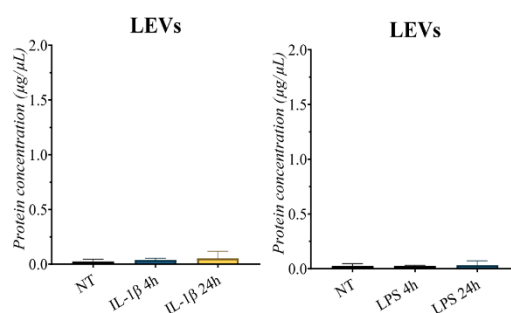
For each individual EV preparation, the amount of EVs-related protein was assessed using the MicroBCA assay. For each sample, using a spectrophotometer, the absorbance at 562 nm was measured, and from the value obtained, the final protein concentration of our EVs suspension was calculated by interpolating the measured value with a previously constructed calibration line using known concentrations of BSA. Results reported in **Figure 2-14** show that the different stimuli resulted in different amounts of collected EVs.

A.

LEVs	Mean Protein Concentration (µg/µL)	SD
NT	0,025	0,0212
IL-1β 4h	0,04	0,0141
IL-1β 24h	0,055	0,0636
LPS 4h	0,025	0,0071
LPS 24h	0,0325	0,0389

SEVs	Mean Protein Concentration (µg/µL)	SD
NT	0,7311	1,05885
IL-1β 4h	1,0944	1,9957
IL-1β 24h	1,3507	1,2869
LPS 4h	0,8093	1,2252
LPS 24h	0,4811	0,5152

B.



C.

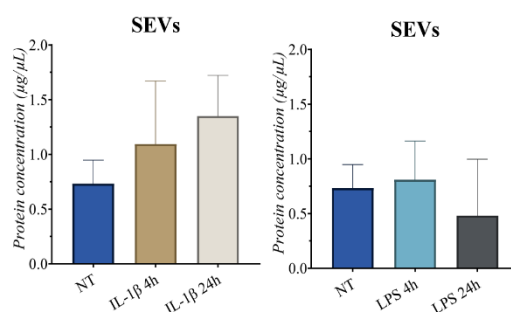


Figure 2-14 Panel A: Protein quantification of EVs from inflamed or untreated C20 cells, gathered with G protocol. EVs were quantified with MicroBCA assay. Data are expressed as mean $\pm$ SD, $n=12$. Panel B-C: Visual representation of data in Panel A, expressed as mean $\pm$ SEM.

From the results expressed in **Figure 2-14**, for each type of EVs derivation, it is observed that the protein concentration of LEVs (**Panel B, Figure 2-14**) is much lower than that obtained for SEVs (**Panel C, Figure 2-14**) in the various samples. This finding is in agreement with what has been reported in the literature. In fact, according to some authors, this different protein content

between the two types of vesicles is mainly related to the structure and different distribution of membrane proteins of the type of EVs (Zendrini et al., 2022). Actually, LEVs have a lower surface/volume ratio than SEVs; it is possible that the portion of protein detected with our protein assay refers to membrane protein rather than protein found in EVs lumen.

Furthermore, while the EV protein quantities measured after IL-1 β well fit with the number of EVs estimated with DLS (see **Panel C, Figure 2-11**), the same is not valid for the samples stimulated with LPS, for which the amount of protein detected in the pellet was not increased with respect to the untreated control. While this could at least in part be explained by the smaller diameter of EVs collected by LPS stimulation (**Panel B, Figure 2-11**), these data corroborated the idea that LPS stimulation could be problematic for the downstream experiments for a number of different reasons. First of all, mRNA expression on parental cells showed how the LPS is less able to induce a pro-inflammatory phenotype than IL-1 β in both C20 cells and corresponding EVs. Moreover, for LPS, the estimate of the protein content could not correctly predict the amount of EVs to be administered as treatment. For all these reasons, we decided to continue the experiments using only IL-1 β as the inflammatory stimulus.

2.10.2 Biological Effect of inflamed microglia-Derived EVs

The following sections will describe our analysis of the biological effect of our EVs samples on two types of cells: human microglia and human glioblastoma. As described in the previous optimizations step, we treated parental cells with IL-1 β 20 ng/mL for 4h or 24h. As a control for the EVs treatments, we used EVs from untreated cells (NT). We also compared the effect of EVs from untreated cells against the administration of just PBS, the buffer used to resuspend all our EV samples. Thus, cells were subjected to 4 possible conditions: PBS, NT, IL-1 β 4h, or IL-1 β 24h.

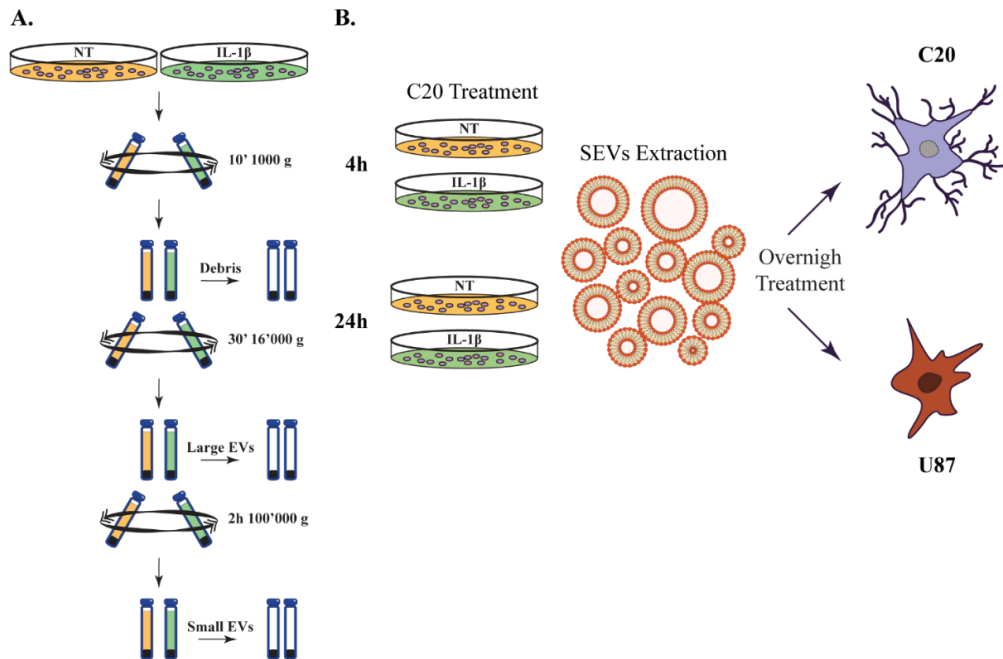


Figure 2-15: Schematics of the Extraction protocol (Panel A) and optimized inflammation stimulus (Panel B). C20 cells were inflamed with 20 ng/mL of IL-1 β for 4h, 24h. SEVs were extracted and administrated to recipient cells for one overnight.

In the scratch test and the invasion assay, all the EVs samples described above were administered to donor cells for one overnight (16-18h). The hours indicated in the nomenclature refer to the hours of treatment on the parental cells.

2.10.3 On microglia

After being extracted and quantified, EVs were used as a treatment to assess their biological function on the same C20 line. In detail, the influence of the different EV categories on recipient cells' migration, invasion capability, and expression of inflammatory markers was assessed.

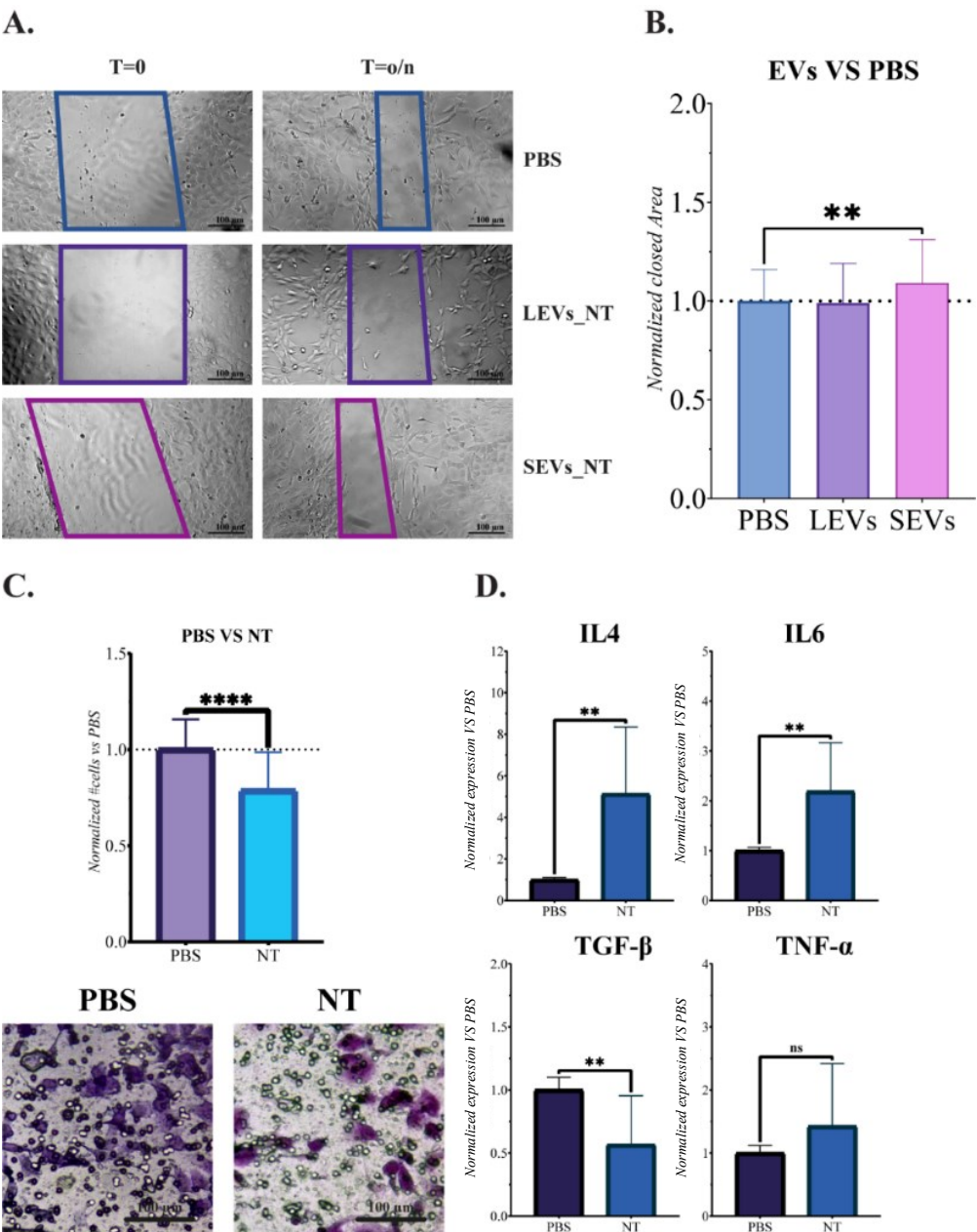


Figure 2-16: Biological effect of untreated microglia-derived EVs on recipient microglia. *Panel A:* EVs were extracted from untreated C20 parental cells (NT) and resuspended in PBS. SEVs or just PBS were administrated to a previously scratched culture of C20 recipient cells for one overnight, representative images show recipient cells after the scratch (T=0) and after one overnight of treatment (T=o/n). *Panel B:* Closed area measured with ImageJ software. Recipient cells were treated with LEVs and SEVs from untreated C20 or just PBS. Images were acquired with an optical microscope (10x magnification) and closed gap measured with ImageJ software. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ vs PBS control, set to 1. *Panel C:* Invasion assay performed by treating recipient C20 with SEVs from untreated C20 or just PBS, recipient cells were seeded on a Matrigel matrix and treated one overnight with SEVs. After treatment, cells were stained, and images acquired with an optical microscope (20x magnification). The number of cells capable of passing through the membrane was counted and normalized against the same value in the PBS control, set to 1. *Panel D:* mRNA expression in recipient C20 treated with SEVs from untreated C20. The significance of the differences was determined by t-test: $**P \leq 0.01$, vs the respective expression in untreated C20, set to 1. All values are expressed as the mean $\pm$ SD of at least two independent experiments.

Firstly, we performed a scratch test on the C20 cell line by administering to them an amount of LEVs equivalent to 250 ng of protein content and an amount of SEVs equivalent to 500 ng of protein content (**Panel A, Figure 2-16**). Then, two images per well were acquired by optical microscopy: at time $t=0$ and after 16-18h incubation with the EVs. The scratched areas were measured at $T=0$, and after one overnight of treatment, data are represented as the percentage of the closed area, normalized against the control, in this case, PBS. A bigger value compared to the control indicate an increase in the mobility of the cells as the closed area increased. Compared to the PBS condition, no significant differences were measured in the closed area of cells incubated with NT LEVs. Conversely, SEVs from NT cells were able to increase the mobility of recipient C20 by a small but significant average of 9.1% (**Panel B, Figure 2-16**).

Microglia is also capable of invading neighbor tissues upon stimulation, and this process is critical to controlling and resolving eventual damage in the brain (Lively and Schlichter, 2013). We decided, then, to analyze the influence of SEVs on the invasion capability of microglia (**Panel C, Figure 2-16**).

We started administering 500 ng protein content of SEVs extracted from NT cells to other C20 cells, and we counted the number of cells capable of penetrating a Matrigel matrix. Our data showed how SEVs from untreated cells

significantly reduce, by 21.2%, the invasion capability of C20 cells treated with just PBS with no EVs. This data seems to be in disagreement with the scratch test; however, the scratch test provides information on the migration capability of large masses of cells (Justus et al., 2014; Zhang et al., 2012); the invasion assay, instead, provides information on the capability of single cells to respond to chemoattractant (Castellone et al., 2011; Junger, 2008; Justus et al., 2014; Lazennec and Richmond, 2010; Peter et al., 2008; Radu et al., 2004; Yang et al., 2005; Zhang et al., 2012). Our data suggest that SEVs from untreated cells can reduce the sensitivity to chemoattractant while not interfering with the scanning capability of microglia cells.

To better understand the causes of the biological effect detected in the two migration assays, we decided to evaluate, by RT-PCR, the expression of two pro- and two anti-inflammatory marker genes in the cells recipient the SEVs. We extracted SEVs after all the treatments, and we administered 1.25 μ g protein content of EVs to recipient cells overnight, the same administration time used in both motility assays (*Panel A-B, Figure 2-16*).

SEVs were able to induce significant changes in the expression of the chosen markers, even from untreated cells. IL4 and IL6 showed an increase in the NT condition compared to PBS, stronger for IL4 with a 5.12 ± 3.23 -fold increase than for IL6 with a 2.19 ± 0.97 -fold increase. On the contrary, we detected a decrease of 0.57 ± 0.39 -fold in the expression of TGF- β in the NT condition, and TNF- α did not show significant changes.

Overall, these data allow telling that the SEVs, but not LEVs, are able to affect the condition of recipient cells even if extracted from untreated cells. The mRNA signature of recipient C20 suggests a light state of activation in recipient cells, hence the reduction of TGF- β ; this, with the increase expression of IL4, supports the increase in the migratory capability of recipient cells

(Lively and Schlichter, 2013). The reduction of invasion could be explained by SEVs from untreated microglia being able to reduce sensitivity to chemoattractants in the absence of a traumatic event in the environment. In physiological conditions, microglia need to be able to maintain its patrolling capability but, in the absence of an injury, not induce a migratory event; this is essential because pathological activation and chemotaxis of microglia have been reported in various neurodegenerative diseases, such as Alzheimer's Disease (Fan et al., 2017).

These data on untreated C20-derived SEVs motivated us to perform the same set of analyses on C20 treated with EVs derived from inflamed cells.

Even in these conditions, LEVs could not influence the migratory capability of treated cells in the scratch test (*Panel A, Figure 2-17*). Instead, SEVs were able to increase the recovered area by 7.5% in the IL-1 β 24h condition, compared to the NT (*Panel B, Figure 2-17*). This may suggest that SEVs from C20 cells start to acquire pro-inflammatory characteristics after 24h of inflammation of parental cells; indeed, inflamed microglia acquire increased mobility (Lively and Schlichter, 2013). However, this effect is minimal and

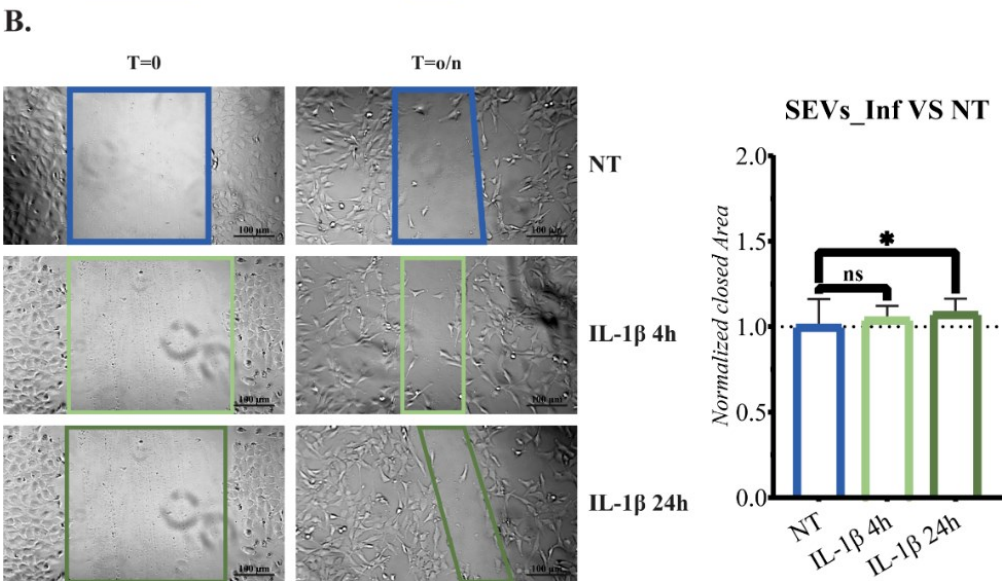
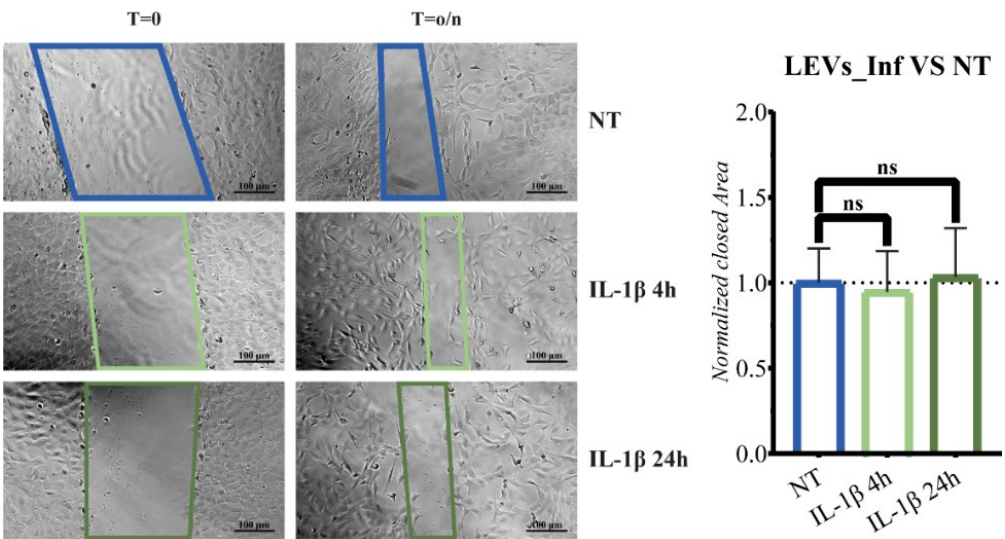


Figure 2-17: Scratch test on recipient C20 treated with EVs from inflamed cells. Panel A: left, C20 recipient cells were treated with LEVs from C20 parental cells inflamed with 20 ng/mL of IL-1 β for 4h or 24h, or untreated. LEVs were administrated to a previously scratched culture of C20 recipient cells for one overnight. Left: representative images show recipient cells after the scratch (T=0) and after one overnight of treatment (T=o/n). Right: images were acquired with an optical microscope (10x magnification) and closed gap measured with ImageJ software. Panel B: C20 were treated with SEVs from C20 cells inflamed with 20 ng/mL of IL-1 β for 4h or 24h, or untreated. SEVs were administrated to a previously scratched culture of C20 recipient cells for one overnight. Left: representative images show recipient cells after the scratch (T=0) and after one overnight of treatment (T=o/n). Right: images were acquired with an optical microscope (10x magnification) and closed gap measured with ImageJ software. All data are expressed as mean $\pm$ SD of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$. vs NT control, set to 1.

does not entirely correlate with our data regarding the expression of pro-inflammatory markers in parental cells inflamed for 24h (**Panel A, Figure 2-12**).

The invasion assay showed that SEVs from C20 inflamed for 4h increased the chemo attractive response of our recipient cells by an average of 17.1% value. This effect was reverted entirely in recipient cells treated with SEVs from C20 inflamed for 24h, which did not show any difference from the NT.

mRNA expression analyses revealed that both treatments on parental C20 cause the secretion of SEVs with more marked pro-inflammatory characteristics than the one extracted from untreated cells. IL4 in recipient cells was notably decreased, with a fold change of 0.7 ± 0.37 in the IL-1 β 4h condition and 0.39 ± 0.12 in the 24h condition. TNF- α had a significant increase, 2 ± 0.47 -fold, in the 4h condition compared to the NT; this result, together with the decrease of IL4, supports the motility assays, confirming that SEVs from inflamed microglia can propagate inflammation to other microglia cells, hence the increase in motility and in response to chemoattractant.

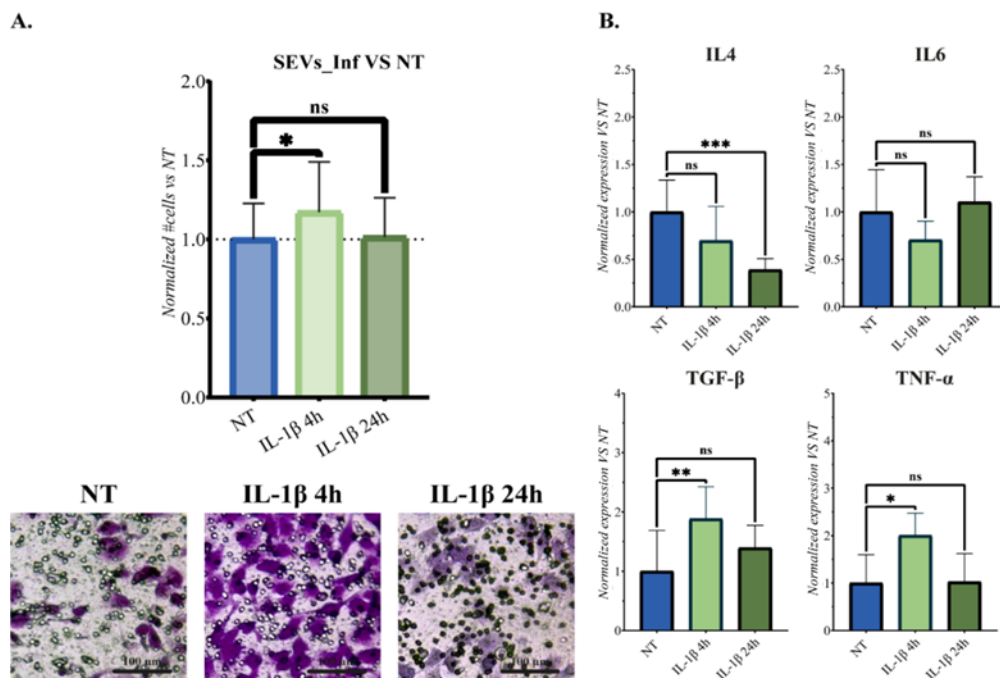


Figure 2-18: Invasion assay on recipient C20 treated with SEVs from inflamed cells. Panel A: Cells were treated with SEVs from C20 inflamed with 20 ng/mL of IL-1 β for 4h or 24h, or untreated. Recipient cells were seeded on a Matrigel matrix and treated one overnight with SEVs. After treatment, cells were stained, and images acquired with an optical microscope (20x magnification). The number of cells capable of passing through the membrane was counted and normalized against the same value in the NT control, set to 1. Bottom, representative images show recipient cells after one overnight of treatment. Panel B: qPCR quantification of mRNA expression in recipient C20 cells treated with SEVs from parental C20 inflamed with 20 ng/mL of IL-1 β for 4h or 24h, or untreated. Recipient cells were treated with 1.25 μ g of SEVs for one overnight, then total RNA was extracted, quantified, reverse-transcribed, and analyzed by qRT-PCR. All data are expressed as mean $\pm$ SD of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, vs NT control, set to 1.

Conversely, TGF- β showed a marked increase in the 4h condition, 1.88 ± 0.54 -fold. This pattern of expression suggests that SEVs from inflamed C20 can propagate pro-inflammatory signals; recipient cells are activated to an inflammatory state after treatment with SEVs from C20 inflamed for 4h, hence the increase in TNF- α and the decrease in IL4. This hypothesis is furthermore supported by the significant increase of miR-155-5p in SEVs from inflamed C20 (**Figure 2-13**). The increase expression of TGF- β could be explained by microglia trying to resolve an inflammatory stimulus and to come back to homeostasis (Norden et al., 2015; Norden and Godbout, 2013). Recipient cells treated for 24h (**Panel A, Figure 2-12**) showed a pattern of inflammatory markers similar to a resolved pro-inflammatory condition; indeed, SEVs produced by these cells showed very little difference from the one produced by untreated cells, meaning a reduced response to chemoattractant and increase motility (**Figure 2-18**).

2.10.4 Preliminary data on the effect of inflamed microglia EVs on Glioblastoma

EVs-mediated intercellular communication is an essential process in the brain environment, not only to maintain brain homeostasis by interacting with resident cells but also with unwanted ones. Therefore, we performed analyses to assess the influence of microglia-derived EVs on a glioblastoma cell line: U87MG.

We extracted EVs from C20 cells in untreated conditions and, after inflammatory stimulus, quantified them and administered them to U87MG, 250 µg protein content of LEVs, and 500 µg protein content of SEVs.

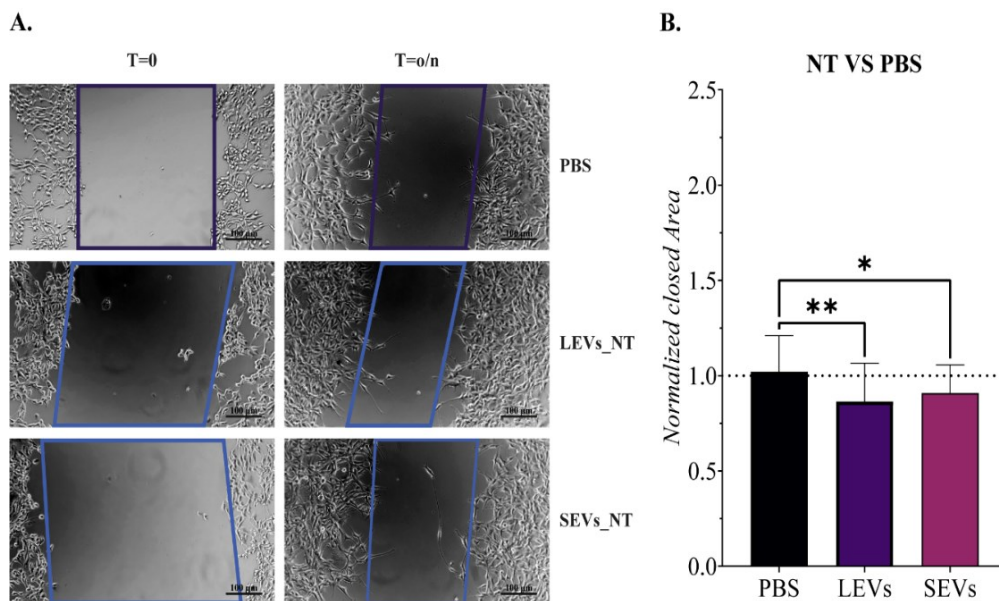
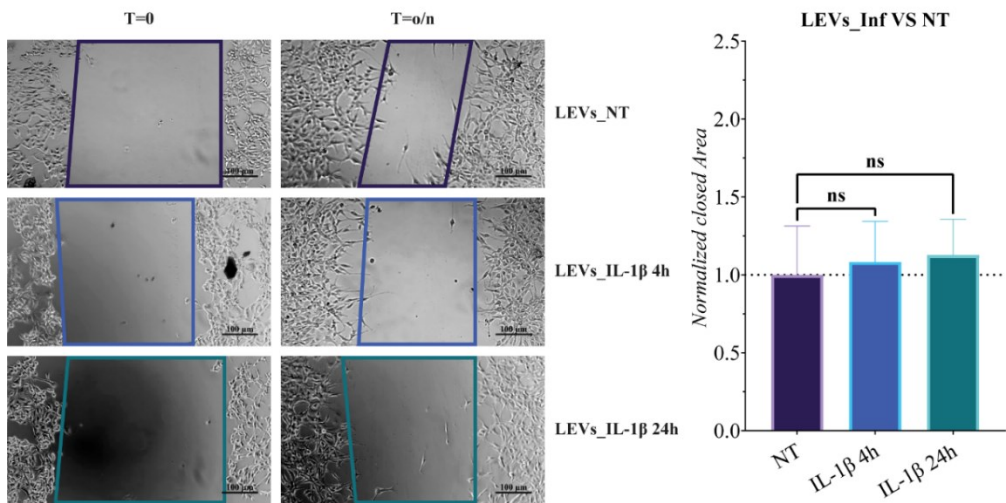


Figure 2-19 Scratch test on Recipient U87MG. Panel A: EVs were extracted from untreated C20 parental cells (NT) and resuspended in PBS. SEVs or just PBS were administrated to a previously scratched culture of U87MG recipient cells for one overnight, representative images show recipient cells after the scratch (T=0) and after one overnight of treatment (T=o/n). Panel B: Closed area measured with ImageJ software. Recipient cells were treated with LEVs and SEVs from untreated C20 or just PBS. Images were acquired with an optical microscope (5x magnification) and closed gap measured with ImageJ software. Data are expressed as means $\pm$ SD of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, ** $P \leq 0.01$ vs PBS control, set to 1.

Results showed that both LEVs and SEVs from NT C20 can reduce the mobility of U87MG cells, respectively, by 13.5% and by 9.11% (**Figure 2-19**). These results suggest that homeostatic microglia can stem the metastatic capability of glioblastoma cells by reducing their mobility, which adds to the previous ability of the same SEVs to slow down the cell cycle progression of the same cells (**Figure 2-8**).

Conversely, LEVs from inflamed C20 did not show any significant effect on

A.



B.

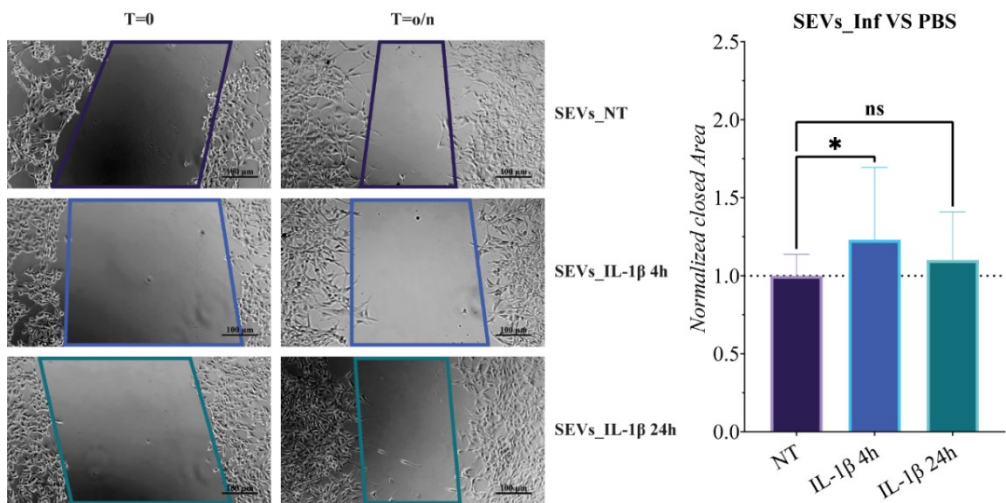


Figure 2-20 Scratch test on recipient U87MG treated with EVs from inflamed cells. Panel A: left, U87MG recipient cells were treated with LEVs from C20 parental cells inflamed with 20 ng/mL of IL-1 β for 4h or 24h, or untreated. LEVs were administrated to a previously scratched culture of C20 recipient cells for one overnight. Left: representative images show recipient cells after the scratch (T=0) and after one overnight of treatment (T=o/n). Right: images were acquired with an optical microscope (10x magnification) and closed gap measured with ImageJ software. Panel B: U87MG were treated with SEVs from C20 cells inflamed with 20 ng/mL of IL-1 β for 4h or 24h, or untreated. SEVs were administrated to a previously scratched culture of C20 recipient cells for one overnight. Left: representative images show recipient cells after the scratch (T=0) and after one overnight of treatment (T=o/n). Right: images were acquired with an optical microscope (10x magnification) and closed gap measured with ImageJ software. All data are expressed as mean $\pm$ SD of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$. vs NT control, set to 1.

the migratory capability of glioblastoma. SEVs from inflamed cells could increase the mobility of our glioblastoma cell line by 23.1%, but just in the IL-1 β 4h condition; no significant effect was detected in the 24h condition.

Our hypothesis at the moment is that C20 inflamed for 4h secrete pro-inflammatory SEVs, meaning that the effect of these SEVs could be to spread inflammation in the brain environment. At the moment, our data regarding these effects are limited, and this set of experiments is not enough to thoroughly understand the cause of these observed effects on glioblastoma. There will be the need to complete our experimentation with the invasion assay. Moreover, we could analyze the change in the expression of some critical EMT genes.

2.11 Materials and Methods

2.11.1 Cell culture

All materials for cell culture were purchased by Corning, New York, USA. HMC3 cells (ATCC, Manassas, Virginia) were cultured in MEM (10-009-CV) added with 10% FBS (35-089-CV), 100 U/mL Penicillin, 100 µg /mL Streptomycin. C20 cells, initially generated by David Alvarez-Carbonell et al. (Case Western Reserve University) (Garcia-Mesa et al., 2017) and kindly gifted by Christian H. Wetzel, were cultured in DMEM F12 50/50 (10-092-CV) added with 10% FBS (35-089-CV), 100 U/mL Penicillin, 100 µg /mL Streptomycin, and 600 µg /mL Neomycin. The cells were maintained at 37°C in a 5% CO₂ incubator. Before EVs production, both cell lines were seeded in 150 mm Petri dishes or 100 mm Petri dishes, and, once confluency reached 90-95%, the complete medium was removed, cells were washed once with PBS with ions (D8662) and added with the same medium without FBS (Starvation Medium) for many valid reasons, according to MISEV2018 indications, mainly: i) FBS contains high levels of EVs, ii) serum deprivation increases EVs production (Théry et al., 2018a), iii) FBS contains high levels of bovine serum albumin (BSA) and other soluble nanoparticles (e.g., lipoproteins) that could be co-isolated with EVs and are considered exogenous contaminants; iv) microglia grown without serum showed a closer similarity to homeostatic microglia and low levels of activation (Bohlen et al., 2017; Verderio et al., 2012).

U87MG cells (CLS Cell Lines Service GmbH, Eppelheim, Germany), a cell line of human glioblastoma, were cultured in RPMI 1640 (15-040-CV) added with 10% FBS, 100 U/mL Penicillin, 100 µg /mL Streptomycin, and maintained at 37°C in a 5% CO₂ incubator.

2.11.2 EVs isolation From untreated Cells

HMC3 and C20 cells were maintained in Starvation Medium one over-night (16-18h), the supernatant was collected, and the cells detached and counted. Cells were maintained in 150 mm Petri dishes with 16 mL of complete medium or 12 mL of the starvation medium; the mean amount of cells relative to one 150 mm Petri dish is 12.8×10^6 for C20 cells and 18.1×10^6 for HMC3 cells. Supernatants were centrifuged 10' at 4°C at 1000 xg to remove any cellular debris. Two isolation protocols were used to gather EVs from the cell supernatant: EVs were extracted by single ultracentrifugation at 4°C at 100'000 xg for 2h or extracted in two steps, 30' at 4°C 16,000 xg to isolate the LEVs and 2h at 4°C 100,000 xg to isolate the SEVs. All EV pellets were washed with an excess (3 mL) of PBS with ions (Sigma cat. D8662) 0.22 µm filtered and centrifuged again in the same condition used to extract them. The pellets obtained were suspended in lysis buffer with protease inhibitors or PBS with ions 0.22 µm filtered, depending on the specific use.

2.11.3 EVs isolation from inflamed Cells

C20 WT cells are seeded in six 100 mm Petri dishes, each with one million cells, and maintained for 72 hours in a 37°C incubator with a humidity-saturated atmosphere and 5% CO₂. When the culture has reached about 90-95% confluence, treatments are carried out. The following scheme is used for each experiment: two plates are untreated, two are treated with interleukin-1β (IL-1β) 20 ng/mL, and two with lipopolysaccharide (LPS) 10 ng/mL. For each treatment, one plate is incubated for 4h and one for 24h. After the time of the treatment, the supernatant was collected, and the cells detached and counted. Supernatants were centrifuged 10' at 4°C at 1000 xg to remove any cellular debris. EVs were extracted in two steps, 30' at 4°C 16,000 xg to isolate the LEVs and 2h at 4°C 100,000 xg to isolate the SEVs. All EV pellets were washed with an excess (3 mL) of PBS with ions (Sigma cat. D8662) 0.22 µm

filtered and centrifuged again in the same condition used to extract them. The pellets obtained were suspended in lysis buffer with protease inhibitors or PBS with ions 0.22 μ m filtered, depending on the specific use.

2.11.4 Western Blot Analysis of Cells and EVs

Cell pellets were suspended in Ripa Buffer added by Protease inhibitors (Merck KGaA), lysed, and protein content quantified with DC Protein Assay (Biorad) (LOWRY et al., 1951), 1 μ g of total protein content was loaded on a gel for the detection of α -Actinin-4 and 30 μ g for each of the other proteins. EVs pellets were suspended and lysed in Ripa Buffer added by Protease inhibitor, and the equivalent quantity of total protein suspension coming from one 100% confluent 150 mm Petri dish or from one 100 mm Petri dish after the inflammatory stimulus was loaded in each lane. All samples were added with Laemmli Solution and boiled 5' at 95°C, then an SDS-PAGE was performed in a 4-20% polyacrylamide gel. The following antibodies were used: mouse anti- α -Actinin-4 (sc-390205) 1:500, mouse anti-CD81 (sc-166029) 1:2000, mouse anti-CD63 (sc-5275) 1:250, mouse anti-GM130 (BD Bioscience, 610822) 1:1000, rabbit anti-GAPDH (Sigma, G9545) 1:2500, anti-rabbit and anti-mouse-HRP (Merk) 1:5000. The band intensity was acquired with ChemiDoc (Biorad) and quantified using Image Lab Software (Biorad). The intensity of the lanes was compared after normalization to the total protein loaded in each lane estimated with the Stain-free technology of the Biorad precast gels.

2.11.5 Purity assessment of C20 and HMC3 EV subpopulations

The absence of soluble single and aggregated proteins not belonging to EVs (hereafter referred to as exogenous SAPs) was assessed by using the Colorimetric NANoplasmonic assay (CONAN), following the open-access protocol described elsewhere (Zendrini et al., 2020). The purity grade of the

16'000 g and 100'000 g EV preparations derived from untreated HMC3 cells and from untreated or inflamed C20 were characterized using a 6 nM solution of gold nanoparticles (AuNPs) with diameters of 13 nm. LSPR spectra of three technical replicates for each sample were analyzed by a Perkin-Elmer Ensign Multimode Plate Reader and used to determine the mean relative Aggregation Index (AI).

2.11.6 TEM Analysis

EVs pellets derived from two 150 mm Petri dishes were suspended in 100 μ L PBS with ions, maintained at -80°C, and thawed only once immediately before analysis. Samples were prepared using a two-step protocol for negative staining as described elsewhere (Anastasi et al., 2020). Briefly, EVs suspension has been adsorbed onto carbon-coated 300 mesh copper grids (Electron Microscope Science, Hatfield, PA, USA). Adsorption time has been changed between 30 and 45 minutes dependently on sample size and suspension concentration. Then grids were washed three times with pure water and stained for 30 s with our homemade X solution diluted 1/5 or 1/10 (v/v) in pure water (Moscardini et al., 2020). Dilution of the staining solution is required to reduce EVs shrinkage. The grids were then paper-drained and directly analyzed with a ZEISS Libra 120 Plus transmission electron microscope, operating at 120 kV and equipped with an in-column omega filter for energy-filtered imaging. EM micrographs are collected with a 16-bit CCD camera (Zeiss, Oberkichen, Germany) at 20.000-25.000 X. Images were finally analyzed with ImageJ software (NIH) for the morphological characterization. Each EV diameter was estimated by measuring the perimeter (p), approximating it as a circumference, and using it to calculate the diameter with the formula: $d=p/\pi$.

2.11.7 Dynamic Light Scattering (DLS) Analysis

EVs pellets derived from two 150 mm were suspended in 100 μ L PBS with ions, maintained at -80°C, and thawed only once immediately before analysis. Dynamic light scattering measurements were performed with a ZetaSizer Nano-ZS90 (Malvern Instruments Inc., Malvern, UK). The Instrument laser wavelength is 633 nm and, as scattering angle (θ), 90° was chosen. The mean hydrodynamic diameter was calculated from the autocorrelation function of the intensity of scattered light by DTS Nano software (version 1.41, Malvern Instruments Inc., Malvern, UK). Briefly, the intensity fluctuations are used to obtain an intensity-weighted distribution (% I_a). % I_a is linked to the size of the macromolecules as shown in equation 1:

$$\%I_a = \frac{a^6 N_a * 100}{N_a a^6 + N_b b^6} \quad (1)$$

where N_a represents the population of molecules with size a and N_b is the population of macromolecules with size b . Summing, the intensity distribution is approximately proportional to (size)⁶. Starting from the intensity distribution, a number-weighted distribution (% N_a) can be obtained (Stetefeld et al., 2016):

$$\%N_a = \frac{N_a * 100}{N_a + N_b} \quad (2)$$

where only the number of vesicles constituting each population is taken into account. The % N_a distributions obtained for each replica of SEVs sample (G_100K samples) were fitted with a mono-peak gaussian function:

$$y = y_0 + A * \exp(-0.5 * ((x - x_c)/w)^2) \quad (3)$$

Moreover, the x_c value used to calculate the average hydrodynamic diameter of the distribution. The mean $\pm$ SD of all the obtained peak values were plotted

and analyzed. 50 μL of each sample were diluted in PBS to a final volume of 130 μL and measured 5 times.

For a better evaluation of the obtained EV populations, the polydispersity index (PDI) of each population was also calculated using equation (4). Notably, the ZetaSizer software permits to estimate the vesicle concentration in a sample by knowing the size of a population, its PDI, and its count rate (intensity of the scattered light). The concentration values obtained for the different replicas were used to plot the graphs.

$$PDI = \left(\frac{\text{Std Dev.}}{\text{Mean of the population}} \right)^2 \quad (4)$$

2.11.8 U87MG cell proliferation assay

U87MG cells were seeded in 96-well (3000 cells per well) and maintained in a complete medium. After 24h, cells were treated with sequential dilutions of SEVs pellet or LEVs pellet extracted from untreated HMC3 and C20. EVs were previously suspended in sterile-filtered PBS with ions, and protein content was assessed using Micro BCA Protein Assay Kit (ThermoFisher). EVs were diluted in the same buffer and administered to cells starting from 0 ng to 500 ng of total EV protein for the SEVs pellet and from 0 to 31.25 ng for the LEVs pellet. Treated U87 cells were maintained in a complete medium for 48 or 72 hours, and then proliferation was quantified using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega) following the supplier's instructions.

2.11.9 U87MG cell cycle assay

U87MG cells were seeded in 12-well (31000 cells per well) and maintained in a complete medium. After 24h, cells were treated with 2.5 μg of SEVs sample quantified with Micro BCA assay. Total cells deriving from two 12-well with the same treatment were pooled and analyzed. The quantification of the

percentage of cells in the different cell phases was performed using the Muse Cell Cycle Kit (MCH100106) using the Muse Cell Analyzer (Giacomelli et al., 2016) following the supplier's instructions.

2.11.10 U87MG cleaved caspase-3 assay

U87MG cells were seeded on an 8-well chamber slide at medium-high density. The following day cells were treated for 48 h with 25 µg/ml SEVs extracted from untreated C20 cells, with the same PBS volume as a negative control, or with 20 mM DTT for 2 hours as positive apoptosis control (Xiang et al., 2016). Samples were subjected to immunofluorescence for cleaved caspase-3, as described (Marchetti et al., 2019b). Briefly, cells were fixed in cold 1:1 acetone:methanol solution for 15 min at – 20°C, washed three times in PBS, permeabilized with 0.5% Triton X-100 plus 2.5 % BSA/PBS for 5 min, blocked for 1 h at room temperature with 5% BSA/PBS, incubated overnight at 4°C with anti-cleaved caspase-3 (1:300, 9664 Cell Signaling Technology) antibody in 2.5% BSA/PBS and stained 1 h at room temperature with an anti mouseAlexa488 secondary antibody (1:100, ThermoFisher). Samples were then mounted with Fluoroshield-DAPI (Sigma-Aldrich) and imaged with a laser scanning confocal microscope (Nikon A1, Eclipse Ti) using a 20x air-objective (NA 0.5) and pinhole set to 1 Airy Unit. Images were acquired at 1024 × 1024 pixel resolution using a 405 nm laser (425-475 emission window) and a 488 nm laser (500-600 emission window). Cleaved caspase-3 positive cells were manually counted using the ImageJ software, as DAPI positive cells displayed a mean intensity above an intensity threshold in the Alexa488 channel set according to evidently apoptotic cells in the DTT-treated sample.

2.11.11 miRNAs quantification (qRT-PCR)

EVs pellets from untreated HMC3 and untreated or inflamed C20 were suspended and lysed in Qiazol buffer (QIAGEN); total RNA was extracted

using miRNeasy Micro Kit (QIAGEN, cat. 217084) by adding 5 µl of 5 nM cel-miR-39 (GenePharma) after the Qiazol step and then following the manufacturer's protocol, extracted RNA was then quantified using NanoDrop (Thermofisher). 200 ng of total RNA was used for the reverse transcriptase reaction, according to the manufacturer's recommendation, using miR-X miRNA first-strand synthesis kit (Takara) to retrotranscribe mature miRNAs. The quantification of mature miRNAs was performed with Rotor-Gene Q 2plex (Qiagen), using the SsoAdvanced SYBR Green Supermix (BIO-RAD) according to the manufacturer's protocol. The relative quantification was performed using the Rotor-Gene Q Software by normalizing it to cel-miR-39. All reactions were performed in triplicate, and the results are expressed as the mean of three biological replicates. The list of primers for the analyzed miRNA is reported in **Table 2-1**.

Table 2-1 Primers used for miRNA and mRNA expression.

miRNA ID	Primers FW
hsa-mir-21-5p	TAGCTTATCAGACTGATGTTGA
hsa-mir-26a-5p	TTCAAGTAATCCAGGATAGGCT
hsa-mir-146a-5p	TGAGAACTGAATTCCATGGGTT
hsa-mir-153-3p	TTGCATAGTCACAAAAGTGATC
hsa-mir-155-5p	TTAATGCTAATCGTGATAGGGGTT
hsa-mir-200b-3p	TAATACTGCCTGGTAATGATGA
hsa-mir-223-3p	TGTCAGTTTGTCAAATACCCCA
hsa-let-7c-3p	CTGTACAACCTTCTAGCTTTCC
IL4_74 bp_55°C	FW 5'-ACTTTGAACAGCCTCACAGAG-3'
	RV 5'-TTGGAGGCAGCAAAGATGTC-3'
IL6_165 bp_55°C	FW 5'-TCCTCGACGGCATCTTCA-3'
	RV 5'-TTTTTACCAGGCAAGTCTCCT-3'
TGF- β _218 bp_55°C	FW 5'-ACTGCAAGTGGACATCAACG-3'
	RV 5'-TGCGGAAGTCAATGTACAGC-3'
TNF- α _101 bp_55°C	FW 5'-AGGGACCTCTCTCTAATCAGCC-3'
	RV 5'-GCTTGAGGGTTTGCTACAACA-3'
β -Actin_254 bp_55°C	FW 5'-GCACTCTTCCAGCCTTCCTTCC-3'
	RV 5'-GAGCCGCCGATCCACACG-3'
GAPDH_107 bp_55°C	FW 5'-GAGAAGTATGACAACAGCCT-3'
	RV 5'-CCTTCCACGATACCAAAGTT-3'

2.11.12 Gene expression analysis

Gene expression was evaluated in C20 parental cells both in untreated and inflamed conditions, in C20 recipient SEVs from inflamed C20, and in HMC3 parental cells in untreated conditions.

For parental cells in untreated conditions, C20 and HMC3 cells were seeded in 100 mm Petri dishes and grown up to 100% confluency in a complete medium. After overnight starvation, cells were collected, and total RNA was extracted using Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions.

For parental C20 in inflamed conditions, cells were seeded and treated as described in section 2.6.3. After treatment and overnight starvation, cells were

collected, and total RNA was extracted using Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions.

For recipient C20, cells were seeded in 24-well, 80,000 cells in each well, and maintained in a complete medium for 24h. After that, cells were treated with 1.25 μ g of EVs extracted in all the conditions described in section 2.10.1 for one overnight; every treatment was performed in double point. After overnight treatment with SEVs, cells were collected, and total RNA was extracted using Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions.

cDNA synthesis was performed with 2 μ g of RNA using an i-Script cDNA synthesis kit (BioRad) following the manufacturer's instructions. Real-time RT-PCR reactions mix consisted of 10 μ L SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.5 μ L of 10 μ M forward and reverse primers, 5 μ L cDNA (75 ng), and 4 μ L H₂O. Reactions were performed for 40 cycles using this temperature profile: 98 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 3 seconds. β -actin and GAPDH were. The relative mRNA levels of each gene in each sample were derived from analysis of the Ct value normalized to both β -actin and GAPDH used as housekeeping genes by using the CFX Maestro Software (Bio-Rad). The list of the used primers is reported in Table 2-1.

2.11.13 Scratch test

The scratch test, or wound healing assay, is an assay that is performed to evaluate cell migration on monolayer cell cultures. 20'000 cells per well are seeded in a 96-well multiwell and left in culture for 24h in the complete medium until a monolayer is achieved. Then, the medium is removed, and the monolayers are "scratched" gently using a 10 μ L tip. The detached cells are removed by washing the monolayers with PBS with ions, and then the cells are grown in a culture medium containing 1% FBS, in which treatment with LEVs

or SEVs extracted from C20 cells is carried out as described in section 2.10.1. After treatment, two bright-field photos are acquired for each well using a 10x objective for the C20 line, or 5x, for the U87 line. These photos will represent time zero (T=0) referenced to evaluate the percentage of reduction of the ‘wound’ area after an overnight incubation, where two images per well will again be acquired. The normalized covered area expressed in each graph is calculated as follows:

$$1 - \frac{\text{Area of scratch after one overnight}}{\text{Area of scratch at } T = 0}$$

This value indicates the percentage of the closed area; a lower value indicates a bigger area after one overnight (decreased motility), while a bigger value indicates a lower area after one overnight (increased motility). The experiment is set up by running three technical replicates of the same scratch test for both control and treated samples.

2.11.14 Invasion assay

The cell invasiveness assay is used to assess, in vitro, the ability of cells to penetrate surrounding tissues in response to chemoattractant signal. This assay is based on the tendency of cells to migrate through a semipermeable membrane from a nutrient-poor environment (FBS-free medium) to a more nutrient-rich environment (complete medium).

The assay is performed in a 24-well multiwell, and an 8 µm transwell (Sarstedt, 83.3932.800) is placed in each well, in which a gelatinous matrix (BD Matrigel 354234) is allowed to solidify. C20 cells are suspended in a non-complete medium and treated with SEVs derived from inflamed and noninflamed microglia cells. SEVs were extracted as described in section 2.10.1. Approximately 30,000 cells are seeded in each transwell (100 µL of suspension) and treated by gentle dripping so as to obtain

a monolayer on matrigel. Treatments are made by diluting the suspension containing EVs to a concentration of 0.1 ng/L. Then, 5 μ L of the diluted suspension are administered to the cells. In the chamber below each transwell, 500 μ L of complete medium (containing 10% FBS) are added. This results in two chambers for each well separated by a semipermeable membrane consisting of the gelatinous layer. The plates are incubated at 37°C for 24h. At the end of the treatment period, the culture medium is aspirated from the two chambers and washed with PBS with ions. The cells are fixed with 4% PFA + 5 % Sucrose for about 20 min at room temperature; then, they are washed with PBS with ions and stained with crystal violet. Excess dye is removed by washes in PBS with ions; then, observation under a light microscope is performed. For each well, at least 5 images are acquired at an initial magnification of 20x of different areas of the membrane in contact with the nutrient-rich medium. This gives the average number of cells that have migrated through the membrane. The experiment is set up by performing two replicates of the same cell invasiveness test for both control and treated samples.

2.11.15 Statistical Analysis

The Graph-Pad Prism program (GraphPad Software Inc., San Diego, CA, United States) was used for data analysis and graphic presentation. All data are the mean $\pm$ SD of at least two independent experiments. Statistical analysis was performed by ordinary one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test or unpaired t-Test. $P \leq 0.05$ was considered statistically significant.

3 The Neurotrophin system as a possible molecular player of EV delivery and function

3.1 Neurotrophin receptors

Neurotrophins (NTs) are a family of growth factors that in mammals comprise nerve growth factor (NGF) and its orthologs: brain-derived growth factor (BDNF), NT-3, and NT-4, all of which are functionally active as homodimers. NTs are synthesized in the form of long precursors (pre-proNTs), containing a signal peptide necessary for the secretion of proteins (pre-peptide). Once translocated into the endoplasmic reticulum, the latter is cut by releasing protein precursors (proNT) of the size of ~30 kDa, which rapidly associate as a non-covalent homodimer. The proNT is cut at a highly conserved amino acid site at the level of the Golgi or in the extracellular space. This cut produces the mature homodimeric protein of about ~26 kDa, ~13 kDa for each monomer. NT are biologically active both in the proNT form and the mature one (Chao, 2003; Reichardt, 2006).

NTs have a variety of functions interacting with different tyrosine kinase receptors: TrkA, TrkB, TrkC, and their co-receptor p75NTR. These neurotrophic receptors belong to the trans-membrane proteins class; NTs binding activates a signal cascade responsible for the balance between survival and cell death in the nervous system (Pazyra-Murphy et al., 2009).

The Trks receptors display specific and selective high-affinity binding of mature neurotrophins: TrkA binds NGF, TrkB binds BDNF and NT-4, and TrkC binds NT-3, which can also be bound by TrkA and TrkB, although with lower affinity. p75^{NTR} instead displays high-affinity binding of all neurotrophins in either the pro-neurotrophin or the mature form (**Figure 3-1**).

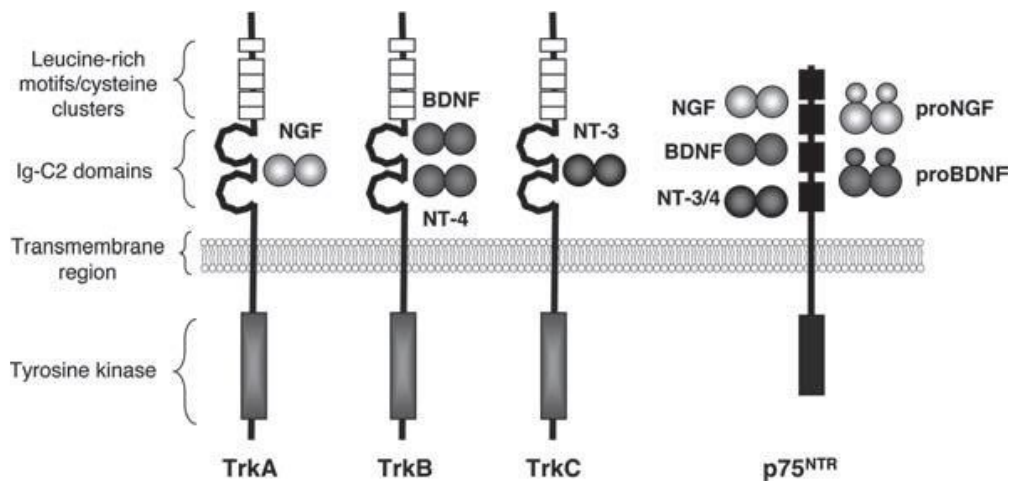


Figure 3-1 Neurotrophins and their receptors. Trks and p75^{NTR} receptors. p75^{NTR} binds with equal affinity all the neurotrophins' dimers, Trks bind preferentially to specific forms. p75^{NTR} can also bind all the pro-neurotrophins, while Trks receptors cannot (Arévalo & Wu, 2006).

The accepted model for activation of Trks and p75^{NTR} by neurotrophin ligands postulates that the different signaling outcome is opposite for the two receptors: whereas Trks generally promote neuronal survival, axonal elongation, and branching, in response to mature neurotrophins, p75^{NTR} promotes growth cone collapse, neuronal degeneration, and inhibition of axonal regeneration in response to pro-neurotrophins (Arévalo and Wu, 2006; Bothwell, 2016; Chowdary et al., 2012). However, in neuronal cells expressing Trks and p75^{NTR}, the latter acts as a co-receptor of the former to form the so-called high-affinity binding sites for neurotrophins and transduce survival signals (Hempstead et al., 1991).

3.2 Tropomyosin-related kinase a (TrkA)

TrkA (Tropomyosin-related Kinase A) receptor is the primary receptor responsible for NGF signaling and, together with TrkB and TrkC, constitutes the Trk subfamily of RTKs. These are type-1 single-pass transmembrane receptors: they pass the plasma membrane once, and orientation leaves the N-terminus outside the cell and the C-terminus on the inside; they are characterized by one intracellular domain containing tyrosine kinase activity and signaling and one extracellular domain, responsive for ligand interaction. The ligand binding domain consists of an immunoglobulin-like domain by which the receptors interact with neurotrophins. Ligand binding results in increased receptor homodimerization and activation, a process that involves ICD transphosphorylation and subsequent phosphorylation of cytoplasmic effectors. However, it is known that RTKs may already be present in the form of dimers, or clusters of dimers, even in the absence of ligands (Casaletto and McClatchey, 2012).

The three members of the Trk family selectively recognize different neurotrophins and, because of that, have different localization in the nervous system and slightly different effects:

- TrkA: has NGF as the higher-affinity ligand, can modulate growth process, differentiation and neuronal survival in the peripheral nervous system and in the central neurons of the basal ganglia (Chao, 2003; Reichardt, 2006).
- TrkB: activated mainly by interaction with BDNF, a crucial growth factor in the survival and function of central nervous system neurons, this receptor triggers signaling for regulation of neuronal development, plasticity, and apoptosis;

- TrkC: binds NT-3 neurotrophin is mainly expressed at the level of proprioceptor sensory neurons and is also involved in growth, metastasis, and survival processes at the level of human melanoma and colorectal cancer (Denkins et al., 2004; Kim et al., 2017);

3.2.1 Structure

The gene coding for TrkA protein is NTRK1, neurotrophic receptor tyrosine kinase 1, localized in Human Chromosome 1q23.1. The NTRK1 locus is roughly 66 kb long and can code for three different transcript variants exploiting alternative splicing. Neuronal tissues typically express the second variant: almost 21 kb on the locus with a cDNA of ~2.3 kb and a final translated protein of 796 aa (<https://www.ncbi.nlm.nih.gov/gene/4914>).

After protein synthesis, TrkA is inserted into the EnR (Endoplasmic Reticulum) membrane with the N-terminus facing the EnR lumen and the C-terminus the cytoplasm; the N-terminus exposes a sequence called signal-peptide which, after being cleaved in the EnR lumen, allows TrkA to be transported from the EnR to the Golgi complex and then to the cell surface. Along this journey, TrkA is heavily glycosylated at the N-terminus, converting a 110 kDa precursor, few glycosylated, into a 140 kDa mature form which is rapidly translocated and exposed on the cell surface (J et al., 2002; Marchetti et al., 2015).

The mature TrkA form on the plasma membrane can be structurally divided into three domains:

(Figure 3-2)

- the extracellular domain (ECD): is characterized by two Immunoglobulin-like domains (IgL-D), the second being responsible for ligand selectivity (Ultsch et al., 1999), and two cysteine-rich domains (CRD), separated by three N-terminal leucine-rich repeats (LRR);
- the transmembrane domain (TM): a single hydrophobic α -helix passing the phospholipid bilayer, which has a sequence involved in receptor recycling (Chen et al., 2005);
- the intracellular domain (ICD): responsible for signaling, presents a tyrosine-kinase domain (TKD). Interaction with ligand induces transphosphorylation of other tyrosine sites in ICD and subsequent interaction with cytoplasmatic effectors.

All the aminoacidic positions described hereafter refer to the human form of the neuronal TrkA isoform.

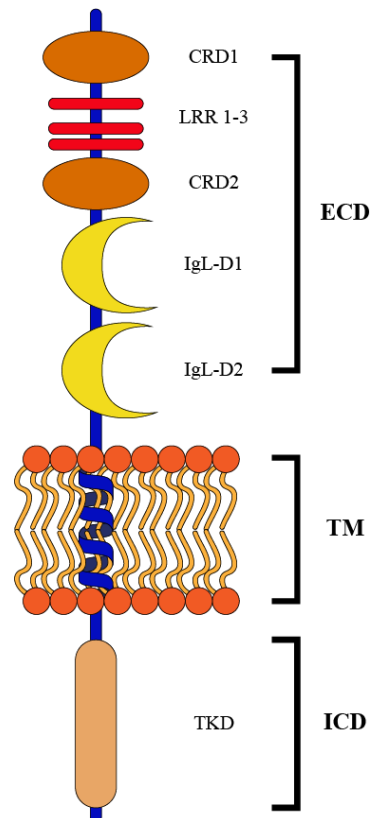


Figure 3-2 Schematic representation of the Trks receptors. In the Trks structure CRD is the cysteine-rich domain, LRR is the leucine-rich repeats, IgL is the immunoglobulin-C2 (Ig) domain and TKD is the tyrosine kinase domain.

3.2.2 Signaling pathways

The Trks-related signaling pathways impact many neuronal functions, including cell survival, differentiation, axonal guidance, dendritic growth,

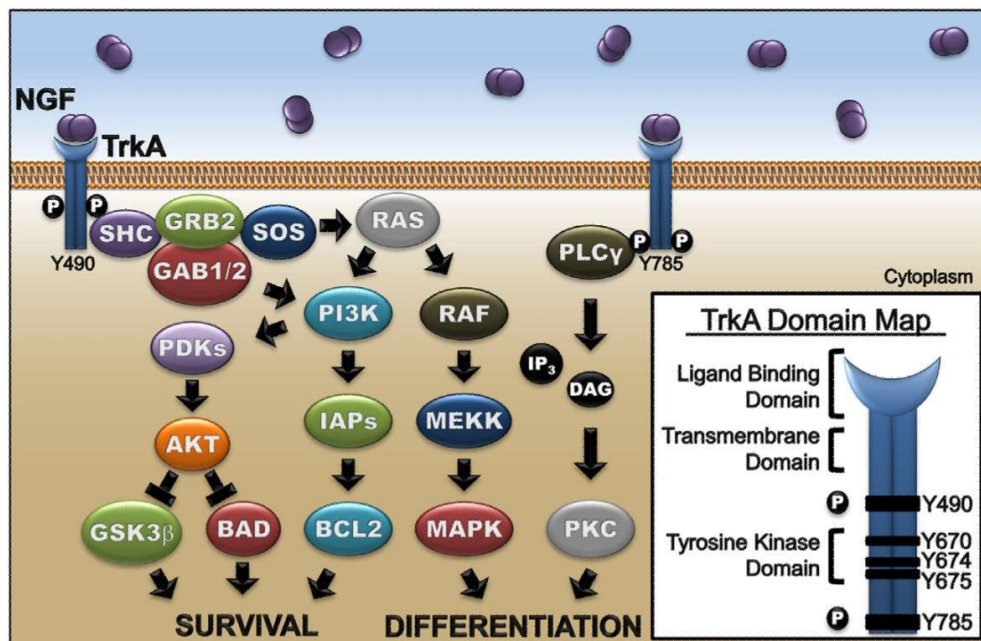


Figure 3-3 NGF/TrkA signal transduction pathways in survival and differentiation of neurons. TrkA signaling is best characterized by the activation of MAPK, PI3K, and PLC γ pathways mediated mainly by the phosphorylation of two tyrosine residues in the ICD, Y490, and Y785. NGF dimers bind to the ECD of two TrkA monomers promoting homodimerization, the tyrosine kinase domain activation, and the trans-phosphorylation of Y490 and Y785. Phosphorylation of these residues recruits intracellular adaptor and signaling molecules and activates the SHC-Ras-MAPK, PI3K-AKT, and PLC γ -PKC signaling pathways (Marlin & Li, 2015).

arborization, synapse formation, and plasticity. Such diversity is achieved via signaling pathways downstream of two different tyrosines: Y490 and Y785, which are phosphorylated in response to neurotrophins and act as significant docking sites for adaptor proteins and enzymes (Arévalo and Wu, 2006). Y490 and Y785 recruit Erk and PLC- γ , respectively (Rm et al., 1994). The trans-phosphorylated tyrosines at the kinase domain, Y670, Y674, and Y675, can

engage adaptor proteins, including SH2B, APS, and Grb2. The most studied and characterized pathways are reported in the following (*Figure 3-3*).

This pathway plays a role in the survival of several neuronal populations during development; it all starts with the phosphorylation of Y490, creating an Shc binding site; the recruited Shc associates with Grb2 and Gab1, leading to the activation of PI3K, phosphorylated inositol phospholipids change the local membrane composition allowing Akt to translocate to the plasma membrane and to become activated. Activated Akt plays a crucial role in enhancing axonal growth and branching in sensory neurons, interacting with mTOR and GSK-3 β and in the neuronal survival inhibiting FKHL, which regulates the expression of pro-apoptotic genes (Arévalo and Wu, 2006).

TrkA receptor stimulated by NGF engages Shc and Grb2 to activate the SOS, which then activates the small GTPase Ras (Rm et al., 1994). Activation of the small GTPase Ras in response to neurotrophins has been linked to signaling and transcriptional regulation implicated in neuronal survival and differentiation due to the fact that activated Ras leads to transient activation of the c-Raf/MEK/Erk cascade (Hefti and Walicke, 2007). Creating an Shc binding site at Y490 also initiates downstream Erk signaling. At last, Erk signaling may also lead to local axonal growth and the initiation of CREB-mediated transcriptional events.

PLC- γ signaling in response to NGF has been implicated in the chemoattraction of axonal growth cones, and prolonged activation in response to an NGF pulse induces the transcription of sodium channels. Phosphorylation of Y785 leads to the recruitment of PLC- γ and its activation, resulting in the generation of IP3 and DAG. Increased concentration of intracellular IP3 leads to the release of Ca²⁺ ions from intracellular stores and subsequent activation of Ca²⁺-dependent enzymes, e.g., CaM kinases; in addition, the release of Ca²⁺

together with DAG activates PKC and stimulates Erk signaling via Raf as well as TRPC, which contributes to the BDNF-induced rise of Ca^{2+} at growth cones and synapses (Arévalo and Wu, 2006; Hefti and Walicke, 2007).

3.2.3 Action mechanism: the signaling endosome hypothesis

Like all tyrosine-kinase receptors, the accepted action mechanism of the TrkA receptor includes the formation of the complex with NGF leading to dimerization and trans-phosphorylation of the intracellular domain, with subsequent phosphorylation of intracellular effectors and consequent activation of the respective signaling pathways. The activated receptor-neurotrophin complex undergoes internalization, diversified according to localization at the neuronal level: at the axon tip, membrane receptor-ligand complexes are internalized in “signal endosomes” and retrogradely transported to the soma, where they will regulate neuronal survival processes (**Figure 3-4**) (Dd and Ra, 2002); if the receptor is instead on the plasma membrane of the soma, activated complexes, once cellular signaling has been activated, will be involved in membrane recycling or conveyed to the lysosome for the degradation process. Another type of internalization was reported, which involves non-activated receptors on the plasma membrane of the soma: these receptors are endocytosed and anterogradely transported to the axon terminals by a transcytotic process (Ascaño et al., 2009). NGF applied to distal axons enhanced the transcytosis of TrkA receptors from cell bodies to distal axons, and this trafficking of Trk receptors was shown to be necessary for complete signaling and growth responses to target-derived neurotrophins (Ascano et al., 2012). The “signaling endosomes” contain activated TrkA and are associated

with adaptors and effector proteins that mediate downstream signaling cascades (Harrington et al., 2011).

Overall, TrkA trafficking in neurons can be subdivided into three main distinct events:

1. constitutive endocytosis, and subsequent transcytosis, are responsible for the

anterograde transport of TrkA to the axon in a positive-feedback loop initiated by NGF signaling (Ascaño et al., 2009; Vaegter et al., 2011);

2. internalization in distal axons after NGF binding forms the signaling endosome; TrkA signaling differs before and after internalization (Zhang et al., 2000), meaning that formation of the signaling endosome is crucial for adequate TrkA signaling: cell surface TrkA receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation (Zhang et al., 2000). It is not known if, after undergoing signaling transcytosis, the internalized TrkA receptor is still signaling;

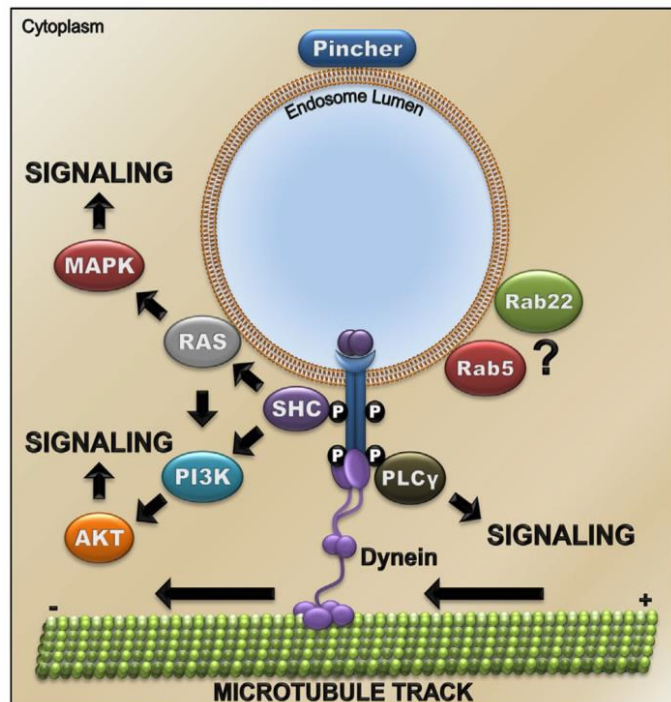


Figure 3-4 A model for NGF/TrkA signaling endosome. Protein dynein promotes retrograde transport on microtubules in the axon. NGF remains bound to TrkA in the signaling endosome and keeps TrkA activated for sustained signaling that facilitates trafficking and leads to neuronal survival and differentiation (Marlin & Li, 2015).

3. the signaling endosome can be exocytosed on the soma membrane and re-internalized (Suo et al., 2014). This third mechanism could be a potential mechanism for lysosomal avoidance and specification of signaling outputs.

3.3 p75 neurotrophin receptor (p75NTR)

p75NTR is the first discovered receptor from the TNFR family. It is also known in the literature as LNGFR (Low-affinity Nerve Growth Factor Receptor) since the first studies on this protein showed how it binds the NGF with lower affinity compared to TrkA, although in more recent times, it has become evident that both p75 NTR and TrkA show very high affinity for NGF (Hempstead et al., 1991; Kaplan et al., 1991; Rodríguez-Tébar et al., 1992). Afterward, it was established that p75NTR could bind all four mammalian neurotrophins, showing a higher affinity for their unprocessed precursor forms, pro-neurotrophins, when complexed with sortilin (Friedman, 2000; Meeker and Williams, 2015). p75NTR constitutes still nowadays a recognized elusive receptor, as it is involved in comprehensive, independent, and somehow contradicting pathways depending on the cellular

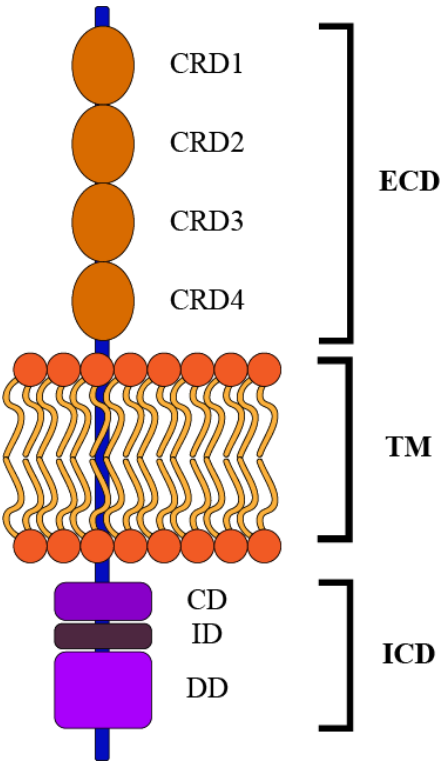


Figure 3-5: Schematic representation of p75 receptor. In the p75 structure are show the four cysteine-rich domain and the intracellular chopper and death domain respectively CD and DD.

context and on the presence of other receptors. For example, it is involved in processes such as axonal growth and degeneration, neuronal proliferation, differentiation, myelination, and synaptic plasticity; it can also assist NT

survival action, interacting with Trks, or induce apoptosis in the absence of Trks in neuronal cells (Meeker and Williams, 2015).

3.3.1 Structure

The human p75NTR gene (Tumor Necrosis Factor Receptor Superfamily Member 16 Precursor) contains six exons, for an approximate length of 23 kb mapping along chromosome 17, precisely in the Human Chromosome 17q21.33. A 3.8 kb long mRNA is transcribed from this gene, which is finally translated into a protein of 427 aa, the first 28 of which constitute the signal-peptide for membrane sorting, and the remaining 399 constitute the actual receptor. After synthesis, p75NTR undergoes processes of N-glycosylation, O-glycosylation, and phosphorylation; these other chains increase the protein molecular weight from about 45 kDa of the native form to 75 kDa of the mature and complete form, from which its name is derived (<https://www.ncbi.nlm.nih.gov/gene/4804>).

p75NTR is a type I single-pass transmembrane receptor, with its N-terminus localized extracellularly while the C-terminus protrudes into the cytoplasm (Hefti and Walicke, 2007; Reichardt, 2006; Roux and Barker, 2002).

Dividing p75NTR as already shown with TrkA, it is possible to analyze its main domains:

(Figure 3-5)

- the Extracellular domain (ECD): is composed of four cysteine-rich domains (CDR1, 2, 3, 4), a distinctive feature of the members of the TNFR family, maintained in tandem thanks to intra-chain disulfide bridges. While CRD1 does not seem to be involved in the link with the ligand, CRD2, 3, and 4 create an interface with the ligand exploiting the formation of several salt- and H-bonds;

- the Intracellular domain (ICD) can be further subdivided in:
 - o Death Domain (DD): thanks to certain interactors, this domain can start a pro-apoptotic pathway, as it will be described in the following section;
 - o Chopper Domain (CD): similarly to the DD, it manages to activate a pro-apoptotic pathway; however, this occurs for CD only after a Cysteine residue is palmitoylated;
 - o Interaction Domain (ID): presents a series of serine and threonine residues as phosphorylation sites.

3.3.2 Signaling pathways and action mechanism

The cellular context, the molecules it interacts with, and the levels of expression are all parts of a delicate balance that, if even slightly altered, can result in paradoxically-different outputs. It is evident that p75 is a highly complex receptor. Fundamental problems still surround how p75 interacts with various signaling pathways and how these affect how p75 functions. For instance, it is currently unknown how p75 activates JNK and caspases in various cell types and how this route counteracts the effects of elevated NF- κ B activity, which can subsequently trigger both pro-inflammatory and pro-survival responses (leading then to cell death). In this setting, activation with NTs or proNTs appears to be necessary, although how precisely the ligands activate or repress p75NTR is still unclear and the subject of ongoing research.

In addition to functioning independently of Trks in regulating neurite outgrowth and morphology, cell cycle withdrawal of growing neural progenitors (Zanin et al., 2016), and viral-related neuropathies, p75NTR binds a number of ligands unconnected to NTs (Langevin et al., 2002; Wang et al.,

2002; Yaar et al., 2002). It is evident that more research is necessary to comprehend the structural underpinnings of p75NTR signaling fully.

3.3.2.1 Regulated Intramembrane Proteolysis (RIP)

Similarly to the amyloid precursor protein (APP), p75NTR undergoes regulated intramembrane proteolysis (Skeldal et al., 2012). The extracellular region of p75NTR is first cleaved by the metalloproteinase TNF α -converting enzyme (TACE, also known as ADAM17), producing a 24 kDa C-terminus which remains bound to the plasma membrane: the p75NTR-CTF (C-terminus fragment). The p75NTR-CTF is then cleaved within its transmembrane region by the γ -secretase complex, releasing the 19 kDa intracellular domain of the receptor named p75NTR-ICD (Kenchappa et al., 2010, 2006; Weskamp et al., 2004).

One functional role of p75NTR cleavage is to facilitate signaling to the nucleus: the release of the p75NTR-ICD may facilitate nuclear translocation of associated factors such as NRIF, which was shown to be required for p75NTR-mediated apoptotic signaling (Franco et al., 2021; Linggi et al., 2005).

The mechanism of NRIF nuclear translocation also depends on TRAF6-mediated ubiquitination. TRAF6 was shown to ubiquitinate NRIF following ligand binding to p75NTR. The ubiquitination of NRIF required p75NTR cleavage, suggesting that receptor proteolysis facilitates interaction between NRIF and TRAF6, enabling ubiquitination of NRIF, which is needed for it to enter the nucleus, and oligomerization of TRAF6, which promotes the activation of JNK. In sympathetic neurons, JNK activation was required for ligand-induced proteolysis of the receptor by both TACE and γ -secretase. The activation of JNK facilitated the transcriptional upregulation of TACE and, through an unknown mechanism, stimulated both TACE and γ -secretase,

inducing p75NTR processing (Kenchappa et al., 2010, p. 201, 2006; Schachtrup et al., 2015).

3.3.2.2 Apoptosis

The apoptotic pathway is a scenario in which immature neurotrophic precursors play an essential role: together with sortilin, they are complexed with p75NTR and then follow two paths.

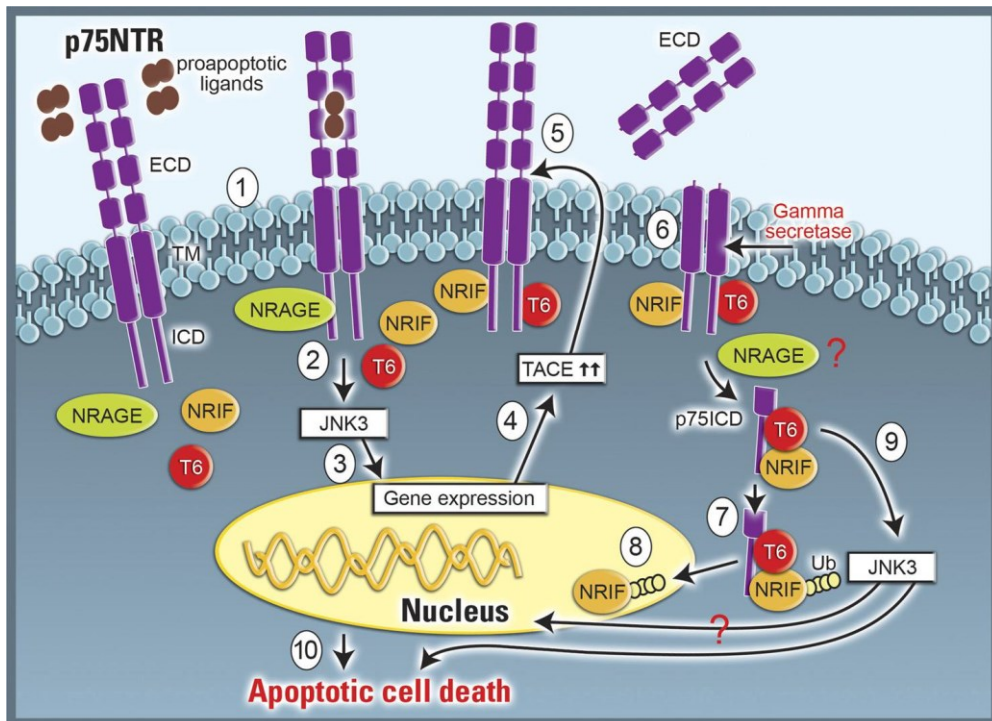


Figure 3-6: Schematic illustration of p75NTR-mediated apoptotic signaling. Binding of pro-BDNF and mature-BDNF to p75NTR recruits cytosolic interactors like NRAGE to the ICD, resulting in activation of JNK3 leading to up-regulation of TACE, which cleaves the extracellular domain of p75NTR and allows secretase mediated release of the p75NTR-ICD. The liberated p75NTR-ICD forms a ternary complex with NRIF and TRAF6 to promote TRAF6-dependent ubiquitination of NRIF and its nuclear translocation; in addition, the complex also activates JNK3, all of which lead to p75NTR-mediated apoptosis (Kenchappa et al., 2006).

The first involves lateral diffusion towards cholesterol-rich lipid rafts where p75NTR is cleaved by an α -secretase. The C-terminus remains in the plasma membrane after the cleavage and activates Rac protein through the Chopper Domain; activated Rac allows the activation of GIRK channels and caspases (Coulson et al., 2008; Underwood et al., 2008).

A second way (**Figure 3-6**) involves the association with intracellular interactors of the juxta membranal domain that activates JNK3, causing over-expression of the TACE enzyme. P75NTR, in this case, is cleaved by a γ -secretase, and its ICD associates with NRIF and TRAF6 in the DD, ubiquitinated NRIF can now move into the nucleus and start apoptosis. At the same time, TRAF6 stimulates JNK3 again to amplify the death signal (Kenchappa et al., 2010, 2006). Incorrect or missing expression of Trk receptors results in increased apoptosis suggesting that the apoptotic pathway is activated if there is an imbalance in the Trk/p75NTR ratio; in a similar way, an imbalance of proNT/NT ratio switches from survival (Trk-driven) to apoptosis (p75-driven) regardless of the Trk/p75NTR ratio (Peng et al., 2004).

Since the initial finding of RIP of p75NTR, it has been demonstrated that this proteolysis occurs through a ligand-dependent mechanism, and exciting experiments were conducted regarding the regulation of apoptosis in sympathetic neurons. Those neurons do not express TrkB, so the biological effect of BDNF can be entirely addressed to its interaction with p75NTR; it has been demonstrated that BDNF induces neuronal apoptosis when p75NTR is activated in the absence of TrkB (Bamji et al., 1998).

3.3.2.3 Cell Survival

This signaling pathway is mediated by a functional interaction of p75NTR with Trk receptors: Trks already have a high affinity for mature neurotrophic factors, but the direct involvement of p75NTR as co-receptor results in a 100-fold increased affinity for the ligand (Hempstead et al., 1991).

Recent studies indicate that this interaction carries on a two-step signaling: Trk phosphorylates the enzyme TACE activating it and leading to a p75NTR cleavage, and the ICD is released promoting and enhancing the Trk-mediated

survival pathway (e.g., Ras pathways, PI3K-Akt, and PLC- γ) (Barker et al., 2010; Light et al., 2011).

Apart from enhancing Trk signaling, there is evidence that p75NTR can activate Trks-independent survival signaling; selective activation of p75NTR prevented the death of specific neuroblastoma and breast cancer cells (Cortazzo et al., 1996; Verbeke et al., 2010).

The molecular mechanisms by which p75NTR promotes survival independent of the Trk receptors are known but not fully understood; however, one of the main targets of these pathways seems to be NF- κ B (Hamanoue et al., 1999), a protein complex that has the task of regulating the transcription of various genes, including those involved in cell survival (Foehr et al., 2000).

3.4 TrkA-NGF-P75 – The ternary complex

Functional interactions between p75NTR and TrkA can be broken into two main categories. The first is derived from studies showing that p75NTR enhances the response of Trk to neurotrophins. The p75NTR has long been regarded as a pan-NT coreceptor of Trk receptors, working with them to create NTs with high binding affinities (Hempstead et al., 1991).

Roux et al. work showed that antibodies directed against p75NTR reduced NGF-mediated TrkA phosphorylation in PC12 cells and primary neurons and demonstrated that co-expression of p75NTR with TrkA in heterologous expression systems enhanced NGF-induced TrkA phosphorylation (Roux and Barker, 2002). A second group of studies showed that p75NTR increases the specificity of the Trk receptors for particular ligands. Studies showed that TrkB is readily activated by BDNF, NT3, and NT4 in the absence of p75NTR; only BDNF can efficiently activate the receptor when p75NTR is co-expressed (Bibel et al., 1999). Other studies have shown that function-perturbing antibodies to p75NTR enhance the response of TrkA to NT3, consistent with the notion that p75NTR acts to suppress TrkA responses to this ligand (Clary and Reichardt, 1994). These and other related studies indicate that p75NTR enhances Trk responses to preferred ligands (e.g., NGF for TrkA, BDNF for TrkB) while attenuating responses to nonpreferred ligands (e.g., NT3 for TrkA).

There is ample evidence showing that these functional collaborations have physiological relevance. Primary dorsal root sensory neurons and sympathetic neurons derived from p75NTR null animals show a 3-fold decrease in survival responses to NGF. This deficit can have severe consequences for a neuron responding to the low quantities of neurotrophins available in target tissues. Indeed, this reduction in responsiveness likely accounts for the defects in

cutaneous sensory innervation originally described in p75NTR null mice (Lee et al., 1994); rich history of work examining the biochemical and kinetic features of neurotrophin binding layered on top of these functional interactions. Early studies indicated that NGF receptors on neurons have two kinetic forms, a high-affinity complex and a low-affinity receptor. The high-affinity binding is formed when both p75 and TrkA are expressed together in neurons and allows for a response to femtomolar concentrations of NGF, a process that is important during development when a limited amount of NGF is available for neurons (SCGs and sensory neurons) to respond (Hempstead et al., 1991). Thus, the notion that p75NTR and TrkA combine to form receptor complexes that give rise to high-affinity NGF binding sites has become well entrenched in the field.

Garcia and colleagues presented a three-dimensional structure of NGF bound to the p75NTR ECD (He and Garcia, 2004). These results showed that the interaction of a single p75NTR ECD with an NGF dimer resulted in conformational changes in NGF that prevented a second molecule of p75NTR from binding to the complex. The conformational change induced in NGF by binding p75NTR does not alter its TrkA binding surface, raising the possibility that NGF bound to p75NTR might simultaneously bind to a TrkA ectodomain. By combining structural solutions for the TrkA-NGF and p75NTR-NGF complexes, it was possible to model putative p75NTR-NGF-TrkA ternary complexes (Wehrman et al., 2007). However, the predicted ternary complex between NTs, Trk, and p75NTR has never been seen using structural biology methods, and the binary nerve growth factor (NGF)-TrkA, NT, and p75NTR complex crystal structures that are currently accessible have opposing orientations: this raises questions about the assembly of ternary complexes unless assuming that the two ECDs could be arranged to create an overlapping

NGF sandwich in which the receptors are arranged in opposite orientations (Barker, 2007; Leloup et al., 2018; Wehrman et al., 2007) (**Figure 3-7**).

A further complication comes from the observation that the NGF-p75NTR cocrystal has a 2:1 stoichiometry (He and Garcia, 2004, p. 75), whereas the NT3-p75NTR cocrystal has a 2:2 stoichiometry (Gong et al., 2008), with no

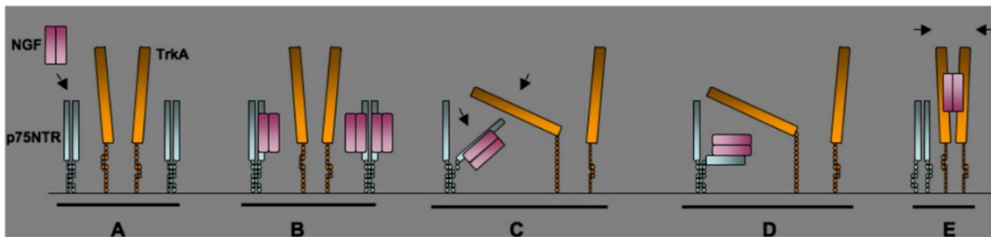


Figure 3-7 *A Ligand-Passing Model for p75NTR and TrkA. (A) In the absence of neurotrophin, p75NTR exists as preformed dimers, but TrkA chains do not form homo- or heterocomplexes. (B) NGF binds p75NTR with a fast association rate; both configurations shown are possible since NGF binding to the p75NTR does not disrupt the p75NTR dimer (Wehrman et al. 2007). Two p75NTR chains cannot simultaneously bind a single NGF dimer (He and Garcia, 2004). (C) NGF bound to p75NTR has a binding interface available for interaction with TrkA. p75NTR and TrkA bind NGF in different orientations, and therefore an ‘NGF sandwich’ that accommodates this requirement is indicated. (D) In this orientation, NGF is presented to TrkA in a conformation that favors rapid association with the receptor tyrosine kinase. This complex is likely to dissociate very rapidly when the ligand is NGF but more slowly when the ligand is NT3. (E) The NGF dimer binds a second chain of TrkA to allow the receptor kinase to form an active homodimer. Adapted from Barker, 2007.*

clear explanation for their difference. As a result, the available NT-p75NTR structures are in disagreement with one another.

The last chapter in this saga is the “inside-out” modulation model proposed by Conroy and Coulson (Conroy and Coulson, 2022). They suggested a model in which the p75ICD interacts with the Trk kinase domain to propagate an allosteric conformational change to the Trk ECD. This model prevents constitutive kinase activation by promoting the formation of inactive receptors that are ready for high-affinity binding instead. As a result, it is conceivable for the Trk receptor to exist in four different conformations: low-affinity

inactive, high-affinity primed inactive, low-affinity active, and high-affinity active.

3.5 The rationale of this work

Extracellular vesicles are becoming more widely recognized as intriguing drug delivery vehicles, but little is understood about the molecular processes that enable them to interact with and transport their payload to particular cell populations. The most widely recognized theory holds that the surface molecules on EVs, also known as the "Surfaceome," play a significant role in mediating their interaction with recipient cells. Therefore, to accomplish precise targeting and delivery, attention has been given to both the identification of surfaceome components and EV engineering (Chen et al., 2021; O'Brien et al., 2020). In this thesis, I am going to examine the potential of achieving a targeted interaction between engineered EVs and recipient cells by using two neurotrophic receptors, namely the TrkA and p75NTR receptors, A.

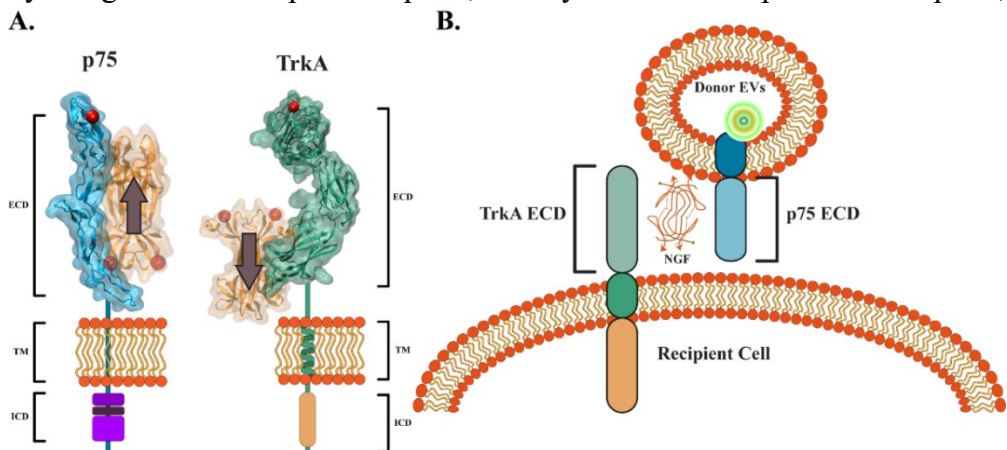


Figure 3-8 Panel A: schematics of the interaction of NGF with TrkA and p75. It is interesting to note that NGF binds to TrkA and p75NTR extracellular domains in an antiparallel fashion in co-crystal structures, indicating the potential of a ternary complex in trans formation Panel B: Schematics of the hypothesis of the trans ternary complex formation between recipient cells and donor EVs.

and their shared ligand, NGF. TrkA and p75NTR can both bind NGF separately, and their combined ability to translate the NGF signal indicates that a ternary complex may form (Covaceuszach et al., 2015). It is interesting to note that NGF binds to TrkA and p75NTR extracellular domains in an

antiparallel fashion in co-crystal structures, indicating the potential of a ternary complex in trans formation (Barker, 2007).

Our hypothesis is formulated thanks to preliminary data showing the presence of both p75 (Escudero et al., 2014) and Trk (Pinet et al., 2016) receptors in EVs. When EVs carrying the p75NTR come into contact with recipient cells that are expressing TrkA in the presence of NGF, we believe that a ternary complex of p75NTR, NGF, and TrkA may form. This interaction may be used to increase the delivery of EVs to target cells (*Figure 3-8*).

3.6 Investigating the role of TrkA-NGF-p75 ternary complex in SEVs uptake and targeted delivery

My journey in the neurotrophins world started during my master's thesis, where I was involved in a study to optimize a labeling technique for membrane receptors, particularly p75NTR, TrkA, and VEGFR2 (Amodeo et al., 2020). I was interested in the interplay between neurotrophin receptors and was determined to introduce it in my main line of research regarding EVs. I wanted to understand if I could exploit their interaction in the targeted delivery of EVs to specific districts.

The following section will describe my work for the investigation of whether the formation of the ternary complex could affect the interaction of SEVs on recipient cells and, consequently, their uptake rate and biological effect. We used two cell lines to conduct these experiments, one commercial (293T/17) and some genome-edited versions of PC12 cells: WT, KO for p75 (KOP), KO for TrkA (KOT) and double KO for p75 and TrkA (KOPT). PC12 cells were genome-edited with CRISPR/Cas9 technology by the group of M. Costa and A. Cattaneo in Pisa, as described in their recent publication (Testa et al., 2022), and were kindly gifted by them. The first step was to evaluate if our chosen model suited this line of experiments; therefore, we confirmed that our extraction protocol could isolate SEVs and LEVs from PC12 cells and, moreover, that each receptor was expressed in SEVs extracted from cells expressing the receptor. To improve the detection of SEVs in recipient cells, we also employed 293T cells transfected with p75-eGFP as donor cells and 293T cells transfected with TrkA as recipients.

All the described experiments were done by the candidate at the Department of Pharmacy, Pisa.

3.6.1 PC12 Extracellular Vesicles

The first step in this line of experiments was to confirm that our protocols, optimized for microglia cells, could isolate pure EVs even from PC12 cells. EVs were extracted from all PC12 genotypes following protocol G described in section 2.11. We performed Western blot analyses on all EVs pellets to identify negative and positive EV markers (*Panel A, Figure 3-9*).

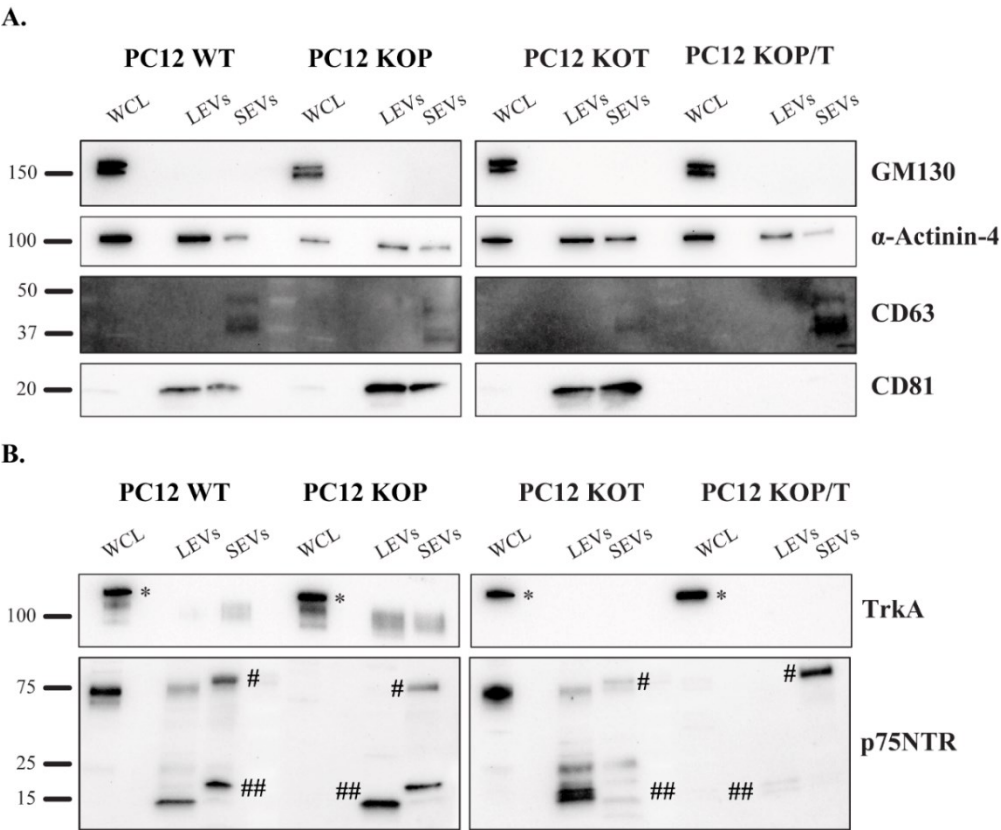


Figure 3-9: Panel A: Expression of EVs markers in PC12-derived EVs. EVs gathered from one 100 mm Petri dish were loaded in each lane, together with 1 μ g (α -Actinin-4 blot) or 30 μ g (for all other blots) of the whole-cell lysate (WCL) of the corresponding cell line. Panel B: TrkA and p75 expression in PC12 cells and PC12-derived EVs. EVs were gathered from each genotype of PC12, lysed, and the proteins separated by SDS-page. EVs gathered from one 100 mm Petri dish were loaded in each lane, together with 20 μ g of whole-cell lysate (WCL) of the corresponding cell line.

GM130 was positive only in the WCL, meaning we had no contamination of intracellular EVs; one ubiquitous marker, α -Actinin-4, was detected in all our samples. This may suggest that Actinin-4 is less able to discriminate LEVs

from SEVs in PC12 cells when compared to microglia cells, as already described in section 2.9.2.

We detected CD63 and CD81 in most of the analyzed EVs samples, with CD63 detected only in SEVs. Interestingly, the presence of CD81 was abolished entirely in EVs extracted from PC12 KOP/T, even though all other positive markers were present, confirming the actual presence of extracted EVs. There is a possible explanation for this which involves how these cellular models were generated. The expression of CD81 could be stopped or significantly impaired by the disruption of the regulation of its gene or protein expression caused by an off-target of the CRISPR/Cas9 system. Indeed, PC12 KOPT were subjected to two rounds of CRISPR/Cas9-mediated knock-down, increasing the possibility for this off-targeting to happen (Zhang et al., 2015).

This marker panel confirmed that our EV extractions were satisfactory to go further with the experiments.

Since we intended to investigate how the presence of TrkA and p75 on the surface of cells and EVs could affect EV uptake and biological effect, we also decided to confirm the presence of these two receptors on the cells and on the cognate extracted EVs (**Panel B, Figure 3-9**). We detected p75 in WT and KOT cells, in cells, LEVs, and SEVs. On the contrary, we saw TrkA in WT, KOP cells, LEVs, and SEVs.

TrkA was correctly detected in cells expressing it (WT and KOP) as a double band running at ~110 and 140 kDa, corresponding to the two more abundant glycosylated forms of the receptor. We also detected one non-specific band at a slightly higher molecular weight. It is easily identifiable as non-specific as it is present in every PC12 genotype, even in the KO for TrkA (*). Interestingly, the LEVs populations seem to display less TrkA in the WT than in the KOP genotype. Whether this is due to some batch-to-batch reproducibility in the EV

content or to the absence of p75 in the latter case remains a matter of future investigation.

For p75, the scenario is more complicated. There is an evident band in all SEVs samples, which is likely non-specific, being present also in KOP and KOPT EVs (less evident for KOT EVs) (#). This band slightly hampers the visualization of the full-length p75 band, which can be definitely appreciated in LEVs from PC12 WT and KOT but not from KOP and KOPT. Moreover, there are bands at a lower molecular weight which are particularly evident in LEVs and SEVs from PC12 WT and KOP, less in KOT, and almost absent in KOPT (##). As for the TrkA case, these bands are also possibly non-specific, as their presence is also in PC12 KO for p75. As for PC12 KOT EVs, they seem to display not only the full-length form of p75 but also high levels of ICD (19 kDa) and CTF (24 kDa) and to sort them preferentially in LEVs. This may suggest higher levels of RIP for p75 in this PC12 genotype, possibly due to the absence of TrkA. Indeed, TrkA was recently shown to regulate endocytosis and further processing of the CTF in basal forebrain neurons (Franco et al., 2021).- It is known that PC12 can produce and secrete low levels of proNGF (Soligo et al., 2019). Our pattern of expression of p75 and TrkA may suggest that these basal levels of proNGF are enough to stabilize p75 on the membrane in the presence of TrkA; however, in the absence of the latter, p75 is extensively internalized and cleaved.

Overall, these results describe peculiar patterns of expression of p75 and TrkA in our chosen model and in the cognate EVs. Since the presence of both receptors in the prepared EVs could be detected, we decided to go on with the experiments.

3.6.2 Internalization of PC12-derived SEVs on the same PC12 cells

Based on the rationale provided above, we hypothesized that the formation of a *trans* ternary p75-NGF-TrkA complex could drive the interaction between EVs and cells and, therefore, affect the efficiency of the uptake of SEVs. We tried to demonstrate this hypothesis by measuring the fluorescence of internalized SEVs, previously treated with a fluorescent lipophilic dye, in recipient cells.

We extracted SEVs from all PC12 genotypes, labeled them with Vybrant-DiD (Carter et al., 2022), and we administered them to each PC12 genotype in the presence of a saturating concentration of NGF (150 ng/mL). We wanted to investigate if the presence of the ternary complex could help or affect the delivery of SEVs, so we used a matrix-like organization of treatments to deliver each type of SEVs to each type of PC12. The results are shown in ***Figure 3-16***.

PC12 WT and KOT did not show any significant differences in the uptake of any SEVs sample; conversely, PC12 KOP and KOP/T did. KOP cells did internalize significantly more SEVs derived from KOP/T cells than any other type. PC12 KOP/T internalized fewer SEVs derived by either KOT cells or KOP. This data may suggest that the internalization of SEVs, in the absence of p75 on recipient cells, could be favored if SEVs did not bear any of the two receptors.

However, as shown by two representative experiments reported in *Panel A*, *Figure 3-10*, the staining of SEVs was not reproducible in our conditions. We

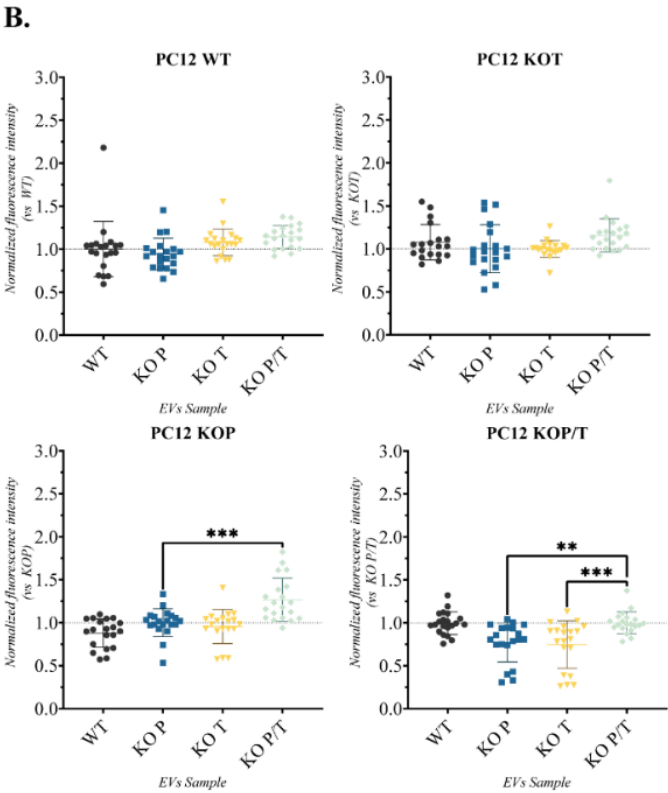
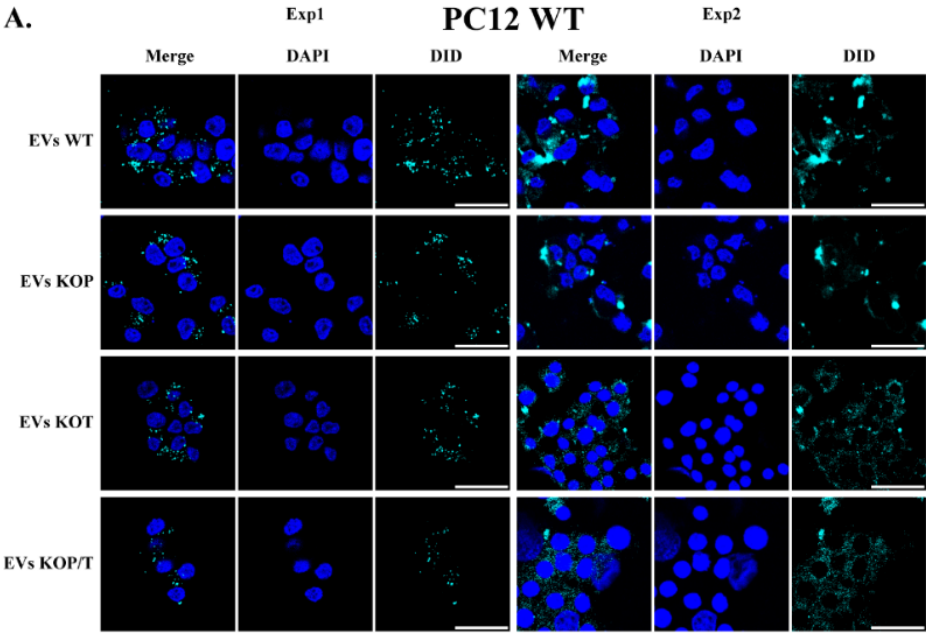


Figure 3-10: Internalization of PC12-derived SEVs in PC12 recipient cells. Panel A: representative images of two labelling experiments. SEVs were gathered from one 100 mm Petri dish of each PC12 genotype, labeled with Vybrant DiD, preincubated with NGF for 15', and administrated to recipient cells for 4h. Then recipient cells were washed, fixed, and stained with DAPI. Panel B: Internalization was quantified measuring intensity of fluorescence in recipient cells from images acquired with a confocal microscope. Data are expressed as mean $\pm$ SD of the pool of at least 20 field from three independent experiments. The intensity of fluorescence was normalized to the value measured in recipient cells treated with SEVs from the same genotype. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: $**P \leq 0.01$, $***P \leq 0.001$ vs control. Scale bars are set to 20 μ m.

performed various experiments with the same protocol of labeling and obtained very different results: sometimes, the signal in recipient cells consists of very bright cytoplasmatic spots, and sometimes in a low and diffused cytoplasmatic signal. We believe this could be a significant cause for not obtaining great differences in most of the analyzed samples. Also, the significance of the differences for the experiment in which KOPT were recipient cells may be due to the fact that we pooled together experiments bearing very different average values, so we cannot be sure, at this step, if they are reliable.

3.6.3 Change in the cell model to better evaluate the uptake rate

The previous experiments suggested that our way of labeling EVs was not reliable enough to robustly evaluate the differences in the uptake of EVs by recipient cells (**Figure 3-10**). We decided, then, to optimize a different labeling technique exploiting a fluorescent construct of the p75 receptor fused with eGFP.

We tried as much as possible to replicate our combination of PC12 by transfecting 293T cells with p75 or TrkA (**Figure 3-11**). Since our reporter was on p75, we transfected parental cells with p75-eGFP, allowing us to use eGFP to mark SEVs. Recipient cells were co-transfected with TrkA and an RFP-laminin nuclear marker to have positive control of transfection or mock transfected.

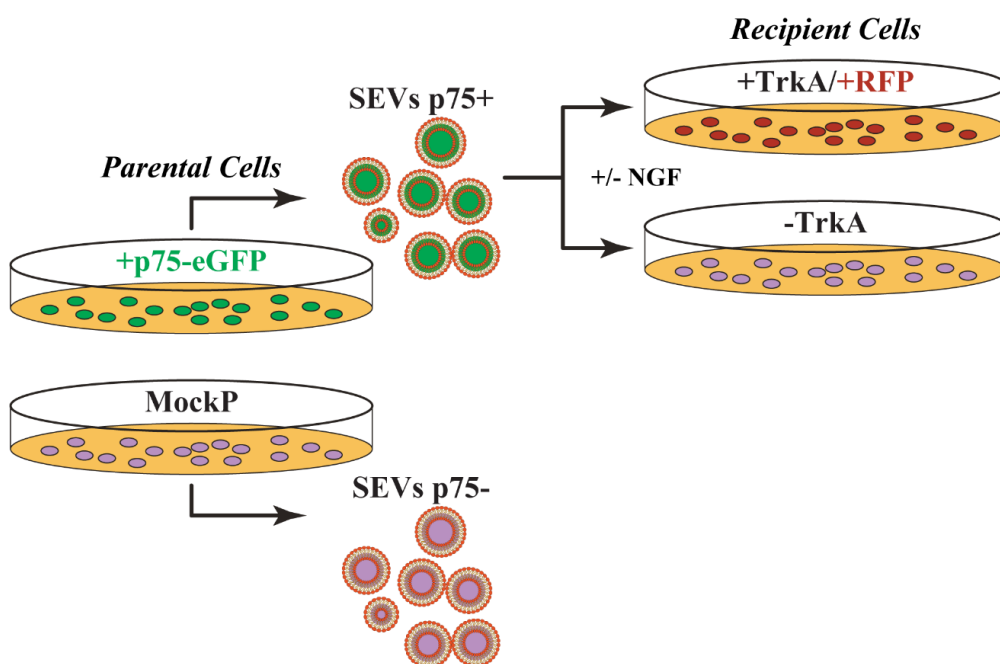


Figure 3-11: Schematics of experiment conducted on 293T cells. SEVs were extracted from parental 293T transfected with p75-eGFP, then administrated to recipient 293T cells transfected with TrkA+RFP or mock transfected, both in presence or absence of 150 ng/mL of NGF.

Since our quantification of uptake was possible only in samples derived from p75+ cells (they express eGFP), we had to compromise on some combinations.

As a first step, we confirmed the presence of the transfected construct in the cells and in EVs produced by transfected cells (**Figure 3-12**). Similar to previous observations (Marchetti et al., 2019b), the p75-egfp construct displayed different bands: the highest one corresponds to the full-length

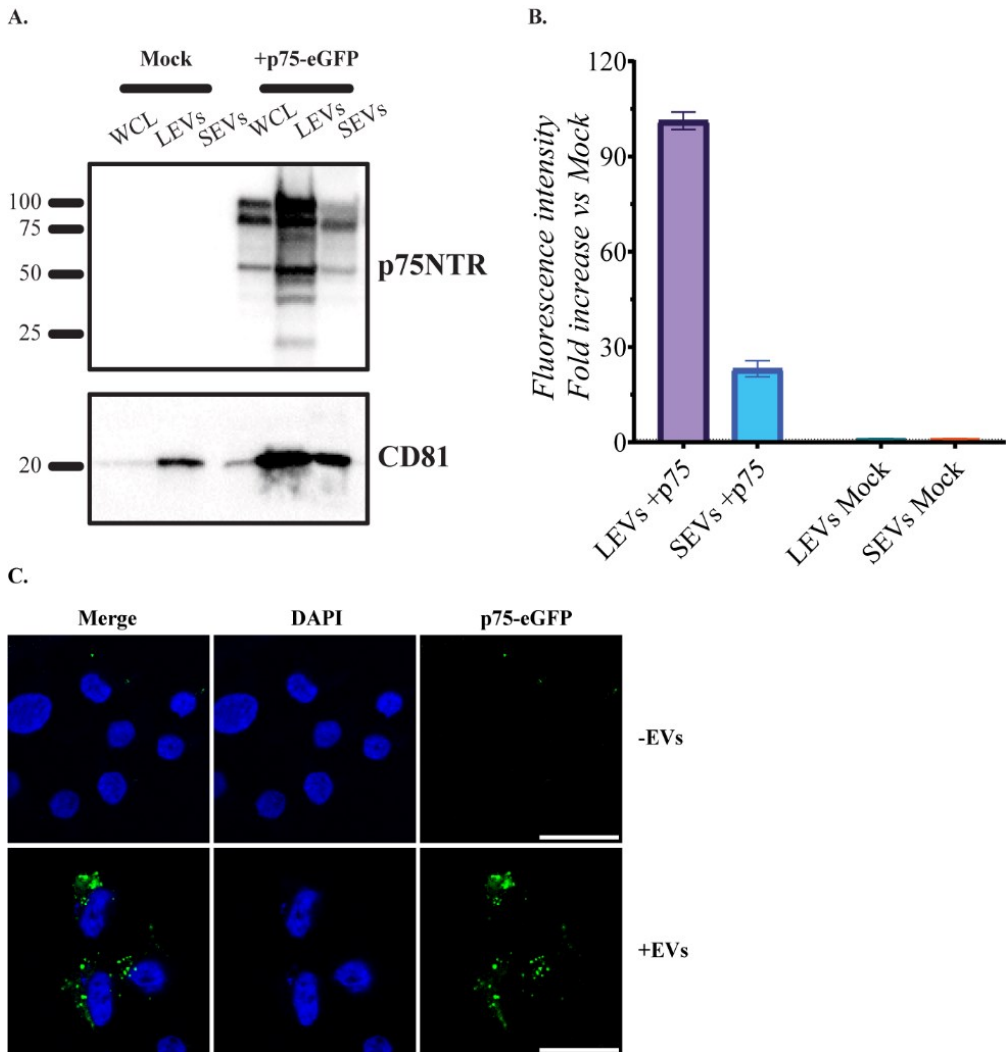


Figure 3-12: Panel A: Expression of transfected p75-eGFP and EVs marker CD81 in 293T-derived EVs. EVs were gathered, lysed, and the proteins separated by SDS-page. EVs gathered from one 100 mm Petri dish were loaded in each lane, together with 30 ng of the whole-cell lysate (WCL) of the corresponding parental cell. Panel B: EVs gathered from 293T cells were analysed with Enight to measure the intensity of fluorescence relative to eGFP. Panel C: representative images of recipient 293T treated with SEVs p75+ or just PBS. SEVs were gathered from one 100 mm Petri dish of 293T transfected with p75-eGFP, preincubated with NGF for 15', and administrated to recipient 293T cells for 4h. Then recipient cells were washed, fixed, and stained with DAPI. Images acquired with a confocal microscope. Scale bars are set to 20 μ m.

construct, and the other ones are likely intermediate glycosylated products. CD81 positive marker confirmed the presence of our construct both in LEVs and SEVs. Moreover, we measured the fluorescence of our extracted samples to confirm the effective fluoro-labeling of the produced EV samples. We detected a fluorescence increase of 101-fold in LEVs and of 23-fold in SEVs for EVs produced from p75-eGFP transfected cells when compared to EVs from mock cells, confirming not only the presence of p75 but also that eGFP was detectable and could be used for our subsequent experiments (**Panel B, Figure 3-12**). Finally, we detected fluorescent signals in cells treated with SEVs extracted from 293T transfected with p75-eGFP (**Panel C, Figure 3-12**).

We administered SEVs from 293T cells p75+ or Mock (p75-) to 293T TrkA+ or TrkA-in presence or absence of saturating concentrations of NGF. After 4h of incubation, cells were washed and fixed for confocal microscopy analysis. As expected (*Panel B left, Figure 3-13*), we did not detect an increase in fluorescence in any sample lacking p75-eGFP; on the contrary, there was a ~ 1.5-fold increase in the signal in all samples treated with +p75 SEVs (*Panel B right, Figure 3-13*).

Interestingly, we detected a significant increase of fluorescence in 293T TrkA+/+NGF, compared to all conditions administered to 293T TrkA-. This may suggest that the presence of TrkA on recipient cells could have a positive effect on the internalization SEVs carrying p75. The possible effect prompted by the addition of NGF, and therefore by the possibility of in trans ternary complex formation, remains so far elusive. Indeed, there were no significant differences between samples treated with a combination of SEVs and NGF and the SEVs alone. It must be pointed out, however, that the increase of fluorescence in recipient cells obtained with this experimental set-up may not be so high to allow the evaluation of nuanced differences. This model could be improved by increasing the quantity of p75 in SEVs, the amount of administered EVs, as well as the rate of transfection of TrkA in 293T cells. It is possible that a low yield of transfection of TrkA contributes to cutting down on any minor differences. It will It is also possible that heterologous expression

of TrkA in 293T could induce the production of NGF, reducing, even more, the differences between the two conditions +/- NGF.

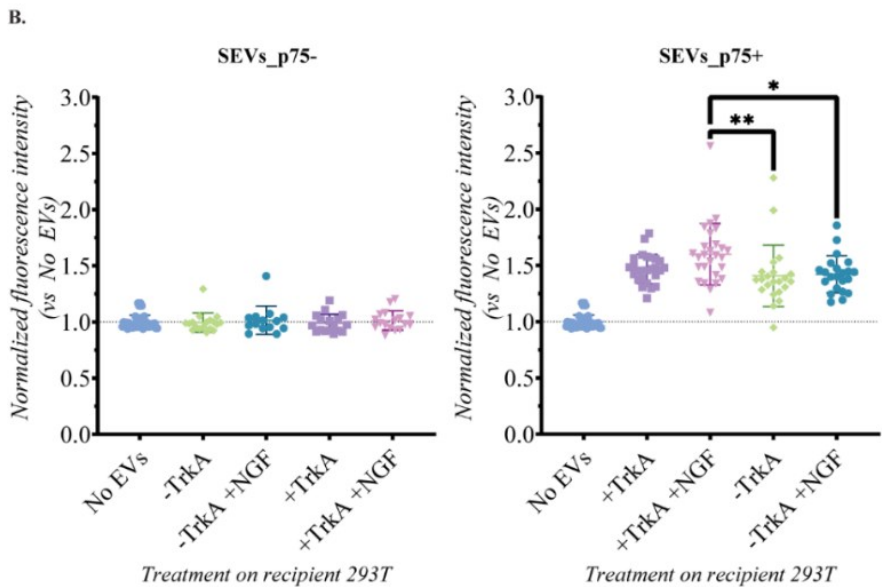
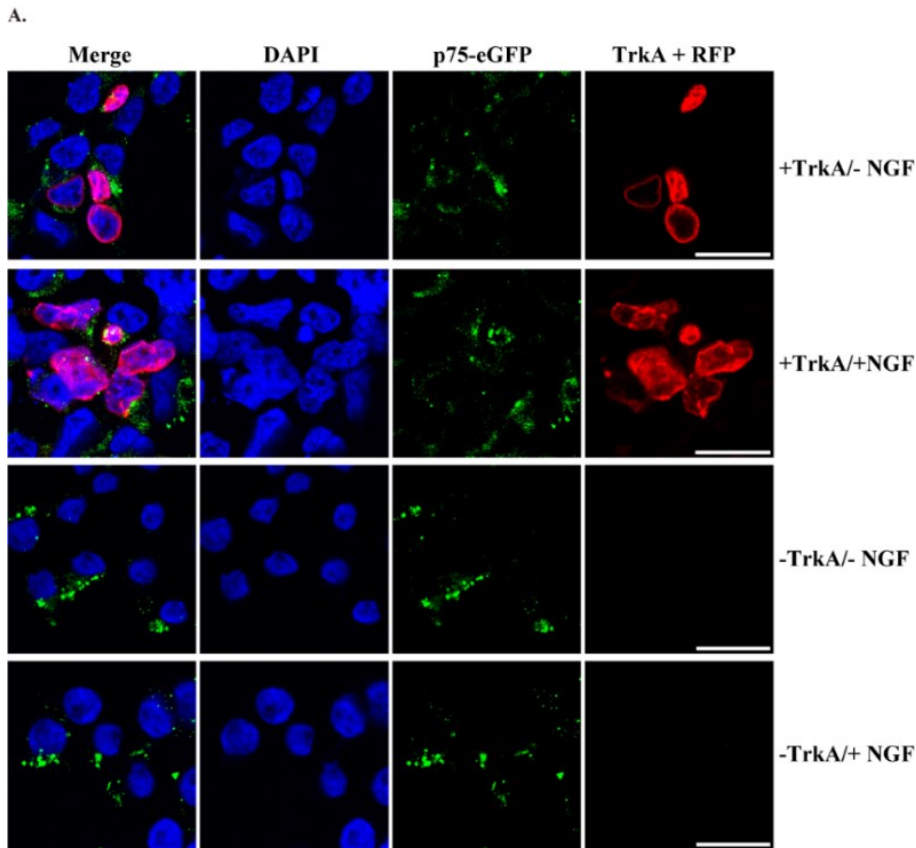


Figure 3-13: *Panel A: Representative images of the internalization experiment of 293T p75+ -derived SEVs on 293T expressing or not TrkA + RFP-laminin. SEVs were gathered from one 100 mm Petri dish of 293T parental cells transfected with p75-eGFP, preincubated with NGF for 15', and administrated to recipient 293T cells transfected with TrkA+RFP or mock transfected for 4h. Then recipient cells were washed, fixed, and stained with DAPI. Panel B: Internalization was quantified by measuring intensity of fluorescence in recipient cells from images acquired with a confocal microscope. Data are expressed as mean $\pm$ SD of the pool of at least three independent experiments (SEVs_p75-: at least 15 field; SEVs_p75+: at least 20 field). The intensity of fluorescence was normalized to the value measured in recipient 293T cells mock transfected and treated with no EVs (just PBS). The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, ** $P \leq 0.01$, vs control with no EVs. Scale bars are set to 20 μ m.*

We could not compare the influence of just p75 on the uptake because our labeling system depended on the presence of the construct p75-eGFP. We tried with the same lipophilic dye used for PC12 cells, but with this cell model, similar to what was shown previously for PC12 cells, the labeling was very inconsistent and unreliable (**Panel A, Figure 3-10**), as it was only partially co-localized with eGFP.

3.6.4 Can SEVs affect the NGF signaling mediated by neurotrophic receptors?

Regardless of the possible formation of the ternary complex and of its influence on the uptake rate of EVs in the short run, we decided to investigate on a longer time scale if the different administered EVs can modulate NGF

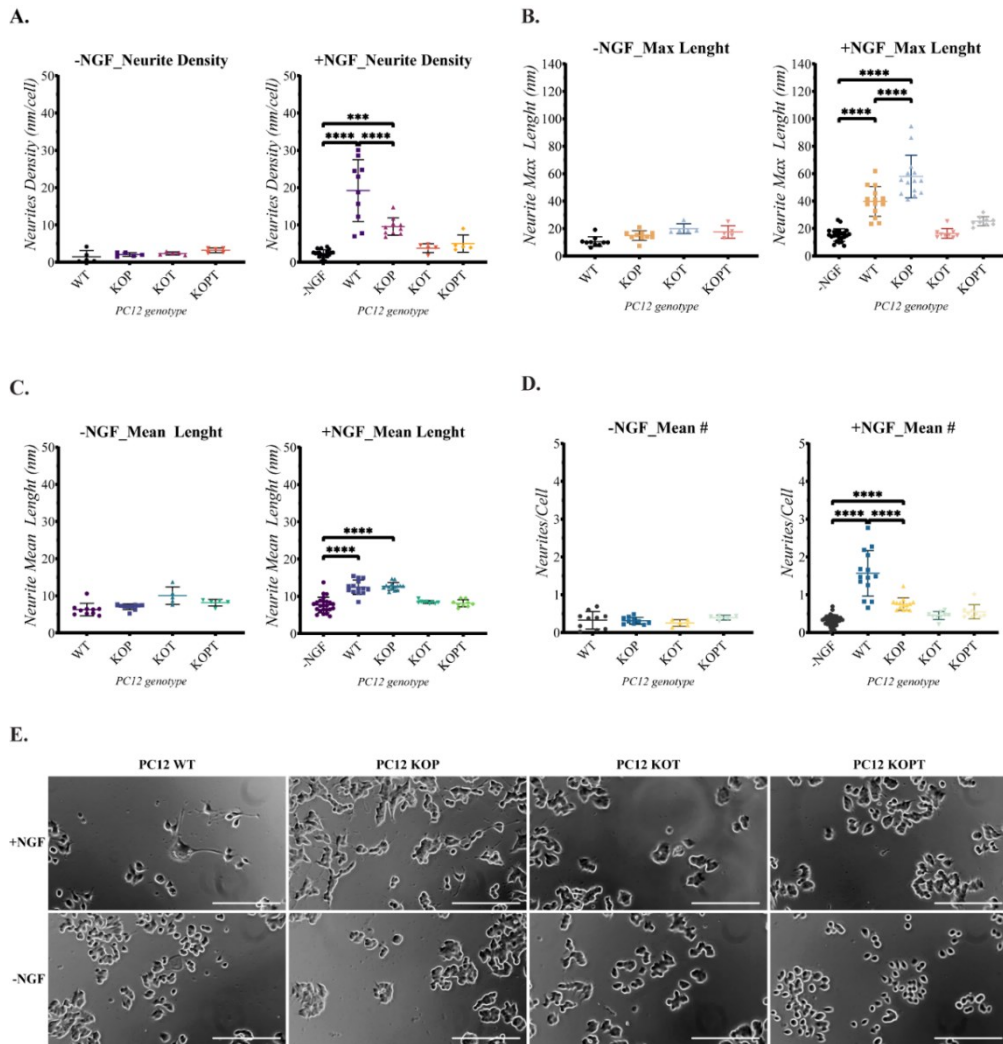


Figure 3-14: Differentiation of each PC12 phenotype without EVs administration. Each cell type was administered with 150 ng/mL of NGF for 48h. Images were acquired with an optical microscope (20x magnification). Neurites were measured with ImageJ software. Data are expressed as mean $\pm$ SD of the pool of at least two independent experiments, at least 5 field for each genotype. 4 parameters of outgrowth were quantified as reported in the Methods (A-D). The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: *** $P \leq 0.001$, **** $P \leq 0.0001$. Panel E: representative images of PC12 after 48h of treatment. Scale bars are set to 100 μ m.

signaling in terms of neurite outgrowth stimulation induced in the recipient cells. We, therefore, prolonged to 48 hours the timeframe of analysis and evaluated if the formation of NGF/EV complexes could potentiate the NGF-mediated signaling in cells expressing NT receptors. The possibility to have both EVs and recipient cells differentially expressing none, one or two of the NT receptors endowed us, again, with the possibility to perform a combinatorial experiment to dissect the EV, NGF, and receptor roles finely.

Our first experiment was to evaluate the differentiating capability of all our PC12 phenotypes (**Figure 3-14**) in response to NGF administration. We measured the neurite density (**Panel A**), the max length (**Panel B**) of formed neurites, the mean length (**Panel C**), and the number of neurites (**Panel D**) per cell. These parameters allowed us to investigate the effect on different processes of neuritogenesis: initiation (neurite density and mean neurite per cell) and elongation (neurite density, max, and mean neurite length). We confirmed that only the cells bearing TrkA could respond to NGF; in particular, we were happy to confirm the results of Testa et al. (Testa et al., 2022), in which these genome-edited cell lines were first described. Our collaborators demonstrated that PC12 KOP showed an increase in the length of the neurites compared to WT cells; our neurite max length quantification confirmed this observation. The mean length also showed a boost for the KOP cells compared to the WT, even though not a significant one.

Moreover, we confirmed that KOT and KOP/T did not show any significant signs of differentiation. We next administered the saturating concentration of NGF to all four PC12 phenotypes in a combination of all SEVs types and measured the differentiation mediated by NGF in the different cases (**Figure 3-15**).

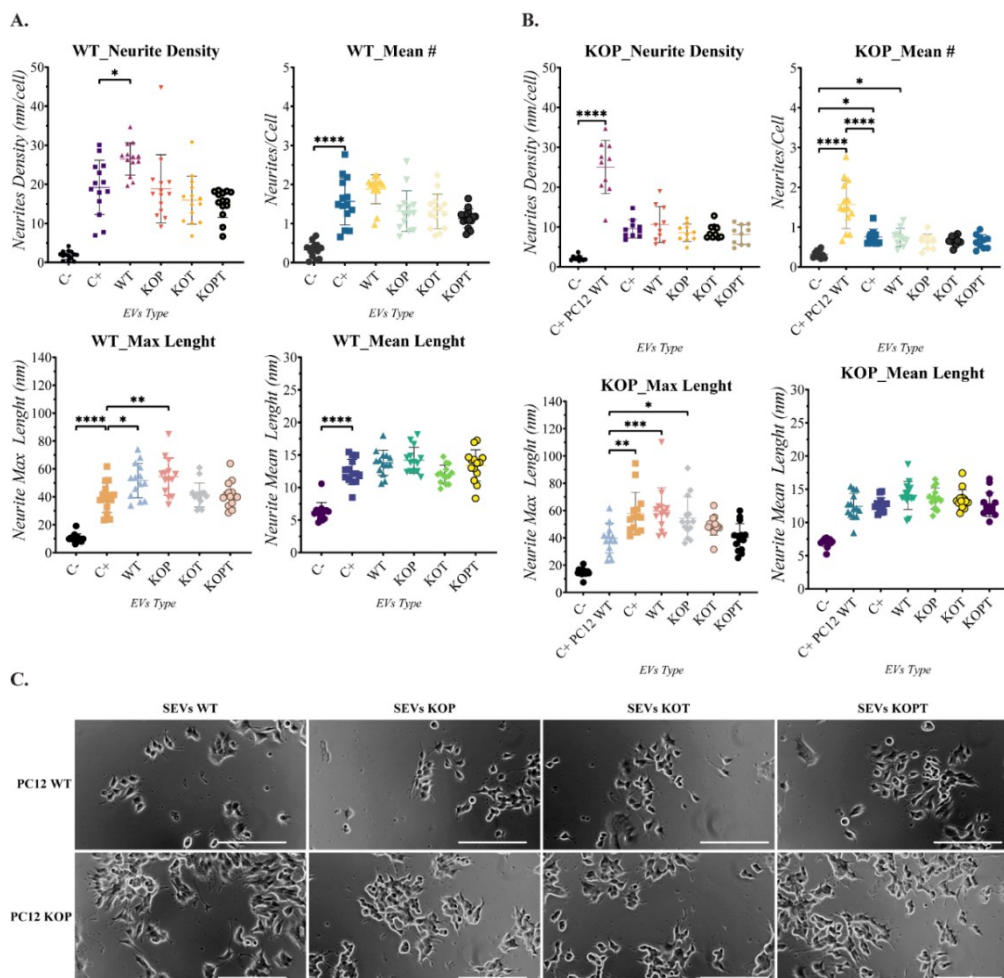


Figure 3-15: Panel A, B: PC12 WT and KOP differentiation upon SEVs treatment. Each cell type was administered with 150 ng/mL of NGF for 48h in combination with SEVs derived from each PC12 phenotype. Images were acquired with an optical microscope (20x magnification). Neurites were measured with ImageJ software. Data are expressed as mean $\pm$ SD of the pool of at least two independent experiments, at least 14 field for each genotype. 4 parameters of outgrowth were quantified as reported in the Methods. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, vs each C+ or vs C+ of PC12 WT. Panel C: representative images of PC12 after 48h of treatment. Scale bars are set to 100 μ m.

WT PC12 showed a significant increase in the neurite density after treatment with NGF + SEVs derived from other WT PC12 compared to NGF alone. This treatment also caused an increase in the max neurite length; this effect was also observed after the administration of KOP SEVs. No significant differences were detected in the mean neurite length nor the number of neurites generated by cells, suggesting that the administration of TrkA (but not necessarily of p75NTR) via SEVs could potentiate more the neurite elongation than the initiation.

The effect on KOP cells of our treatments was compared not only to their positive control (NGF) but also to the positive control on WT cells; this was done to compare the effect of administering receptors via SEVs with the optimal NGF response obtained when both receptors are present in the recipient cells.

In KOP cells, we detected no significant differences in the neurite density in any treatment compared to NGF alone. We saw a substantial increase in the max length of neurites compared to the C+ of PC12 WT after administration of both WT and KOP-derived SEVs; this effect correlates with the longer neurites also found after stimulation with NGF alone, previously described in **Figure 3-14**; and it could be explained just by the presence of NGF in these samples, the role of SEVs may be negligible. No significant differences were detected in the mean neurite length. The differences in neurites per cell reflect what was already described in **Figure 3-14** for NGF alone, with no significant differences compared to the NGF control.

The data shown in **Figure 3-16** describe the effect of our SEVs treatments on PC12 Cells KOT and KOP/T, meaning neither has TrkA expressed. As expected, we detected almost no statistically significant differences between the various treatments, and when we did (PC12 KOT, neurite mean length), the increase in outgrowth was so slight that we decided to consider them just background variation in cells not able to respond to NGF. In combination with

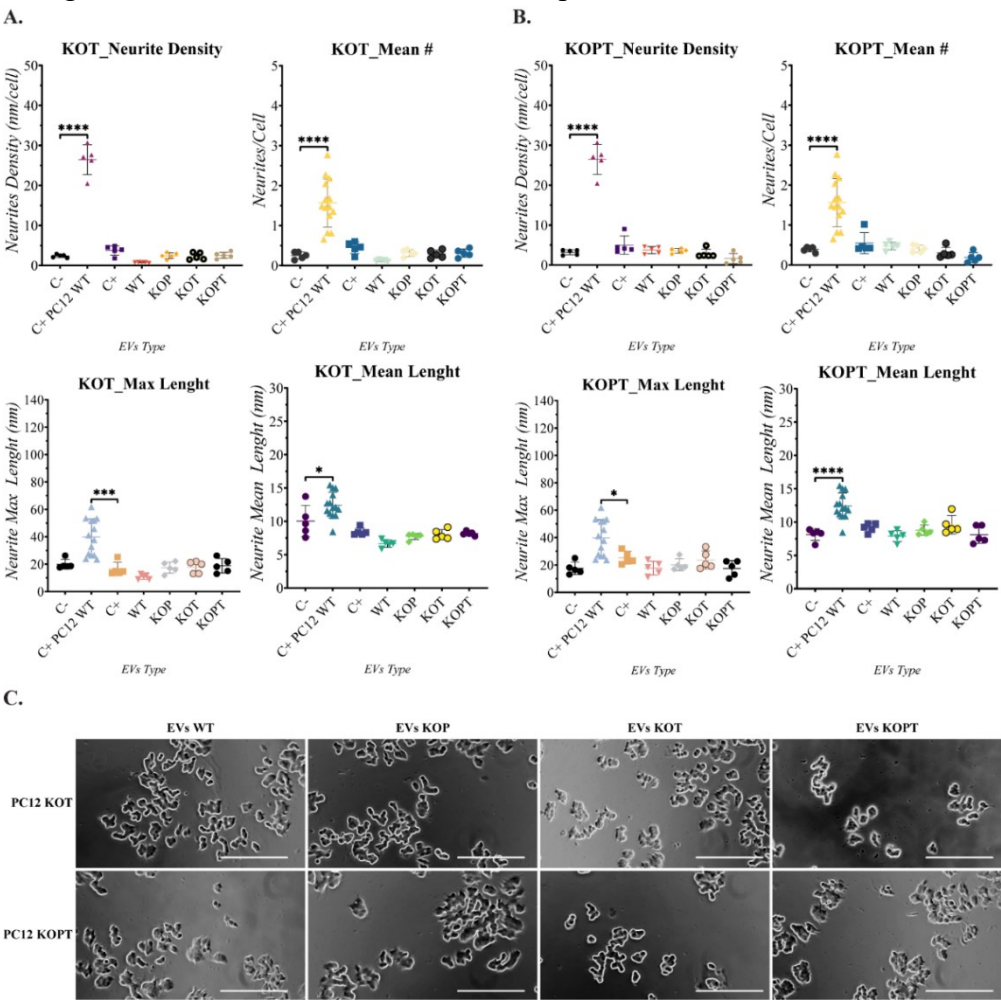


Figure 3-16: Differentiation of PC12 KOT and KOPT upon SEVs treatment. Each cell type was administered with 150 ng/mL of NGF for 48h in combination with SEVs derived from each PC12 phenotype. Images were acquired with an optical microscope (20x magnification). Neurites were measured with ImageJ software. Data are expressed as mean $\pm$ SD of the pool of at least two independent experiments, at least 5 field for each genotype. 4 parameters of outgrowth were quantified as reported in the Methods. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test: * $P \leq 0.05$, *** $P \leq 0.0001$, **** $P \leq 0.0001$, vs each C+ or vs C+ of PC12 WT. Panel E: representative photos of PC12 after 48h of treatment. Scale bars are set to 100 μ m.

EVs, NGF could not affect the differentiation of PC12, lacking expression of the central receptor that guides this process.

3.1 Materials and Methods

3.1.1 Cell culture

WT and genome-edited (KOT, KOP, KOPT) PC12 cells were kindly provided by Dr. Mario Costa (CNR-IN, Pisa) and cultured in RPMI added with 10% horse serum, 5% FBS, 100 U/mL Penicillin, 100 µg /mL Streptomycin.

293T/17 (ATCC: CRL-11268) cells were cultured in DMEM 4.5 g/L glucose added with 10% FBS, 100 U/mL Penicillin, 100 µg /mL Streptomycin.

For EVs production, cells were maintained in a starvation medium for one overnight, and the day after, the medium was collected, and EVs were extracted with protocol G, described in section 2.9.1.

3.1.2 Extraction and Labelling of SEVs

Labeling of SEVs was done during the extraction process. Supernatants were centrifuged 10' at 4°C at 1000 xg to remove any cellular debris, then centrifuged for 30' at 4°C 16,000 xg to isolate the LEVs and 2h at 4°C 100,000 xg to isolate the SEVs. At this point, SEVs were resuspended in PBS with ions added with 5 µl/mL of Vybrant-DID (v22887, Thermofisher) and incubated for 20 min at 37°C. Labeled SEVs were diluted in 5 mL of PBS with ions and centrifuged again for 2h at 100,000 xg to remove excess dye. SEVs pellets were suspended in PBS with ions 0.22 µm filtered, quantified with MicroBCA, and used for further experiments.

3.1.3 Internalization assay on PC12

Parental cells were seeded in 100 mm Petri dishes and cultured until 100% confluence was achieved. Then, the medium was removed, cells washed, and Starvation Medium (a complete medium not containing FBS) was added. After one overnight of starvation, EVs were collected, as described in section 3.1.6.

Each genotype of recipient PC12 cell was seeded on a 24 mm glass coverslip previously treated overnight with poly-D-lysine 50 µg/mL (A38904-01, Gibco) at a confluence of no more than 50%. After 24h culture, the medium was removed, cells were washed with PBS with ions, and SEVs treatment was performed. Each treatment was prepared by mixing 5 µg of SEVs with 150 ng/mL of NGF in 100 µL of complete medium. Treatments were preincubated for 15 min at 37°C to allow binding between NGF and receptors on the EVs; then, 100 µL of the treatment were administered on top of cells. After 4h of incubation at 37°C, treatments were removed, and cells were washed 3 times with PBS with ions.

3.1.4 Internalization assay on 293T cells

Transfection was performed on parental cells with p75-eGFP (Marchetti et al., 2019b).

Transfection on recipient cells was performed with RFP-Laminin (Marchetti et al., 2019a) and SP-TAP-TrkA

The SP-TAP-TrkA construct was produced initially to allow a Tandem Affinity Purification (TAP) of the TrkA receptor together with its interactor. It is composed of the TrkA protein sequence, together with the SP-TAP fragment comprising a His-Tag and a 3xFLAG-Tag separated by a TEV protease cleavage site. Firstly, the TrkA cDNA was fused with the SP-TAP fragment by means of an Overlap extension and, subsequently, a purification PCR. Then, SP-TAP-TrkA was cloned in the multiple cloning site of the pCDNA3.1 (+) commercial plasmid (V79020, Thermofisher) by means of HindIII and XbaI restriction sites.

Transfection on parental 293T cells was performed using cells with a confluence of not more than 70%, seeded in a 150 mm Petri dish.

First, two solutions were prepared:

1. 730 μ L of CaCl_2 (250 mM) with 120 μ g of p75-eGFP plasmid DNA added
2. 730 μ L of HBS 2X pH 7.21 (NaCl 270 mM, Hepes 40 mM, KCl 10 mM, D-glucose 10 mM, Na_2HPO_4 1.5 mM)

The two mixes were combined under continuous stirring, drop by drop, and incubated for 20 min at room temperature. After the incubation time had passed, the transfection mixture was added to the cells in a complete medium. The cells were incubated overnight, then washed with PBS with ions to replace the medium with starvation medium, which was necessary for subsequent vesicle extraction. After one overnight of starvation, EVs were collected, as described in section 3.1.6.

Transfection on recipient 293T cells was performed with JetPei (101-40N, Polyplus) as recommended by the manufacturer.

Recipient 293T +TrkA and Mock transfected were seeded on 24 mm glass coverslip previously treated overnight with poly-D-lysine 50 μ g/mL (A38904-01, Gibco) at a confluence of no more than 50%. After 24h culture, the medium was removed, cells were washed with PBS with ions, and SEVs treatment was performed. Each treatment was prepared by mixing 5 μ g of SEVs with 150 ng/mL of NGF in 100 μ L of complete medium. Treatments were preincubated for 15 min at 37°C to allow binding between NGF and p75/TrkA; then, 100 μ L of the treatment were administered on top of cells. After 4h of incubation at 37°C, treatments were removed, and cells were washed 3 times with PBS with ions.

3.1.5 Confocal microscopy analysis

Fixation of cells was carried out for 20' in 4% PFA in PBS. Samples were then mounted with Fluoroshield-DAPI (Sigma-Aldrich) and imaged with a laser

scanning confocal microscope (Nikon A1, Eclipse Ti) using a 60x air-objective (NA 1.40) and pinhole set to 1 Airy Unit. Images were acquired at 1024 × 1024 pixel resolution using a 405 nm laser (400-500 emission window), a 488 nm laser (475-575 emission window), a 561 nm laser (545-645 emission window), and a 640 nm laser (emission window >645), 5 images each field. Images were converted in 8-bit and analyzed with ImageJ software performing a field fluorescence intensity measurement. Briefly, a threshold was set to create a mask (M1), and the mask was divided by 255 (M2) and measured to evaluate the number of pixels; the integrated density of M1 was divided by the number of pixels to calculate the mean intensity of the pixels with intensity>0.

3.1.6 Biological effect on NGF signaling

Parental cells were seeded in 100 mm Petri dishes and cultured until 100% confluence was achieved. Then, the medium was removed, cells were washed, and Starvation Medium was added. After one overnight of starvation, EVs were collected, as described in section 3.1.6.

PC12 recipient cells were seeded on a 24 mm glass coverslip previously treated overnight with poly-D-lysine 50 µg/mL (A38904-01, Gibco) at a confluence of no more than 50%. The following day treatment was performed as described in table 3-1.

Table 3-1 Treatment on PC12 cells for the evaluation of differentiation.

	C-	C+	WT	KOP	KOT	KOPT
NGF	∅	150 ng/mL	150 ng/mL	150 ng/mL	150 ng/mL	150 ng/mL
SEVs	∅	∅	800 ng	800 ng	800 ng	800 ng
Complete medium	Until 100 µL	Until 100 µL	Until 100 µL	Until 100 µL	Until 100 µL	Until 100 µL

Treatments were administered in a complete medium for 48 hours; then, cells were acquired with an optical microscope in bright field. Each treatment was administered in two technical replicates.

Quantification of neurites' length was performed by means of ImageJ software.

Multiple data were acquired:

- Neurite Density: sum of all neurite lengths divided by the number of cells of each field.
- Max Neurite Length: length of the longest neurite in each field.
- Mean Neurite Length: sum of all neurite lengths divided by the number of neurites in each field.
- Neurites/cell: the number of neurites divided by the number of cells in each field.

3.1.7 Statistical Analysis

The Graph-Pad Prism program (GraphPad Software Inc., San Diego, CA, United States) was used for data analysis and graphic presentation. All data are the mean $\pm$ SD of at least two independent experiments. Statistical analysis was performed by ordinary one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test or unpaired t-Test. $P \leq 0.05$ was considered statistically significant.

4 Concluding remarks and future perspectives

This work describes the approach to the field of research on extracellular vesicles that I undertook during my Ph.D. We decided to tackle this research in two distinct but very interlinked ways. On the one hand, we took a pure biochemical approach to investigate the characteristics of EVs derived from human microglia in physiological and pathological conditions. On the other hand, we use a more biotechnological approach to investigate a possible way to increase EVs' targeted delivery.

The first part of our research fills a void in the field of EVs from microglia, where preparations made from human cells have received scant attention thus far. Most neurodegenerative diseases have neuroinflammation as a common denominator, and microglia is one of the major players in the induction, spreading, as well as resolution of neuroinflammation. For this reason, this kind of investigation is essential for the research field that aims to use EVs as a therapeutic target in any diseases involving microglia and neuroinflammation.

The second part is equally crucial; EVs are recognized as a novel and promising therapeutic agent. Our research in this field aimed to understand better the specificity of EV uptake and a possible molecular tool to control it. We hypothesized that the formation of an NGF-mediated trans ternary complex between p75 and TrkA could increase the specific binding and uptake of EVs on targeted recipient cells.

For what concerns EVs derived from human microglia, we started optimizing our experimental setup by comparing EVs from two human microglia cell lines, HMC3 and C20, in untreated conditions. We provided proof that although most of the morphological, biochemical, and biophysical characteristics of the two types of microglial EVs are comparable, there is a

significant difference in their miRNA cargo, even when the EVs are at rest. The two EV preparations behaved differently in a biological assay, supporting this observation. According to earlier research, a different derivation of the same cell type can drastically alter the cargo of the produced EVs (Yuyama et al., 2015). Moreover, we investigated morphological, biochemical, and biophysical characteristics also on the EVs produced by inflamed microglia, mimicking pathological conditions, confirming that their composition and biological effect display increased pro-inflammatory characteristics. The biological effect obtained on recipient microglia confirms that SEVs could be a way for inflamed microglia to spread inflammation to neighboring cells. We also provided preliminary data on the effect of SEVs from inflamed microglia on glioblastoma cells; our results showed that homeostatic microglia could impair glioblastoma proliferation and motility through signals mediated by SEVs, while SEVs from inflamed microglia seems to affect the tumor cell motility. However, these data are not conclusive; there is a need to investigate better how our observed effect is mediated. Immune cells that have invaded the tumor microenvironment cause oxidative DNA damage, which is thought to promote both genetic and epigenetic changes that take place during glioma evolution; those signals could be mediated by SEVs and could positively affect glioma growth. Glioma cells that use a variety of metabolic modifications, including changes in the metabolism of various amino acids, are positively selected in this environment. Glioma cells use altered metabolism to support tumor cell survival and to limit adaptive immune responses, whereas altered metabolism can accelerate the death of normal tissues (Sowers et al., 2014). As a future perspective on this topic, we think that the evaluation of microglia SEVs' effects on different processes of glioma, such as oxidative damage and altered metabolism, may lead to new mechanistic details of EV function within the tumor microenvironment.

Regarding the experiments in the use of EVs as a valuable fully biological nanoparticle for drug delivery, we inserted ourselves in a two-decade-long saga concerning the seeking of an explanation of the particular ternary complex between TrkA and p75 receptors mediated by NGF. We hypothesized here that this ternary complex could be formed when the two receptors are not on the same membrane but instead in a trans configuration; moreover, we tried to evaluate if the formation of this complex may affect the targeted delivery of SEVs on recipient cells. Although our data are still too preliminary to assess our hypothesis, they suggest that the presence or absence of p75 and TrkA could affect SEVs uptake. Future experiments are needed to improve the EV labeling technique, better optimizing the DID use or increasing the level of p75-eGFP in SEVs (or of TrkA in the recipient cells).

Regardless of that, the biological assay showed that PC12 WT cells were positively affected by the administration of NGF in combination with SEVs from PC12 WT or KOP. Our data showed that the administration of TrkA (but not necessarily of p75) via SEVs to recipient cells increases the response to NGF in cells that express both receptors. Actually, from the WB obtained both in EVs from PC12 and HEK cells, the p75 seems to have a preferential sorting to LEVs than to SEVs, and this may explain why SEVs from PC12 WT and KOP do not behave so differently in the neurite outgrowth assay. Indeed, a more robust and quantitative assessment of either receptor in LEVs and SEVs would be necessary to rule out this hypothesis. Nevertheless, if that was the case, the SEVs biological effect could also be interpreted not as an effect of the presence of TrkA but rather of low levels or absence of p75 therein. Indeed, the capability of p75 to collapse initiation processes in the absence of TrkA is well known (Conroy and Coulson, 2022; Sun et al., 2012). The administration of SEVs from PC12 WT or KOP could cause an imbalance in the relative quantities of TrkA compared to p75. This imbalance could potentiate the

trophic action exerted by TrkA while attenuating the collapsing actions prompted by p75.

In any case, we believe that the NGF signaling potentiation prompted by SEVs may stem from two possibilities: 1) TrkA/p75 are transferred from the EV (via direct fusion or internalization/recycling routes) to the membrane of recipient cells, thus enriching the reservoir of receptors able to respond to NGF; 2) the EV carried by NGF slows down the internalization of the latter via TrkA/p75 on the membrane of the recipient cells, overall reducing their internalization and degradation and potentiating their signaling. Which of the two possibilities is actually occurring represents a fascinating future investigation both for the EV and neurotrophin research fields. Finally, to better understand if these effects are really mediated by TrkA or p75, it could be helpful to investigate the downstream signaling activated by both, e.g., pERK, pAKT, pPLC- γ for TrkA and pJun, RhoA/ROCK for p75.

5 Appendix I: The role of p75 signaling in the proliferating/quiescent phenotype transition of Neuronal Stem Progenitor Cells

This Appendix will present my research dedicated to increase my knowledge of the neurotrophin system, I asked an international expert in the field to be hosted in his lab. I spent my six months abroad at the Institute of Biomedicine of Valencia; I could work with Marçal Vilar's group in the Unit of Molecular Basis of Neurodegeneration and with Helena Mira's group in the Unit of Stem Cells and Ageing.

Both are involved in research regarding the role of p75 and the neurotrophin system in Adult Neurogenesis (Vilar and Mira, 2016). I was involved in some projects that I will describe in further sections.

All experiments described in the following section were done by the candidate at the Institute of Biomedicine of Valencia.

5.1 Kinetics of p75NTR expression in NSPC cells

The cells used for our experiments were rat adult hippocampal Neural Stem and Progenitor Cells (NSPCs) (Mira et al., 2010; Palmer et al., 1997). These cells can switch from an active and proliferating phenotype to a quiescent one upon administration of BMP4 (Mira et al., 2010); this transition is reversible if BMP4 is removed. Preliminary genome-wide microarray data (not shown) on these cells demonstrated a substantial increase in the expression of p75 in the quiescent phenotype compared to the proliferating one; our first experiment was to confirm these preliminary data.

In this experiment, we defined the three states in which NSPCs can be analyzed in vitro: proliferating, quiescent, and quiescent reversion (QRev). The proliferating state is achieved in standard culture conditions with mitogenic stimulation (FGF2), the quiescent state is achieved with the administration of BMP4 in the presence of FGF2 for at least 72h, and the quiescent reversion state is achieved by removing BMP4 from quiescent cells and by culturing them again in FGF2 only for at least 72h. At 8h after removing BMP4 NSPCs are still in quiescence (since they do not express the cell cycle marker Ki67; ***Panel C, Figure 5-1***). Most NSPCs re-enter the cell cycle and become positive for Ki67 at 24h after removing BMP4.

We confirmed an increase in p75 expression from 24h after BMP4; the protein level continued to increase proportionally to the time of BMP4 treatment (*Panel A-B, Figure 5-1*). Furthermore, the expression continued to rise after BMP4 removal for 24h, then drastically decreased. The depletion of p75 in the

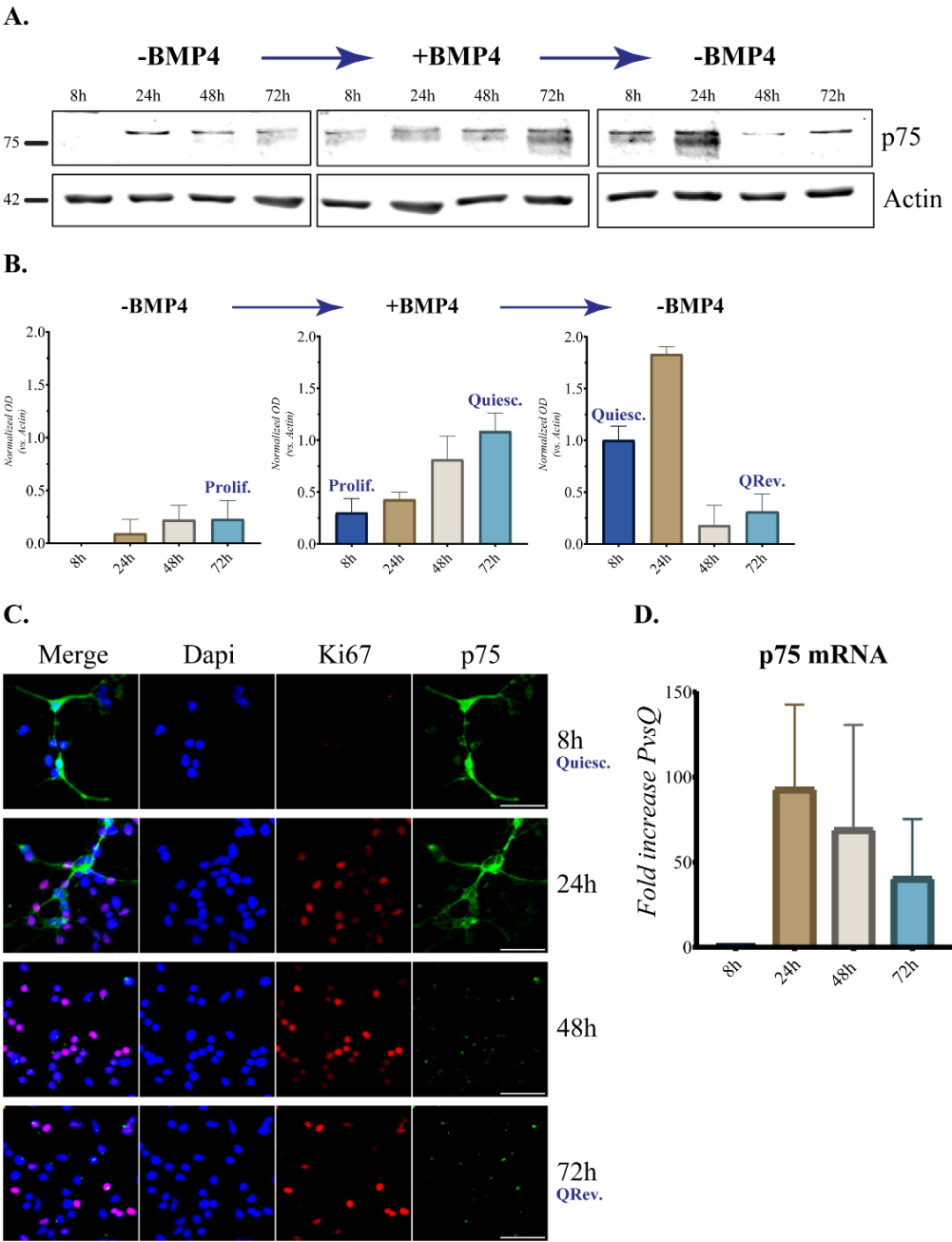


Figure 5-1: p75NTR Expression in quiescent and proliferating cells. Cells were treated with 25 ng/mL of BMP4 for 72h to induce quiescent phenotype, reverted from quiescence by removing BMP4 for the indicated times, and maintained in untreated conditions for the proliferating state. Panel A: cells from each condition were lysed, and protein extracted. 30 μ g of proteins from the whole cell lysate were separated by SDS-page and blotted to detect p75 and actin as a loading control. Panel B: Quantification of Blots showed in panel A. ODs of protein bands were normalized VS the relative OD of Actin. Data are expressed as mean $\pm$ SD of at least two independent experiments. Panel C: Representative images of immunofluorescence of p75 in the transition from quiescent to QRev. Treated cells were fixed, immunostaining against p75 and Ki67 performed, and stained with DAPI. Ki67 is used as a marker of proliferation. Images were acquired with a confocal microscope (63x magnification). Scale bars are set to 50 μ m. Panel D: mRNA expression of p75 was evaluated by means of qPCR. Cells from each condition were lysed, and RNA extracted, quantified, reverse-transcribed, and analyzed by qRT-PCR. Data are expressed as mean $\pm$ SD.

QRev state was also confirmed through immunofluorescence on NPSCs treated as already described (**Panel C, Figure 5-1**). Conversely, the mRNA expression steeply increases after 24h of administering BMP4 to proliferating cells but decreases as time passes (**Panel D, Figure 5-1**).

All our following experiments aimed to understand better if these changes in the expression of p75 could directly impact the state of cells or if they constitute a consequence of the transition between NSPC proliferation and quiescence.

5.2 Influence of p75 signaling on NSPC differentiation into a neuronal phenotype

Published data showed that NSPCs could differentiate into a neuronal phenotype upon administration of GSK3B inhibitor CHIR99021 (Chir) (García-Corzo et al., 2022), which triggers canonical Wnt signaling, a master regulator of adult neurogenesis (Lie et al., 2005). Our first experiment aimed to evaluate the possibility that the increase of expression is needed in quiescent cells and that the signaling mediated by proNGF, a primary ligand of p75, affects the differentiation process into neurons. Published data showed that NSPCs could differentiate into a neuronal phenotype upon administration of GSK3B inhibitor CHIR99021 (Chir) (García-Corzo et al., 2022), which triggers canonical Wnt signaling, a master regulator of adult neurogenesis (Lie et al., 2005). Our first experiment aimed to evaluate the possibility that the increase of expression is needed in quiescent cells and that the signaling mediated by proNGF, a primary ligand of p75, affects the differentiation process into neurons.

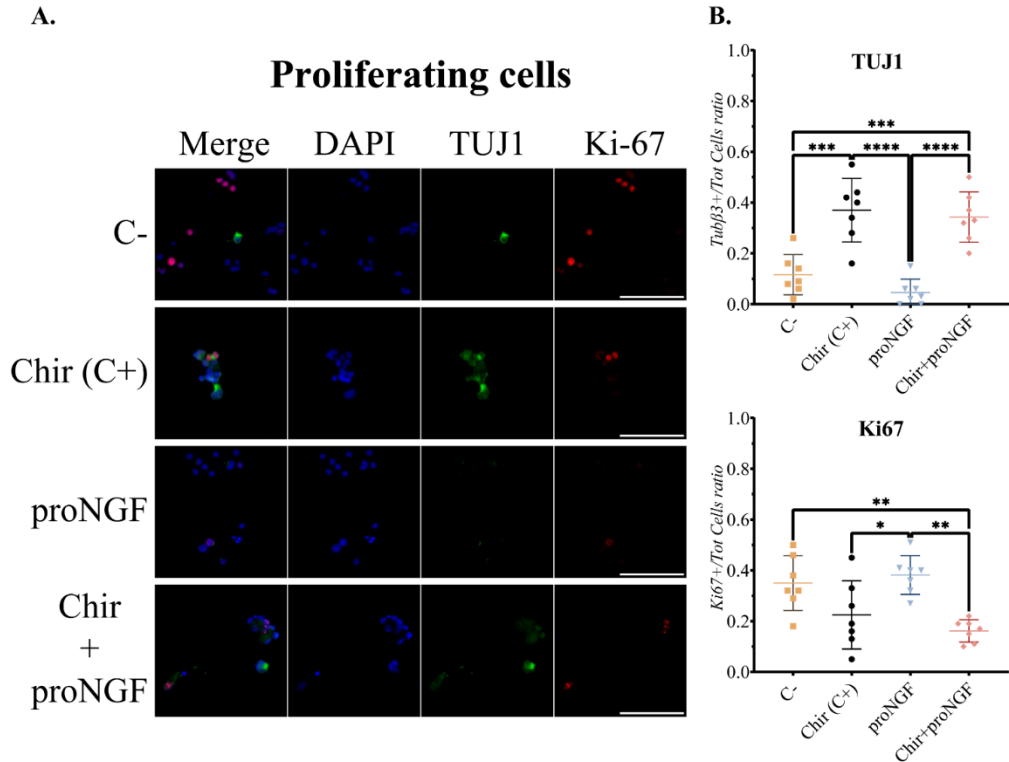


Figure 5-2: NSPCs cells differentiation into neuronal phenotype. Cells were treated with 1.5 μ M GSK3B inhibitor CHIR99021 (Chir) in combination with main ligand of p75, 10 ng/mL proNGF. Treatments were performed on proliferating cells in absence of BMP4. Panel A: Representative images of NSPCs treated in the indicated conditions. Treated cells were fixed, immunostaining performed, and stained with DAPI. Images were acquired with a confocal microscope (63x magnification). Scale bars are set to 100 μ m. Panel B: Expression of markers was quantified with ImageJ software on confocal images. The number of positive cells were normalized to the total DAPI positive nuclei. Data are expressed as mean $\pm$ SD of the pool of at least 7 field from three independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test for comparison of the means of all samples: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

We found that TUJ1 significantly increases upon Chir administration in proliferating cells, while Ki67 did the opposite. These variations are expected in proliferating cells; NSPCs that differentiate into neurons stop dividing, expressing more TUJ1 and less Ki67 (**Figure 5-2**).

proNGF-treated cells did not show significant differences, for any of the two markers, compared to the negative control. These results were also expected as proliferating cells express little to no p75 (**Figure 3-9**). Together these

findings made it easy to understand that combining both treatments have the same effect of the Chir alone since no effect was prompted by proNGF alone.

We performed the same experiment on NPSCs treated with BMP4 for 96h, thus showing a quiescent phenotype.

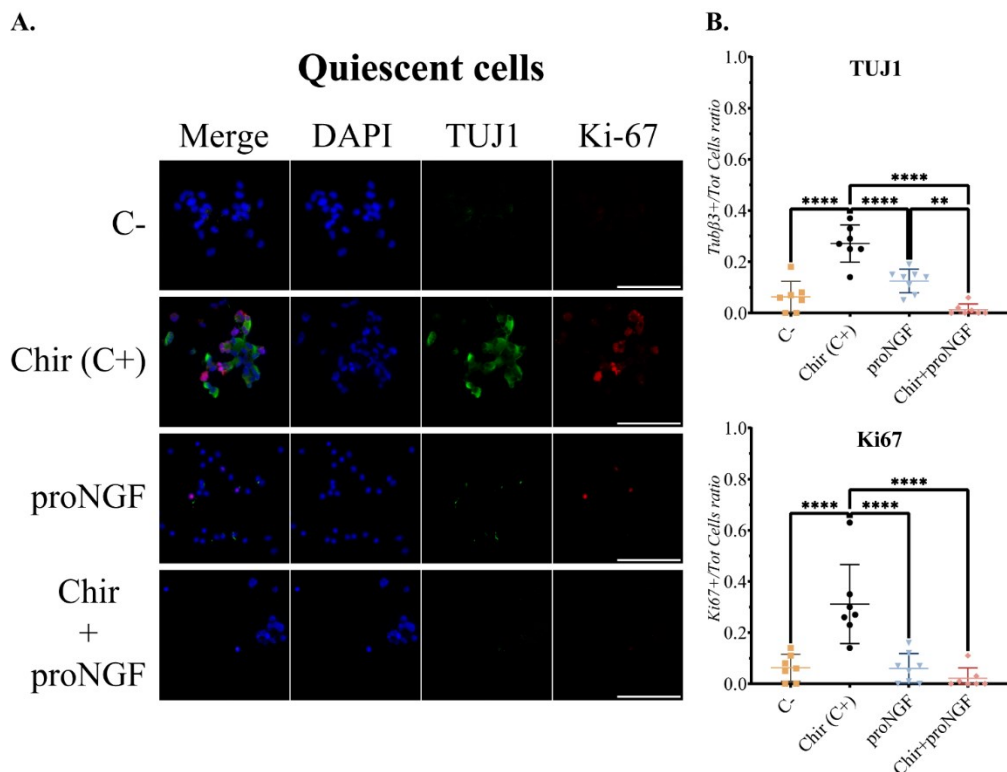


Figure 5-3: NSPCs cells differentiation into neuronal phenotype. Cells were treated with 1.5 μ M GSK3B inhibitor CHIR99021 (Chir) in combination with main ligand of p75, 10 ng/mL proNGF. Treatments were performed on quiescent cells (treated with 25 nl/mL of BMP4 for 96h). Panel A: Representative images of NSPCs treated in the indicated conditions. Treated cells were fixed, immunostaining performed, and stained with DAPI. Images were acquired with a confocal microscope (63x magnification). Scale bars are set to 100 μ m. Panel B: Expression of markers was quantified with ImageJ software on confocal images. The number of positive cells were normalized to the total DAPI positive nuclei. Data are expressed as mean $\pm$ SD of the pool of at least 7 field from three independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test for comparison of the means of all samples: ** $P \leq 0.01$, **** $P \leq 0.0001$.

Interestingly, quiescent cells could still differentiate upon Chir stimulation; in this case, the expression of Ki67 also increases following the same pattern of TUJ1 (**Figure 5-3**). This result could be explained by the fact that quiescent NSPCs need to start proliferating again to generate dividing intermediate progenitor cells that, in turn, differentiate into neuroblasts and then give rise to neurons.

Also, in this case, proNGF alone showed no significant differences compared to the negative control; however, the increase in differentiation mediated by

Chir was entirely abolished when cells were exposed to both proNGF and Chir. This finding may suggest that proNGF-mediated p75 signaling could work against differentiation in quiescent NSPCs, possibly favoring stemness.

This effect could be translated in vivo as a double-edged sword. On the one hand, it could be, in physiological conditions, a way to maintain a reservoir of neuronal precursors in the presence of proNGF, preventing all of them from differentiating to neurons.

On the other hand, it is known that proNGF accumulates in the cortex and hippocampus in AD (Fahnestock and Shekari, 2019); this could prevent NPSCs from exiting quiescence and maturing into neurons, effectively impairing neurogenesis.

5.3 Crosstalk between p75 and ROCK signaling

It has been observed that a variety of substances function as GDI displacement factors for Rho GTPases. For instance, the RhoGDI-1 is immediately bound by the neurotrophin receptor p75NTR, which then triggers RhoA activation by releasing RhoA from RhoGDI-1. Interestingly, the myelin-derived proteins Nogo and myelin-associated glycoprotein have been found to strengthen the formation of the p75NTR-RhoGDI-1 complex for the inhibition of neurite outgrowth, whereas NGF interferes with the binding between p75NTR and RhoGDI-1 that stimulates neurite outgrowth (Schmidt et al., 2022).

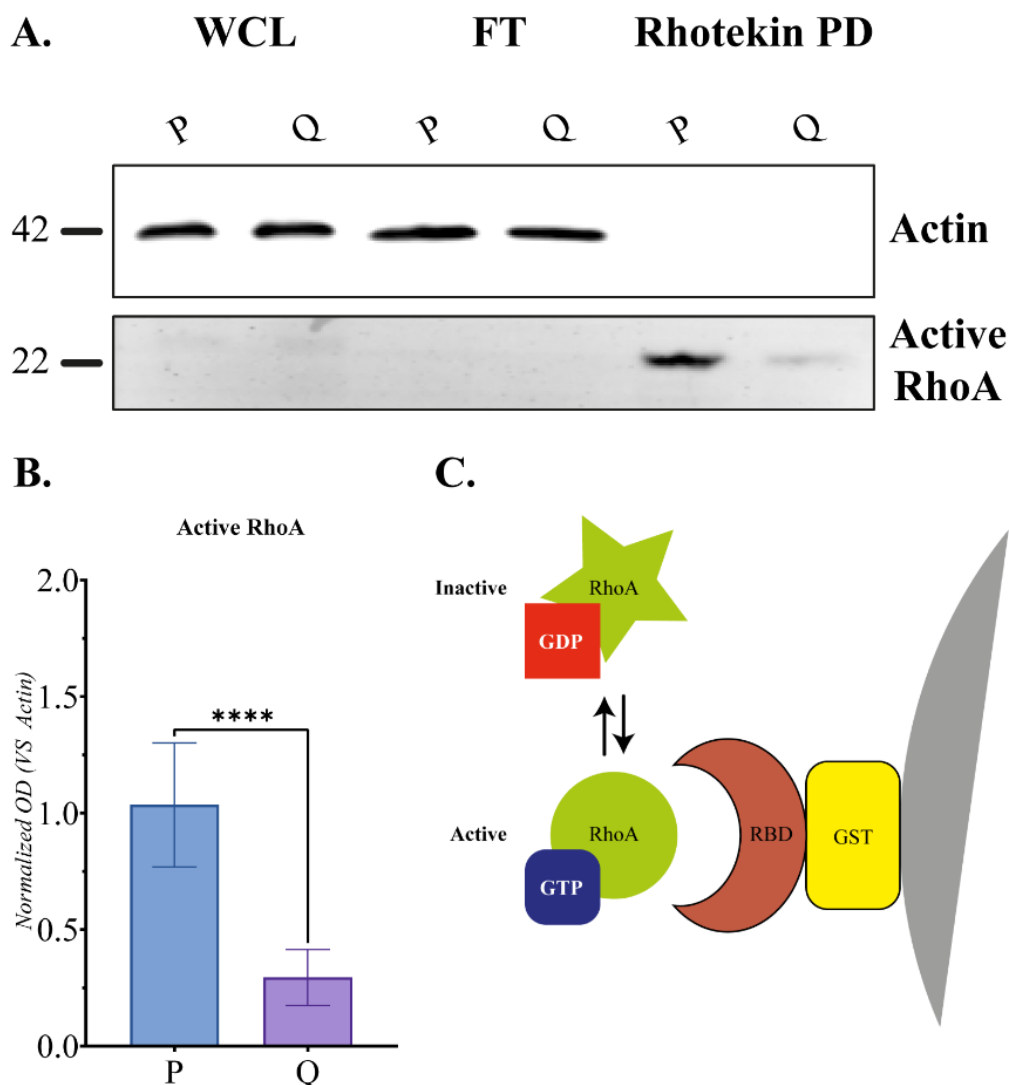


Figure 5-4: Active RhoA expression in NSPCs cells in proliferating (P) and quiescent (Q) state. Cells were treated with 25 ng/mL of BMP4 for 72h to induce quiescent phenotype or maintained in untreated conditions for the proliferating state. Panel A: Cells from each condition were lysed, and protein extracted. 30 μ g of WCL (whole cell extract), and of FT (flowthrough) were separated with SDS-Page together with 250 μ g of samples pulled down with Rhotekin RBD (Rhotekin PD). Panel B: quantification of pulled down Active RhoA in proliferating (P) and quiescent (Q) cells by western blot (panel A), OD values are expressed as mean $\pm$ SD. of at least two independent experiments. ODs of protein bands were normalized VS the relative OD of Actin in the respective WCL. The significance of the differences was determined by t-test: **** $P \leq 0.0001$, vs P.

ROCK signaling is crucial in controlling the cell cycle; it has been demonstrated that the activation of RhoA causes ROCK-mediated cell cycle arrest (Amin et al., 2013). Neurotrophin-mediated signaling of p75 includes the activation of the ROCK pathway (Liu et al., 2015). This data prompted us

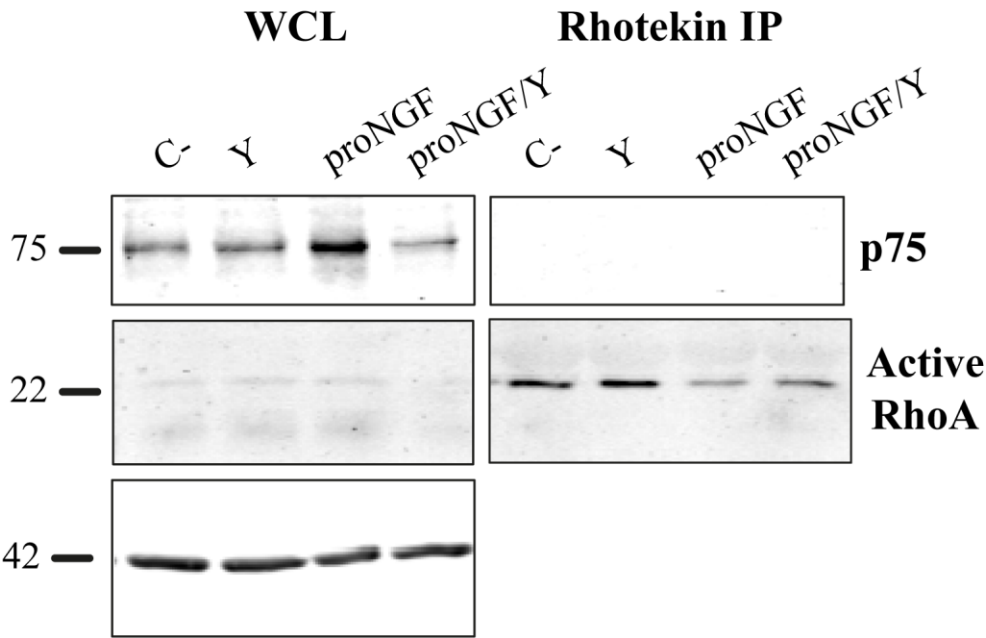
to investigate how the expression of p75 was affected by ROCK signaling and how the signaling of proNGF through p75 may affect ROCK signaling by measuring the active RhoA levels (**Figure 5-4**). We compared active RhoA levels in proliferating and quiescent cells to indicate their basal levels. We extracted total proteins and immunoprecipitated them to isolate the portion of active RhoA. We used beads functionalized with GST-Rhotekin-RBD (<https://www.cytoskeleton.com/rt02>).

The human Rhotekin protein's Rho binding domain (RBD) binds specifically to GTP-bound, not GDP-bound, RhoA, RhoB, and RhoC proteins. Therefore, the domain can be used to precipitate active, GTP-bound Rho proteins.

We detected a significant decrease in active RhoA in quiescent cells compared to proliferating ones. This variation does not correlate with the more abundant p75 expression in quiescent than in proliferating cells. Indeed, active RhoA levels can be reliably unrelated to the expression of p75, as RhoA is a mediator of many different pathways that may discriminate proliferating from quiescent cells.

After this evaluation in basal condition, we decided to modulate RhoA signaling with ROCK inhibitor Y-27632 (Y) and p75 signaling through the administration of proNGF (*Figure 5-5*).

A.



B.

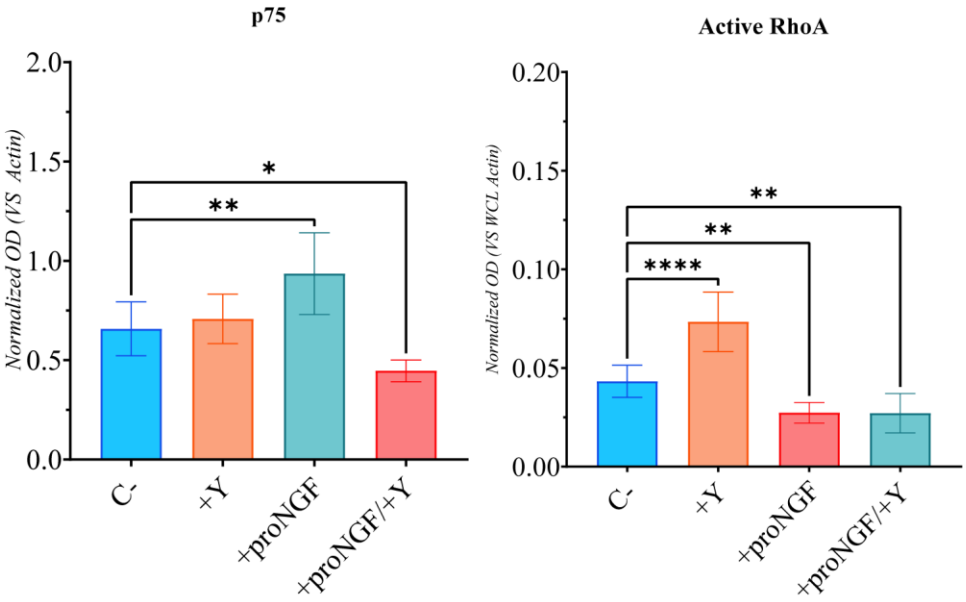


Figure 5-5: Crosstalk between p75 and RhoA-mediated signaling. Panel A: NPSC were treated with 25 ng/mL BMP4 for 96h to induce quiescence, then treatment with 10 μ M Y-27632 (Y), 10 ng/mL proNGF, or both were carried on for 2h, cells were lysed, and protein separated through SDS-page. Panel B: quantification of pulled down Active RhoA by western blot, OD values are expressed as mean $\pm$ SD of at least two independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post-test for comparison of the means of all samples: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$.

We wanted to investigate the role of p75 in this signaling pathway, so we decided to concentrate our experiments on quiescent cells since, in proliferating, there is a negligible expression of p75.

After 96h of treatment to induce quiescence, NPSCs were treated for 2h with proNGF, Y, and a combination of both.

The expression of p75 was affected by our treatments. We detected a significant increase after administering proNGF. This increase may be explained by a constant internalization and degradation of p75 in the quiescent state of NSPCs, and by the previous observation that proNGF prompts a ligand-mediated immobilization of the receptor on the surface of cells, resulting in reduced endocytosis and, possibly, degradation (Marchetti et al., 2019a). Indeed, the sharp and fast increase of expression of p75 after just 2h of administration of proNGF could be explained by a decrease in protein degradation more than an increase in protein expression. No significant changes were detected after the administration of just Y; however, upon administration of a combination of proNGF and Y, we detected a significant reduction of the expression of p75. This could suggest an interplay between ROCK inhibition and the ligand-mediated internalization and degradation of p75: it could be that once ROCK signaling is inhibited, p75's response to proNGF is enhanced to compensate for such inhibition, resulting in internalization/degradation of p75.

The expression of active RhoA also was affected by our treatments. We observed a significant increase upon administration of just Y, which could be

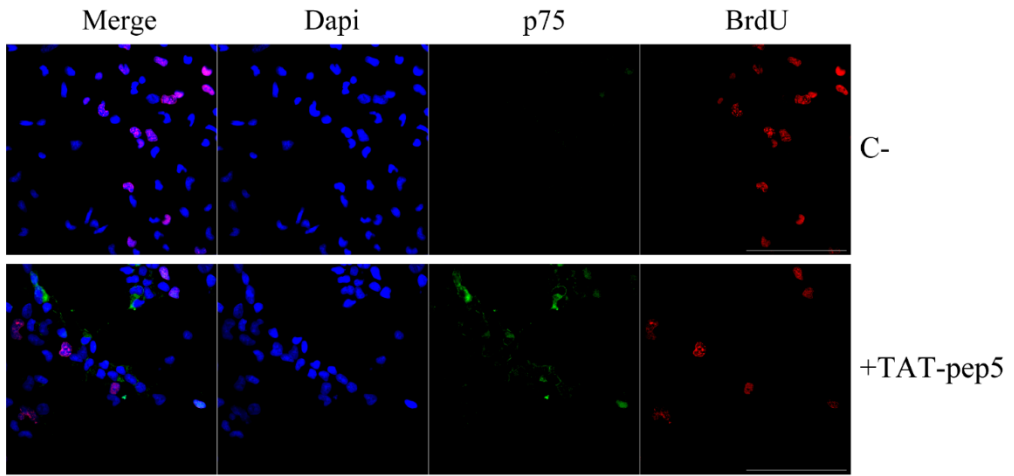
interpreted as a compensating mechanism to counteract the inhibition of ROCK, a RhoA downstream target. Conversely, the decrease in active RhoA in both conditions with proNGF confirms that activating ligand-dependent p75 signaling can reduce RhoA signaling (*Pane A, Figure 5-5*).

Overall, our data confirmed that the experimental conditions align with what was reported in the literature; however, they did not unveil significant insight into the possibility that ROCK-p75 interplay could affect our process of interest. This data may suggest that RhoA signaling is not playing the leading role in this system. In light of this, we decided to upstream as much as possible in the p75-mediated signaling by inhibiting p75 directly with a peptide, TAT-pep5 (Yamashita and Tohyama, 2003).

5.4 Preliminary data and future perspectives

This experiment was first done on proliferating cells, meaning they express shallow levels of p75. We administrated to them an inhibitor of p75, TAT-pep5, and we evaluated any change in the expression of p75 and the percentage of proliferating cells marked with a BrdU proliferating marker. TAT-pep5 is an inhibitor of the direct interaction between Rho-GDI and p75. This means

A.



B.

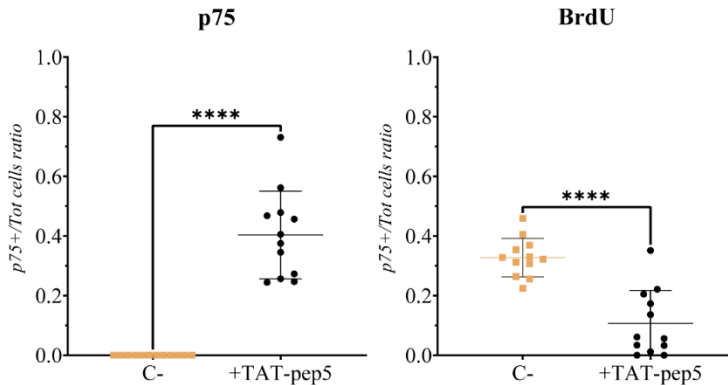


Figure 5-6: Effect on proliferating cells of p75 inhibition. Panel A: Representative images of NSPCs maintained proliferating and treated with 0.1 μM of TAT-pep5 for 96h. Treated cells were fixed, immunostaining performed, and stained with DAPI. Images were acquired with a confocal microscope (63x magnification). Scale bars are set to 100 μm. Panel B: Expression of markers was quantified with ImageJ software on confocal images. The number of positive cells were normalized to the total DAPI positive nuclei. Data are expressed as mean ± SD of the pool of at least 12 field from three independent experiments. The significance of the differences was determined by t-test: ****P ≤ 0.0001.

that with the peptide, Rho-GDI is released from p75 and keeps RhoA in an inactivated state.

Interestingly, p75 levels increase significantly after the administration of TAT-pep5, while BrdU⁺ cells drastically decrease (**Figure 5-6**). Low proliferation levels and high expression of p75 is a characteristic of the quiescent state of NSPCs; this may suggest that the p75 inhibitor has the effect of inducing the entrance in quiescence of those cells even in the absence of BMP4.

Since TAT-pep5 is specific for p75, this effect must be mediated by the shallow expression of p75 in proliferating cells.

These findings may suggest that low levels of p75 can maintain the proliferating phenotype in the absence of proNGF and that inhibition of the ligand-independent p75 activity breaks the brake and induce cells to enter quiescence.

However, data at the moment are limited. There is a need to repeat the same experiment on quiescent cells and QRev and test if the presence of proNGF can increase or reduce the inhibitor effect. In any case, Q cells display high levels of p75, which, as shown in **Figure 5-1**, concur to maintain the quiescent state; in the presence of proNGF, entrance in the proliferating state and further differentiation are inhibited. Overall, the NPSCs model offered another opportunity to detect unexpected functions for p75, which depend on the expression level changes and the absence/presence of ligands. Future investigations are certainly needed to fully unveil the role of this receptor in regulating this critical cell type fate in the brain.

5.5 Materials and Methods

5.5.1 p75 kinetics

For proliferation, quiescence, and differentiation assays, we used rat adult hippocampal Neural Stem and Progenitor Cells (NSPCs) (H et al., 2010; Palmer et al., 1997). Cells were maintained in DMEM F12 added with 0.6% Glucose, 0.1% NaHCO₃, 100 u/mL Pen/Strep, 5 mM HEPES, and 1% N2 supplement, without FBS. All cultures were added with 10 ng/mL of FGF2.

Proliferating cells → Cells were maintained 72h in culture untreated.

Quiescent cells → Cells were maintained for 72h in culture in the presence of 25 ng/mL of BMP4.

QRev cells → Cells were maintained for 72h in culture in the presence of 25 ng/mL of BMP4; after 72h, BMP4 was removed, and cells were maintained for 72h more untreated.

Cells were recovered at the indicated time points, and cells from the same well were divided in half: ½ lysed with Lysis Buffer for WB and ½ lysed with Tri-Reagent for RNA extraction.

The following antibodies were used for Western Blot analyses: rabbit α -p75 (Millipore, 07-476) 1:10000, mouse α -Actin 1:5000. Membranes were incubated overnight at 4°C in a linear oscillator with the primary antibody. Secondary α -mouse 1:10000 and α -rabbit 1:10000 were incubated for 1h30' at room temperature.

mRNA expression was evaluated through qPCR using primers for the p75 gene; expression was normalized on the SDHA housekeeping gene.

For immunofluorescent NSPCS cells were seeded on Matrigel-treated glasses, 50000 cells each 24-well, and treated as described above. Cells were fixed at the indicated time points.

Fixed cells were incubated for 1h in 200 μ L of Blocking Solution (0.1 M PB + 0.5% Tx-100 + 10% FBS) at room temperature to reduce as much as possible unspecific binding of the primary antibody. After the blocking step, cells were incubated for 1 overnight in 120 μ L of the primary antibodies mix: mouse α -Ki67 1:150 and rabbit α -p75 1:100, diluted in Blocking Solution. After 1 overnight, cells were washed 3 times in 0.1 M PB and incubated for 1h at room temperature with the secondary antibodies mix: donkey α -mouse-Cy3 1:500, and donkey α -rabbit-a488 1:500, diluted in 0.1 M PB. Nuclei were stained with DAPI 1:1000 in 0.1 M PB for 5 min; cells were washed 3 times in 0.1 M PB and mounted and coverslipped with Mowiol.

Fluorescence images were acquired with Confocal SP8 (LEICA) 63x magnification and analyzed with ImageJ Software.

5.5.2 Differentiation NSPC

NSPC cells were seeded on Matrigel-treated glasses, 50000 cells each 24-well and maintained without FGF2.

Proliferating cells $\rightarrow$ cells were seeded and maintained in a culture medium without FGF2. Treatments were carried on as follows: +DMSO (C-), 1.5 μ M GSK3B inhibitor CHIR99021 (Chir), 10 ng/mL proNGF, and the combination of both. After 96h, cells were fixed with 4% PFA and stored in 0.1 M PB + 0.05% Sodium Azide.

Quiescent cells $\rightarrow$ cells were seeded and cultured in medium added with 25 ng/mL BMP4 and 20 ng/mL FGF2; after 96h, the medium was removed, cells washed, and a new medium was added with just FGF2 and not BMP4.

Treatments were performed as described above. After 96h, cells were fixed with 4% PFA and stored in 0.1 M PB + 0.05% Sodium Azide.

Fixed cells were incubated for 1h in 200 μ L of Blocking Solution (0.1 M PB + 0.5% Tx-100 + 10% FBS) at room temperature to reduce as much as possible unspecific binding of the primary antibody. After the blocking step, slices were incubated for 1 overnight in 120 μ L of the primary antibodies mix: rabbit α -Ki67 1:150 and mouse α -TUB3 1:100, diluted in Blocking Solution. After 1 overnight, cells were washed 3 times in 0.1 M PB and incubated for 1h at room temperature with the secondary antibodies mix: donkey α -rabbit-Cy3 1:500, and donkey α -mouse-a488 1:500, diluted in 0.1 M PB. Nuclei were stained with DAPI 1:1000 in 0.1 M PB for 5 min; cells were washed 3 times in 0.1 M PB and mounted and coverslipped with Mowiol.

Fluorescence images were acquired with Confocal SP8 (LEICA) 63x magnification and analyzed with ImageJ Software.

5.5.3 RhoA Expression (Pull Down)

Cells were maintained in DMEM F12 added with 0.6% Glucose, 0.1% NaHCO₃, 100 u/mL Pen/Strep, 5 mM HEPES, and 1% N2 supplement, without FBS. All cultures were added with 20 ng/mL of FGF2. Where indicated, cells were treated with 10 μ M Rock inhibitor Y-27632 for the indicated amount of time.

Proliferating cells → Cells were maintained 96h in culture untreated. After 96h, cells were lysed with Lysis Buffer, and total protein was quantified with Bradford Assay.

Quiescent cells → Cells were maintained 96h in culture in the presence of 25 ng/mL of BMP4. After 96h, cells were added with 10 ng/mL proNGF, 10 μ M

Y-27632, or a combination of both for 2h., lysed in Lysis Buffer, and total protein was quantified with Bradford Assay.

250 μ L of the least concentrated sample, and the corresponding amount of protein for every other, were used for pull-down with beads functionalized with GST-Rhotekin-RBD:

- 15 μ L beads
- 10 mM $MgCl_2$ final
- TNE until 500 μ L final volume

2-3h 4°C on the wheel.

Beads were washed twice with TNE and once with TNE+0.1% TritonX100. Each sample was added with 20 μ L of Gel loading buffer 2x, boiled for 10 min at 95°C, and loaded on 10% acrylamide gel.

The following antibodies were used for western Western Blot analyses: rabbit α -p75 (Millipore, 07-476) 1:10000, mouse α -Actin 1:5000, and rabbit α -RhoA 1:1000. Membranes were incubated overnight at 4°C in a linear oscillator with the primary antibody. Secondary α -mouse 1:10000 and α -rabbit 1:10000 were incubated for 1h30' at room temperature.

5.5.4 TAT-mediated inhibition of p75

Cells were maintained in DMEM F12 added with 0.6% Glucose, 0.1% $NaHCO_3$, 100 u/mL Pen/Strep, 5 mM HEPES, and 1% N2 supplement, without FBS. All cultures were added with 20 ng/mL of FGF2. Where indicated, cells were treated with 10 μ M TAT-pep5 (506181, Sigma).

Proliferating cells → Cells were maintained for 96h in culture without BMP4. Meanwhile, treatment with 10 μ M TAT-pep5 was carried on. After 96h, cells

were treated with BrdU for 2h and fixed. BrdU primary antibody needs antigen retrieval with HCl 2N treatment for 20' 37°C.

Fixed cells were incubated for 1h in 200 μ L of Blocking Solution (0.1 M PB + 0.5% Tx-100 + 10% FBS) at room temperature to reduce as much as possible unspecific binding of the primary antibody. After the blocking step, cells were incubated for 1 overnight in 120 μ L of the primary antibodies mix: rat α -BrdU 1:100 and rabbit α -p75 1:100, diluted in Blocking Solution. After 1 overnight, cells were washed 3 times in 0.1 M PB and incubated for 1h at room temperature with the secondary antibodies mix: donkey α -rat-a55 1:500, and donkey α -rabbit-a488 1:500, diluted in 0.1 M PB. Nuclei were stained with DAPI 1:1000 in 0.1 M PB for 5 min; cells were washed 3 times in 0.1 M PB and mounted and coverslipped with Mowiol.

Fluorescence images were acquired with Confocal SP8 (LEICA) 63x magnification and analyzed with ImageJ Software.

References

- Abels, E.R., Breakefield, X.O., 2016. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell. Mol. Neurobiol.* 36, 301–312. <https://doi.org/10.1007/s10571-016-0366-z>
- Abud, E.M., Ramirez, R.N., Martinez, E.S., Healy, L.M., Nguyen, C.H.H., Newman, S.A., Yeromin, A.V., Scarfone, V.M., Marsh, S.E., Fimbres, C., Caraway, C.A., Fote, G.M., Madany, A.M., Agrawal, A., Kaye, R., Gyls, K.H., Cahalan, M.D., Cummings, B.J., Antel, J.P., Mortazavi, A., Carson, M.J., Poon, W.W., Blurton-Jones, M., 2017. iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* 94, 278-293.e9. <https://doi.org/10.1016/j.neuron.2017.03.042>
- Aili, Y., Maimaitiming, N., Mahemuti, Y., Qin, H., Wang, Y., Wang, Z., 2021. The Role of Exosomal miRNAs in Glioma: Biological Function and Clinical Application. *Front. Oncol.* 11.
- Amin, E., Dubey, B.N., Zhang, S.-C., Gremer, L., Dvorsky, R., Moll, J.M., Taha, M.S., Nagel-Steger, L., Piekorz, R.P., Somlyo, A.V., Ahmadian, M.R., 2013. Rho-kinase: regulation, (dys)function, and inhibition. *Biol. Chem.* 394, 1399–1410. <https://doi.org/10.1515/hsz-2013-0181>
- Amodeo, R., Convertino, D., Calvello, M., Ceccarelli, L., Bonsignore, F., Ravelli, C., Cattaneo, A., Martini, C., Luin, S., Mitola, S., Signore, G., Marchetti, L., 2020. Fluorolabeling of the PPTase-Related Chemical Tags: Comparative Study of Different Membrane Receptors and Different Fluorophores in the Labeling Reactions. *Front. Mol. Biosci.* 7.
- Amor, S., Puentes, F., Baker, D., van der Valk, P., 2010. Inflammation in neurodegenerative diseases. *Immunology* 129, 154–169. <https://doi.org/10.1111/j.1365-2567.2009.03225.x>

Anastasi, F., Greco, F., Dilillo, M., Vannini, E., Cappello, V., Baroncelli, L., Costa, M., Gemmi, M., Caleo, M., McDonnell, L.A., 2020. Proteomics analysis of serum small extracellular vesicles for the longitudinal study of a glioblastoma multiforme mouse model. *Sci. Rep.* 10. <https://doi.org/10.1038/s41598-020-77535-8>

André, F., Chaput, N., Schartz, N.E.C., Flament, C., Aubert, N., Bernard, J., Lemonnier, F., Raposo, G., Escudier, B., Hsu, D.-H., Tursz, T., Amigorena, S., Angevin, E., Zitvogel, L., 2004. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J. Immunol. Baltim. Md* 172, 2126–2136. <https://doi.org/10.4049/jimmunol.172.4.2126>

Andreu, Z., Yáñez-Mó, M., 2014. Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* 5, 442. <https://doi.org/10.3389/fimmu.2014.00442>

Arab, T., Raffo-Romero, A., Van Camp, C., Lemaire, Q., Le Marrec-Croq, F., Drago, F., Aboulouard, S., Slomianny, C., Lacoste, A.-S., Guigon, I., Touzet, H., Salzet, M., Fournier, I., Lefebvre, C., Vizioli, J., Sautière, P.-E., 2019. Proteomic characterisation of leech microglia extracellular vesicles (EVs): comparison between differential ultracentrifugation and OptiprepTM density gradient isolation. *J. Extracell. Vesicles* 8, 1603048. <https://doi.org/10.1080/20013078.2019.1603048>

Arévalo, J.C., Wu, S.H., 2006. Neurotrophin signaling: many exciting surprises! *Cell. Mol. Life Sci. CMLS* 63, 1523–1537. <https://doi.org/10.1007/s00018-006-6010-1>

- Ascano, M., Bodmer, D., Kuruvilla, R., 2012. Endocytic trafficking of neurotrophins in neural development. *Trends Cell Biol.* 22, 266–273. <https://doi.org/10.1016/j.tcb.2012.02.005>
- Ascaño, M., Richmond, A., Borden, P., Kuruvilla, R., 2009. Axonal Targeting of Trk Receptors via Transcytosis Regulates Sensitivity to Neurotrophin Responses. *J. Neurosci.* 29, 11674–11685. <https://doi.org/10.1523/JNEUROSCI.1542-09.2009>
- Bamji, S.X., Majdan, M., Pozniak, C.D., Belliveau, D.J., Aloyz, R., Kohn, J., Causing, C.G., Miller, F.D., 1998. The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J. Cell Biol.* 140, 911–923. <https://doi.org/10.1083/jcb.140.4.911>
- Barker, P.A., 2007. High Affinity Not in the Vicinity? *Neuron* 53, 1–4. <https://doi.org/10.1016/j.neuron.2006.12.018>
- Bernimoulin, M., Waters, E.K., Foy, M., Steele, B.M., Sullivan, M., Falet, H., Walsh, M.T., Barteneva, N., Geng, J.-G., Hartwig, J.H., Maguire, P.B., Wagner, D.D., 2009. Differential stimulation of monocytic cells results in distinct populations of microparticles. *J. Thromb. Haemost. JTH* 7, 1019–1028. <https://doi.org/10.1111/j.1538-7836.2009.03434.x>
- Bibel, M., Hoppe, E., Barde, Y.A., 1999. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. *EMBO J.* 18, 616–622. <https://doi.org/10.1093/emboj/18.3.616>
- Bohlen, C.J., Bennett, F.C., Tucker, A.F., Collins, H.Y., Mulinyawe, S.B., Barres, B.A., 2017. Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures. *Neuron* 94, 759–773.e8. <https://doi.org/10.1016/j.neuron.2017.04.043>

Boriachek, K., Islam, M.N., Möller, A., Salomon, C., Nguyen, N.-T., Hossain, M.S.A., Yamauchi, Y., Shiddiky, M.J.A., 2018. Biological Functions and Current Advances in Isolation and Detection Strategies for Exosome Nanovesicles. *Small* Weinh. Bergstr. Ger. 14. <https://doi.org/10.1002/sml.201702153>

Bothwell, M., 2016. Recent advances in understanding neurotrophin signaling. *F1000Research* 5, F1000 Faculty Rev-1885. <https://doi.org/10.12688/f1000research.8434.1>

Brenna, S., Krisp, C., Altmeyden, H.C., Magnus, T., Puig, B., 2021. Brain-Derived Extracellular Vesicles in Health and Disease: A Methodological Perspective. *Int. J. Mol. Sci.* 22, 1365. <https://doi.org/10.3390/ijms22031365>

Budnik, V., Ruiz-Cañada, C., Wendler, F., 2016. Extracellular vesicles round off communication in the nervous system. *Nat. Rev. Neurosci.* 17, 160–172. <https://doi.org/10.1038/nrn.2015.29>

Busatto, S., Morad, G., Guo, P., Moses, M.A., 2021. The role of extracellular vesicles in the physiological and pathological regulation of the blood–brain barrier. *FASEB BioAdvances* 3, 665–675. <https://doi.org/10.1096/fba.2021-00045>

Capra, E., Lange-Consiglio, A., 2020. The Biological Function of Extracellular Vesicles during Fertilization, Early Embryo—Maternal Crosstalk and Their Involvement in Reproduction: Review and Overview. *Biomolecules* 10, 1510. <https://doi.org/10.3390/biom10111510>

Carter, N., Mathiesen, A.H., Miller, N., Brown, M., Colunga Biancatelli, R.M.L., Catravas, J.D., Dobrian, A.D., 2022. Endothelial cell-derived extracellular vesicles impair the angiogenic response of coronary artery

endothelial cells. *Front. Cardiovasc. Med.* 9, 923081. <https://doi.org/10.3389/fcvm.2022.923081>

Casaleto, J.B., McClatchey, A.I., 2012. Spatial regulation of receptor tyrosine kinases in development and cancer. *Nat. Rev. Cancer* 12, 387–400. <https://doi.org/10.1038/nrc3277>

Castellone, R.D., Leffler, N.R., Dong, L., Yang, L.V., 2011. Inhibition of tumor cell migration and metastasis by the proton-sensing GPR4 receptor. *Cancer Lett.* 312, 197–208. <https://doi.org/10.1016/j.canlet.2011.08.013>

Catalano, M., O'Driscoll, L., 2020. Inhibiting extracellular vesicles formation and release: a review of EV inhibitors. *J. Extracell. Vesicles* 9, 1703244. <https://doi.org/10.1080/20013078.2019.1703244>

Catalano, M., Serpe, C., Limatola, C., 2022. Microglial Extracellular Vesicles as Modulators of Brain Microenvironment in Glioma. *Int. J. Mol. Sci.* 23, 13165. <https://doi.org/10.3390/ijms232113165>

Ceccarelli, L., Giacomelli, C., Marchetti, L., Martini, C., 2021a. Microglia extracellular vesicles: focus on molecular composition and biological function. *Biochem. Soc. Trans.* 49, 1779–1790. <https://doi.org/10.1042/BST20210202>

Ceccarelli, L., Marchetti, L., Giacomelli, C., Martini, C., 2021b. Advances in microglia cellular models: focus on extracellular vesicle production. *Biochem. Soc. Trans.* 49, 1791–1802. <https://doi.org/10.1042/BST20210203>

Chalmin, F., Ladoire, S., Mignot, G., Vincent, J., Bruchard, M., Remy-Martin, J.-P., Boireau, W., Rouleau, A., Simon, B., Lanneau, D., De Thonel, A., Multhoff, G., Hamman, A., Martin, F., Chauffert, B., Solary, E., Zitvogel, L., Garrido, C., Ryffel, B., Borg, C., Apetoh, L., Rébé, C., Ghiringhelli, F., 2010. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-

dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J. Clin. Invest.* 120, 457–471. <https://doi.org/10.1172/JCI40483>

Chang, C., Lang, H., Geng, N., Wang, J., Li, N., Wang, X., 2013. Exosomes of BV-2 cells induced by alpha-synuclein: important mediator of neurodegeneration in PD. *Neurosci. Lett.* 548, 190–195. <https://doi.org/10.1016/j.neulet.2013.06.009>

Chao, M.V., 2003. Neurotrophins and their receptors: A convergence point for many signalling pathways. *Nat. Rev. Neurosci.* 4, 299–309. <https://doi.org/10.1038/nrn1078>

Chen, H., Wang, L., Zeng, X., Schwarz, H., Nanda, H.S., Peng, X., Zhou, Y., 2021. Exosomes, a New Star for Targeted Delivery. *Front. Cell Dev. Biol.* 9.

Chen, X., Ye, H., Kuruvilla, R., Ramanan, N., Scangos, K.W., Zhang, C., Johnson, N.M., England, P.M., Shokat, K.M., Ginty, D.D., 2005. A chemical-genetic approach to studying neurotrophin signaling. *Neuron* 46, 13–21. <https://doi.org/10.1016/j.neuron.2005.03.009>

Chowdary, P.D., Che, D.L., Cui, B., 2012. Neurotrophin signaling via long-distance axonal transport. *Annu. Rev. Phys. Chem.* 63, 571–594. <https://doi.org/10.1146/annurev-physchem-032511-143704>

Clary, D.O., Reichardt, L.F., 1994. An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. *Proc. Natl. Acad. Sci. U. S. A.* 91, 11133–11137. <https://doi.org/10.1073/pnas.91.23.11133>

Cocucci, E., Meldolesi, J., 2015. Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends Cell Biol.* 25, 364–372. <https://doi.org/10.1016/j.tcb.2015.01.004>

Colonna, M., Butovsky, O., 2017. Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu. Rev. Immunol.* 35, 441–468. <https://doi.org/10.1146/annurev-immunol-051116-052358>

Conroy, J.N., Coulson, E.J., 2022. High-affinity TrkA and p75 neurotrophin receptor complexes: A twisted affair. *J. Biol. Chem.* 298, 101568. <https://doi.org/10.1016/j.jbc.2022.101568>

Cortazzo, M.H., Kassis, E.S., Sproul, K.A., Schor, N.F., 1996. Nerve growth factor (NGF)-mediated protection of neural crest cells from antimitotic agent-induced apoptosis: the role of the low-affinity NGF receptor. *J. Neurosci. Off. J. Soc. Neurosci.* 16, 3895–3899. <https://doi.org/10.1523/JNEUROSCI.16-12-03895.1996>

Coulson, E.J., May, L.M., Osborne, S.L., Reid, K., Underwood, C.K., Meunier, F.A., Bartlett, P.F., Sah, P., 2008. p75 neurotrophin receptor mediates neuronal cell death by activating GIRK channels through phosphatidylinositol 4,5-bisphosphate. *J. Neurosci. Off. J. Soc. Neurosci.* 28, 315–324. <https://doi.org/10.1523/JNEUROSCI.2699-07.2008>

Covaceuszach, S., Konarev, P.V., Cassetta, A., Paoletti, F., Svergun, D.I., Lamba, D., Cattaneo, A., 2015. The Conundrum of the High-Affinity NGF Binding Site Formation Unveiled? *Biophys. J.* 108, 687–697. <https://doi.org/10.1016/j.bpj.2014.11.3485>

Cunha, C., Gomes, C., Vaz, A.R., Brites, D., 2016. Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. *Mediators Inflamm.* 2016, 6986175. <https://doi.org/10.1155/2016/6986175>

Da Pozzo, E., Tremolanti, C., Costa, B., Giacomelli, C., Milenkovic, V.M., Bader, S., Wetzels, C.H., Rupprecht, R., Taliani, S., Da Settimo, F., Martini, C., 2019. Microglial Pro-Inflammatory and Anti-Inflammatory Phenotypes Are Modulated by Translocator Protein Activation. *Int. J. Mol. Sci.* 20, 4467. <https://doi.org/10.3390/ijms20184467>

Davis, R.L., Buck, D.J., McCracken, K., Cox, G.W., Das, S., 2018. Interleukin-1 β -induced inflammatory signaling in C20 human microglial cells. *Neuroimmunol. Neuroinflammation* 5, 50. <https://doi.org/10.20517/2347-8659.2018.60>

Dd, G., Ra, S., 2002. Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr. Opin. Neurobiol.* 12. [https://doi.org/10.1016/s0959-4388\(02\)00326-4](https://doi.org/10.1016/s0959-4388(02)00326-4)

De Felice, M., Germelli, L., Piccarducci, R., Da Pozzo, E., Giacomelli, C., Baccaglioni-Frank, A., Martini, C., 2023. Intermittent hypoxia treatments cause cellular priming in human microglia. *J. Cell. Mol. Med.* <https://doi.org/10.1111/jcmm.17682>

de Jong, B., Barros, E.R., Hoenderop, J.G.J., Rigalli, J.P., 2020. Recent Advances in Extracellular Vesicles as Drug Delivery Systems and Their Potential in Precision Medicine. *Pharmaceutics* 12, 1006. <https://doi.org/10.3390/pharmaceutics12111006>

Dellar, E.R., Hill, C., Melling, G.E., Carter, D.R.F., Baena-Lopez, L.A., 2022. Unpacking extracellular vesicles: RNA cargo loading and function. *J. Extracell. Biol.* 1, e40. <https://doi.org/10.1002/jex2.40>

Dello Russo, C., Cappoli, N., Coletta, I., Mezzogori, D., Paciello, F., Pozzoli, G., Navarra, P., Battaglia, A., 2018. The human microglial HMC3 cell line:

where do we stand? A systematic literature review. *J. Neuroinflammation* 15, 259. <https://doi.org/10.1186/s12974-018-1288-0>

Delpech, J.-C., Herron, S., Botros, M.B., Ikezu, T., 2019. Neuroimmune crosstalk through extracellular vesicles in health and disease. *Trends Neurosci.* 42, 361–372. <https://doi.org/10.1016/j.tins.2019.02.007>

Deng, Z., Poliakov, A., Hardy, R.W., Clements, R., Liu, C., Liu, Y., Wang, J., Xiang, X., Zhang, S., Zhuang, X., Shah, S.V., Sun, D., Michalek, S., Grizzle, W.E., Garvey, T., Mobley, J., Zhang, H.-G., 2009. Adipose Tissue Exosome-Like Vesicles Mediate Activation of Macrophage-Induced Insulin Resistance. *Diabetes* 58, 2498–2505. <https://doi.org/10.2337/db09-0216>

Denkins, Y., Reiland, J., Roy, M., Sinnappah-Kang, N.D., Galjour, J., Murry, B.P., Blust, J., Aucoin, R., Marchetti, D., 2004. Brain metastases in melanoma: Roles of neurotrophins. *Neuro-Oncol.* 6, 154. <https://doi.org/10.1215/S115285170300067X>

Dimarino, A.M., Caplan, A.I., Bonfield, T.L., 2013. Mesenchymal stem cells in tissue repair. *Front. Immunol.* 4, 201. <https://doi.org/10.3389/fimmu.2013.00201>

Douvaras, P., Sun, B., Wang, M., Kruglikov, I., Lallo, G., Zimmer, M., Terrenoire, C., Zhang, B., Gandy, S., Schadt, E., Freytes, D.O., Noggle, S., Fossati, V., 2017. Directed Differentiation of Human Pluripotent Stem Cells to Microglia. *Stem Cell Rep.* 8, 1516–1524. <https://doi.org/10.1016/j.stemcr.2017.04.023>

Drago, F., Lombardi, M., Prada, I., Gabrielli, M., Joshi, P., Cojoc, D., Franck, J., Fournier, I., Vizioli, J., Verderio, C., 2017. ATP Modifies the Proteome of Extracellular Vesicles Released by Microglia and Influences Their Action on

Astrocytes. Front. Pharmacol. 8, 910.
<https://doi.org/10.3389/fphar.2017.00910>

EL Andaloussi, S., Mäger, I., Breakefield, X.O., Wood, M.J.A., 2013. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* 12, 347–357. <https://doi.org/10.1038/nrd3978>

Escudero, C.A., Lazo, O.M., Galleguillos, C., Parraguez, J.I., Lopez-Verrilli, M.A., Cabeza, C., Leon, L., Saeed, U., Retamal, C., Gonzalez, A., Marzolo, M.-P., Carter, B.D., Court, F.A., Bronfman, F.C., 2014. The p75 neurotrophin receptor evades the endolysosomal route in neuronal cells, favouring multivesicular bodies specialised for exosomal release. *J. Cell Sci.* 127, 1966–1979. <https://doi.org/10.1242/jcs.141754>

Fabbiano, F., Corsi, J., Gurrieri, E., Trevisan, C., Notarangelo, M., D’Agostino, V.G., 2020. RNA packaging into extracellular vesicles: An orchestra of RNA-binding proteins? *J. Extracell. Vesicles* 10, e12043. <https://doi.org/10.1002/jev2.12043>

Fahnestock, M., Shekari, A., 2019. ProNGF and Neurodegeneration in Alzheimer’s Disease. *Front. Neurosci.* 13.

Fan, Y., Xie, L., Chung, C.Y., 2017. Signaling Pathways Controlling Microglia Chemotaxis. *Mol. Cells* 40, 163–168. <https://doi.org/10.14348/molcells.2017.0011>

Felsky, D., Roostaei, T., Nho, K., Risacher, S.L., Bradshaw, E.M., Petyuk, V., Schneider, J.A., Saykin, A., Bennett, D.A., De Jager, P.L., 2019. Neuropathological correlates and genetic architecture of microglial activation in elderly human brain. *Nat. Commun.* 10, 409. <https://doi.org/10.1038/s41467-018-08279-3>

Foehr, E.D., Lin, X., O'Mahony, A., Geleziunas, R., Bradshaw, R.A., Greene, W.C., 2000. NF-kappa B signaling promotes both cell survival and neurite process formation in nerve growth factor-stimulated PC12 cells. *J. Neurosci. Off. J. Soc. Neurosci.* 20, 7556–7563. <https://doi.org/10.1523/JNEUROSCI.20-20-07556.2000>

Franco, M.L., García-Carpio, I., Comaposada-Baró, R., Escribano-Saiz, J.J., Chávez-Gutiérrez, L., Vilar, M., 2021. TrkA-mediated endocytosis of p75-CTF prevents cholinergic neuron death upon γ -secretase inhibition. *Life Sci. Alliance* 4, e202000844. <https://doi.org/10.26508/lsa.202000844>

Frankel, E.B., Audhya, A., 2018. ESCRT-dependent cargo sorting at multivesicular endosomes. *Semin. Cell Dev. Biol.* 74, 4–10. <https://doi.org/10.1016/j.semcdb.2017.08.020>

Friedman, W.J., 2000. Neurotrophins induce death of hippocampal neurons via the p75 receptor. *J. Neurosci. Off. J. Soc. Neurosci.* 20, 6340–6346. <https://doi.org/10.1523/JNEUROSCI.20-17-06340.2000>

García-Corzo, L., Calatayud-Baselga, I., Casares-Crespo, L., Mora-Martínez, C., Julián Escribano-Saiz, J., Hortigüela, R., Asenjo-Martínez, A., Jordán-Pla, A., Ercoli, S., Flames, N., López-Alonso, V., Vilar, M., Mira, H., 2022. The transcription factor LEF1 interacts with NFIX and switches isoforms during adult hippocampal neural stem cell quiescence. *Front. Cell Dev. Biol.* 10.

García-Manrique, P., Matos, M., Gutiérrez, G., Pazos, C., Blanco-López, M.C., 2018. Therapeutic biomaterials based on extracellular vesicles: classification of bio-engineering and mimetic preparation routes. *J. Extracell. Vesicles* 7, 1422676. <https://doi.org/10.1080/20013078.2017.1422676>

Garcia-Mesa, Y., Jay, T.R., Checkley, M.A., Luttge, B., Dobrowolski, C., Valadkhan, S., Landreth, G.E., Karn, J., Alvarez-Carbonell, D., 2017.

Immortalization of primary microglia: a new platform to study HIV regulation in the central nervous system. *J. Neurovirol.* 23, 47–66. <https://doi.org/10.1007/s13365-016-0499-3>

Gassama, Y., Favereaux, A., 2021. Emerging Roles of Extracellular Vesicles in the Central Nervous System: Physiology, Pathology, and Therapeutic Perspectives. *Front. Cell. Neurosci.* 15.

Gehrmann, J., Matsumoto, Y., Kreutzberg, G.W., 1995. Microglia: intrinsic immune effector cell of the brain. *Brain Res. Brain Res. Rev.* 20, 269–287. [https://doi.org/10.1016/0165-0173\(94\)00015-h](https://doi.org/10.1016/0165-0173(94)00015-h)

Germelli, L., Da Pozzo, E., Giacomelli, C., Tremolanti, C., Marchetti, L., Wetzel, C.H., Barresi, E., Taliani, S., Da Settimo, F., Martini, C., Costa, B., 2021. De novo Neurosteroidogenesis in Human Microglia: Involvement of the 18 kDa Translocator Protein. *Int. J. Mol. Sci.* 22, 3115. <https://doi.org/10.3390/ijms22063115>

Giacomelli, C., Natali, L., Trincavelli, M.L., Daniele, S., Bertoli, A., Flamini, G., Braca, A., Martini, C., 2016. New insights into the anticancer activity of carnosol: p53 reactivation in the U87MG human glioblastoma cell line. *Int. J. Biochem. Cell Biol.* 74, 95–108. <https://doi.org/10.1016/j.biocel.2016.02.019>

Glebov, K., Löchner, M., Jabs, R., Lau, T., Merkel, O., Schloss, P., Steinhäuser, C., Walter, J., 2015. Serotonin stimulates secretion of exosomes from microglia cells. *Glia* 63, 626–634. <https://doi.org/10.1002/glia.22772>

Gogoleva, V.S., Drutskaya, M.S., Atretkhany, K.S.-N., 2019. [The Role of Microglia in the Homeostasis of the Central Nervous System and Neuroinflammation]. *Mol. Biol. (Mosk.)* 53, 790–798. <https://doi.org/10.1134/S0026898419050057>

Gong, Y., Cao, P., Yu, H., Jiang, T., 2008. Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. *Nature* 454, 789–793. <https://doi.org/10.1038/nature07089>

Gosselin, D., Link, V.M., Romanoski, C.E., Fonseca, G.J., Eichenfield, D.Z., Spann, N.J., Stender, J.D., Chun, H.B., Garner, H., Geissmann, F., Glass, C.K., 2014. Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. *Cell* 159, 1327–1340. <https://doi.org/10.1016/j.cell.2014.11.023>

Greco, V., Hannus, M., Eaton, S., 2001. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* 106, 633–645. [https://doi.org/10.1016/s0092-8674\(01\)00484-6](https://doi.org/10.1016/s0092-8674(01)00484-6)

Grimaldi, A., Serpe, C., Chece, G., Nigro, V., Sarra, A., Ruzicka, B., Relucenti, M., Familiari, G., Ruocco, G., Pascucci, G.R., Guerrieri, F., Limatola, C., Catalano, M., 2019. Microglia-Derived Microvesicles Affect Microglia Phenotype in Glioma. *Front. Cell. Neurosci.* 13, 41. <https://doi.org/10.3389/fncel.2019.00041>

Groot, M., Lee, H., 2020. Sorting Mechanisms for MicroRNAs into Extracellular Vesicles and Their Associated Diseases. *Cells* 9, 1044. <https://doi.org/10.3390/cells9041044>

Gurunathan, S., Kang, M.-H., Kim, J.-H., 2021. A Comprehensive Review on Factors Influences Biogenesis, Functions, Therapeutic and Clinical Implications of Exosomes. *Int. J. Nanomedicine* 16, 1281–1312. <https://doi.org/10.2147/IJN.S291956>

H, M., Z, A., H, S., Dc, L., S, J., A, C., J, S.E., R, H., Ma, M.-T., K, N., D, C., M, G., I, F., Fh, G., 2010. Signaling through BMPR-IA regulates quiescence

and long-term activity of neural stem cells in the adult hippocampus. *Cell Stem Cell* 7. <https://doi.org/10.1016/j.stem.2010.04.016>

Hallal, S., Mallawaarachy, D.M., Wei, H., Ebrahimkhani, S., Stringer, B.W., Day, B.W., Boyd, A.W., Guillemín, G.J., Buckland, M.E., Kaufman, K.L., 2019. Extracellular Vesicles Released by Glioblastoma Cells Stimulate Normal Astrocytes to Acquire a Tumor-Supportive Phenotype Via p53 and MYC Signaling Pathways. *Mol. Neurobiol.* 56, 4566–4581. <https://doi.org/10.1007/s12035-018-1385-1>

Hamanoue, M., Middleton, G., Wyatt, S., Jaffray, E., Hay, R.T., Davies, A.M., 1999. p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. *Mol. Cell. Neurosci.* 14, 28–40. <https://doi.org/10.1006/mcne.1999.0770>

Harrington, A.W., St Hillaire, C., Zweifel, L.S., Glebova, N.O., Philippidou, P., Halegoua, S., Ginty, D.D., 2011. Recruitment of actin modifiers to TrkA endosomes governs retrograde NGF signaling and survival. *Cell* 146, 421–434. <https://doi.org/10.1016/j.cell.2011.07.008>

Hasselmann, J., Blurton-Jones, M., 2020. Human iPSC-derived microglia: A growing toolset to study the brain's innate immune cells. *Glia* 68, 721–739. <https://doi.org/10.1002/glia.23781>

He, X., Garcia, K.C., 2004. Structure of Nerve Growth Factor Complexed with the Shared Neurotrophin Receptor p75. *Science* 304, 870–875. <https://doi.org/10.1126/science.1095190>

Hefti, F.F., Walicke, P.A., 2007. Neurotrophic Factors, in: *Handbook of Contemporary Neuropharmacology*. John Wiley & Sons, Ltd. <https://doi.org/10.1002/9780470101001.hcn053>

- Heidarzadeh, M., Gürsoy-Özdemir, Y., Kaya, M., Eslami Abriz, A., Zarebkohan, A., Rahbarghazi, R., Sokullu, E., 2021. Exosomal delivery of therapeutic modulators through the blood–brain barrier; promise and pitfalls. *Cell Biosci.* 11, 142. <https://doi.org/10.1186/s13578-021-00650-0>
- Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., Chao, M.V., 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* 350, 678–683. <https://doi.org/10.1038/350678a0>
- Hessvik, N.P., Llorente, A., 2018. Current knowledge on exosome biogenesis and release. *Cell. Mol. Life Sci. CMLS* 75, 193–208. <https://doi.org/10.1007/s00018-017-2595-9>
- Huang, S., Dong, M., Chen, Q., 2022. Tumor-Derived Exosomes and Their Role in Breast Cancer Metastasis. *Int. J. Mol. Sci.* 23, 13993. <https://doi.org/10.3390/ijms232213993>
- Hurwitz, S.N., Conlon, M.M., Rider, M.A., Brownstein, N.C., Meckes, D.G., 2016. Nanoparticle analysis sheds budding insights into genetic drivers of extracellular vesicle biogenesis. *J. Extracell. Vesicles* 5, 31295. <https://doi.org/10.3402/jev.v5.31295>
- Hwang, H.S., Kim, H., Han, G., Lee, J.W., Kim, K., Kwon, I.C., Yang, Y., Kim, S.H., 2021. Extracellular Vesicles as Potential Therapeutics for Inflammatory Diseases. *Int. J. Mol. Sci.* 22, 5487. <https://doi.org/10.3390/ijms22115487>
- J, J., V, G., Lf, R., Bb, R., 2002. Molecular kinetics of nerve growth factor receptor trafficking and activation. *J. Biol. Chem.* 277. <https://doi.org/10.1074/jbc.M202348200>

Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., Turbide, C., 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262, 9412–9420.

Juan, T., Fürthauer, M., 2018. Biogenesis and function of ESCRT-dependent extracellular vesicles. *Semin. Cell Dev. Biol.* 74, 66–77. <https://doi.org/10.1016/j.semcdb.2017.08.022>

Junger, W.G., 2008. Purinergic regulation of neutrophil chemotaxis. *Cell. Mol. Life Sci. CMLS* 65, 2528–2540. <https://doi.org/10.1007/s00018-008-8095-1>

Justus, C.R., Leffler, N., Ruiz-Echevarria, M., Yang, L.V., 2014. In vitro Cell Migration and Invasion Assays. *J. Vis. Exp. JoVE* 51046. <https://doi.org/10.3791/51046>

Kalluri, R., LeBleu, V.S., 2020. The biology, function, and biomedical applications of exosomes. *Science* 367, eaau6977. <https://doi.org/10.1126/science.aau6977>

Kamerkar, S., LeBleu, V.S., Sugimoto, H., Yang, S., Ruivo, C.F., Melo, S.A., Lee, J.J., Kalluri, R., 2017. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 546, 498–503. <https://doi.org/10.1038/nature22341>

Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V., Parada, L.F., 1991. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* 252, 554–558. <https://doi.org/10.1126/science.1850549>

Keller, S., Sanderson, M.P., Stoeck, A., Altevogt, P., 2006. Exosomes: from biogenesis and secretion to biological function. *Immunol. Lett.* 107, 102–108. <https://doi.org/10.1016/j.imlet.2006.09.005>

Kenchappa, R.S., Tep, C., Korade, Z., Urra, S., Bronfman, F.C., Yoon, S.O., Carter, B.D., 2010. p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-alpha-converting enzyme/ADAM17. *J. Biol. Chem.* 285, 20358–20368. <https://doi.org/10.1074/jbc.M109.082834>

Kenchappa, R.S., Zampieri, N., Chao, M.V., Barker, P.A., Teng, H.K., Hempstead, B.L., Carter, B.D., 2006. Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. *Neuron* 50, 219–232. <https://doi.org/10.1016/j.neuron.2006.03.011>

Kim, M.S., Suh, K.W., Hong, S., Jin, W., 2017. TrkC promotes colorectal cancer growth and metastasis. *Oncotarget* 8, 41319. <https://doi.org/10.18632/oncotarget.17289>

Kluszczyńska, K., Czyz, M., 2023. Extracellular Vesicles-Based Cell-Cell Communication in Melanoma: New Perspectives in Diagnostics and Therapy. *Int. J. Mol. Sci.* 24, 965. <https://doi.org/10.3390/ijms24020965>

Kofler, J., Wiley, C.A., 2011. Microglia: key innate immune cells of the brain. *Toxicol. Pathol.* 39, 103–114. <https://doi.org/10.1177/0192623310387619>

Kogure, A., Yoshioka, Y., Ochiya, T., 2020. Extracellular Vesicles in Cancer Metastasis: Potential as Therapeutic Targets and Materials. *Int. J. Mol. Sci.* 21, 4463. <https://doi.org/10.3390/ijms21124463>

Kordelas, L., Rebmann, V., Ludwig, A.-K., Radtke, S., Ruesing, J., Doeppner, T.R., Epple, M., Horn, P.A., Beelen, D.W., Giebel, B., 2014. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 28, 970–973. <https://doi.org/10.1038/leu.2014.41>

- Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J.P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., Théry, C., 2016. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl. Acad. Sci. U. S. A.* 113, E968-977. <https://doi.org/10.1073/pnas.1521230113>
- Kumar, Alok, Stoica, B.A., Loane, D.J., Yang, M., Abulwerdi, G., Khan, N., Kumar, Asit, Thom, S.R., Faden, A.I., 2017. Microglial-derived microparticles mediate neuroinflammation after traumatic brain injury. *J. Neuroinflammation* 14, 47. <https://doi.org/10.1186/s12974-017-0819-4>
- Kumar, S., Matthews, Q.L., Sims, B., 2020. Effects of Cocaine on Human Glial-Derived Extracellular Vesicles. *Front. Cell Dev. Biol.* 8, 563441. <https://doi.org/10.3389/fcell.2020.563441>
- Langevin, C., Jaaro, H., Bressanelli, S., Fainzilber, M., Tuffereau, C., 2002. Rabies virus glycoprotein (RVG) is a trimeric ligand for the N-terminal cysteine-rich domain of the mammalian p75 neurotrophin receptor. *J. Biol. Chem.* 277, 37655–37662. <https://doi.org/10.1074/jbc.M201374200>
- Lazennec, G., Richmond, A., 2010. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol. Med.* 16, 133–144. <https://doi.org/10.1016/j.molmed.2010.01.003>
- Lee, K.F., Davies, A.M., Jaenisch, R., 1994. p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* 120, 1027–1033. <https://doi.org/10.1242/dev.120.4.1027>
- Lee, Y., Lee, S.-R., Choi, S.S., Yeo, H.-G., Chang, K.-T., Lee, H.J., 2014. Therapeutically targeting neuroinflammation and microglia after acute

ischemic stroke. *BioMed Res. Int.* 2014, 297241.
<https://doi.org/10.1155/2014/297241>

Leloup, N., Chataigner, L.M.P., Janssen, B.J.C., 2018. Structural insights into SorCS2-Nerve Growth Factor complex formation. *Nat. Commun.* 9, 2979.
<https://doi.org/10.1038/s41467-018-05405-z>

Leng, F., Edison, P., 2021. Neuroinflammation and microglial activation in Alzheimer disease: where do we go from here? *Nat. Rev. Neurol.* 17, 157–172.
<https://doi.org/10.1038/s41582-020-00435-y>

Li, B., Yang, C., Zhu, Z., Chen, H., Qi, B., 2022. Hypoxic glioma-derived extracellular vesicles harboring MicroRNA-10b-5p enhance M2 polarization of macrophages to promote the development of glioma. *CNS Neurosci. Ther.* 28, 1733–1747. <https://doi.org/10.1111/cns.13905>

Li, Q., Barres, B.A., 2018. Microglia and macrophages in brain homeostasis and disease. *Nat. Rev. Immunol.* 18, 225–242.
<https://doi.org/10.1038/nri.2017.125>

Li, S., Lin, Z., Jiang, X., Yu, X., 2018. Exosomal cargo-loading and synthetic exosome-mimics as potential therapeutic tools. *Acta Pharmacol. Sin.* 39, 542–551. <https://doi.org/10.1038/aps.2017.178>

Li, T.-R., Wang, X.-N., Sheng, C., Li, Y.-X., Li, F.Z.-T., Sun, Y., Han, Y., 2019. Extracellular vesicles as an emerging tool for the early detection of Alzheimer's disease. *Mech. Ageing Dev.* 184, 111175.
<https://doi.org/10.1016/j.mad.2019.111175>

Lie, D.-C., Colamarino, S.A., Song, H.-J., Désiré, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R., Gage, F.H., 2005. Wnt

signalling regulates adult hippocampal neurogenesis. *Nature* 437, 1370–1375.
<https://doi.org/10.1038/nature04108>

Linggi, M.S., Burke, T.L., Williams, B.B., Harrington, A., Kraemer, R., Hempstead, B.L., Yoon, S.O., Carter, B.D., 2005. Neurotrophin receptor interacting factor (NRIF) is an essential mediator of apoptotic signaling by the p75 neurotrophin receptor. *J. Biol. Chem.* 280, 13801–13808.
<https://doi.org/10.1074/jbc.M410435200>

Liu, J., Gao, H., Wang, X., 2015. The role of the Rho/ROCK signaling pathway in inhibiting axonal regeneration in the central nervous system. *Neural Regen. Res.* 10, 1892–1896. <https://doi.org/10.4103/1673-5374.170325>

Lively, S., Schlichter, L.C., 2013. The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J. Neuroinflammation* 10, 843. <https://doi.org/10.1186/1742-2094-10-75>

Lötvall, J., Hill, A.F., Hochberg, F., Buzás, E.I., Di Vizio, D., Gardiner, C., Ghossein, Y.S., Kurochkin, I.V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M.H., Witwer, K.W., Théry, C., 2014. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J. Extracell. Vesicles* 3, 10.3402/jev.v3.26913.
<https://doi.org/10.3402/jev.v3.26913>

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275. [https://doi.org/10.1016/s0021-9258\(19\)52451-6](https://doi.org/10.1016/s0021-9258(19)52451-6)

Maiolo, D., Paolini, L., Di Noto, G., Zendrini, A., Berti, D., Bergese, P., Ricotta, D., 2015. Colorimetric nanoplasmonic assay to determine purity and

titrate extracellular vesicles. *Anal. Chem.* 87, 4168–4176.
<https://doi.org/10.1021/ac504861d>

Man, K., Brunet, M.Y., Jones, M.-C., Cox, S.C., 2020. Engineered Extracellular Vesicles: Tailored-Made Nanomaterials for Medical Applications. *Nanomater. Basel Switz.* 10, 1838.
<https://doi.org/10.3390/nano10091838>

Marchetti, L., Bonsignore, F., Gobbo, F., Amodeo, R., Calvello, M., Jacob, A., Signore, G., Schirripa Spagnolo, C., Porciani, D., Mainardi, M., Beltram, F., Luin, S., Cattaneo, A., 2019a. Fast-diffusing p75NTR monomers support apoptosis and growth cone collapse by neurotrophin ligands. *Proc. Natl. Acad. Sci.* 116, 21563–21572. <https://doi.org/10.1073/pnas.1902790116>

Marchetti, L., Bonsignore, F., Gobbo, F., Amodeo, R., Calvello, M., Jacob, A., Signore, G., Spagnolo, C.S., Porciani, D., Mainardi, M., Beltram, F., Luin, S., Cattaneo, A., 2019b. Fast-diffusing p75NTR monomers support apoptosis and growth cone collapse by neurotrophin ligands. *Proc. Natl. Acad. Sci. U. S. A.* 116, 21563–21572. <https://doi.org/10.1073/pnas.1902790116>

Marchetti, L., Luin, S., Bonsignore, F., de Nadai, T., Beltram, F., Cattaneo, A., 2015. Ligand-induced dynamics of neurotrophin receptors investigated by single-molecule imaging approaches. *Int. J. Mol. Sci.* 16, 1949–1979.
<https://doi.org/10.3390/ijms16011949>

Marrone, M.C., Morabito, A., Giustizieri, M., Chiurchiù, V., Leuti, A., Mattioli, M., Marinelli, Sara, Riganti, L., Lombardi, M., Murana, E., Totaro, A., Piomelli, D., Ragozzino, D., Oddi, S., Maccarrone, M., Verderio, C., Marinelli, Silvia, 2017. TRPV1 channels are critical brain inflammation detectors and neuropathic pain biomarkers in mice. *Nat. Commun.* 8, 15292.
<https://doi.org/10.1038/ncomms15292>

- Mathieu, M., Martin-Jaular, L., Lavieu, G., Théry, C., 2019. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol.* 21, 9–17. <https://doi.org/10.1038/s41556-018-0250-9>
- Meeker, R.B., Williams, K.S., 2015. The p75 neurotrophin receptor: at the crossroad of neural repair and death. *Neural Regen. Res.* 10, 721–725. <https://doi.org/10.4103/1673-5374.156967>
- Men, Y., Yelick, J., Jin, S., Tian, Y., Chiang, M.S.R., Higashimori, H., Brown, E., Jarvis, R., Yang, Y., 2019. Exosome reporter mice reveal the involvement of exosomes in mediating neuron to astroglia communication in the CNS. *Nat. Commun.* 10, 4136. <https://doi.org/10.1038/s41467-019-11534-w>
- Mira, H., Andreu, Z., Suh, H., Lie, D.C., Jessberger, S., Consiglio, A., San Emeterio, J., Hortigüela, R., Marqués-Torrejón, M.A., Nakashima, K., Colak, D., Götz, M., Fariñas, I., Gage, F.H., 2010. Signaling through BMPR-IA regulates quiescence and long-term activity of neural stem cells in the adult hippocampus. *Cell Stem Cell* 7, 78–89. <https://doi.org/10.1016/j.stem.2010.04.016>
- Monfared, H., Jahangard, Y., Nikkhah, M., Mirnajafi-Zadeh, J., Mowla, S.J., 2019. Potential Therapeutic Effects of Exosomes Packed With a miR-21-Sponge Construct in a Rat Model of Glioblastoma. *Front. Oncol.* 9, 782. <https://doi.org/10.3389/fonc.2019.00782>
- Montis, C., Caselli, L., Valle, F., Zendrini, A., Carlà, F., Schweins, R., Maccarini, M., Bergese, P., Berti, D., 2020. Shedding light on membrane-templated clustering of gold nanoparticles. *J. Colloid Interface Sci.* 573, 204–214. <https://doi.org/10.1016/j.jcis.2020.03.123>

- Moscardini, A., Di Pietro, S., Signore, G., Parlanti, P., Santi, M., Gemmi, M., Cappello, V., 2020. Uranium-free X solution: a new generation contrast agent for biological samples ultrastructure. *Sci. Rep.* 10, 1–11. <https://doi.org/10.1038/s41598-020-68405-4>
- Mulcahy, L.A., Pink, R.C., Carter, D.R.F., 2014. Routes and mechanisms of extracellular vesicle uptake. *J. Extracell. Vesicles* 3, 10.3402/jev.v3.24641. <https://doi.org/10.3402/jev.v3.24641>
- Murgoci, A.-N., Cizkova, D., Majerova, P., Petrovova, E., Medvecký, L., Fournier, I., Salzert, M., 2018. Brain-Cortex Microglia-Derived Exosomes: Nanoparticles for Glioma Therapy. *Chemphyschem Eur. J. Chem. Phys. Phys. Chem.* 19, 1205–1214. <https://doi.org/10.1002/cphc.201701198>
- Muriel, O., Tomas, A., Scott, C.C., Gruenberg, J., 2016. Moesin and cortactin control actin-dependent multivesicular endosome biogenesis. *Mol. Biol. Cell* 27, 3305–3316. <https://doi.org/10.1091/mbc.E15-12-0853>
- Nayak, D., Roth, T.L., McGavern, D.B., 2014. Microglia development and function. *Annu. Rev. Immunol.* 32, 367–402. <https://doi.org/10.1146/annurev-immunol-032713-120240>
- Norden, D.M., Godbout, J.P., 2013. Review: microglia of the aged brain: primed to be activated and resistant to regulation. *Neuropathol. Appl. Neurobiol.* 39, 19–34. <https://doi.org/10.1111/j.1365-2990.2012.01306.x>
- Norden, D.M., Muccigrosso, M.M., Godbout, J.P., 2015. Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. *Neuropharmacology* 96, 29–41. <https://doi.org/10.1016/j.neuropharm.2014.10.028>

- O'Brien, K., Breyne, K., Ughetto, S., Laurent, L.C., Breakefield, X.O., 2020. RNA delivery by extracellular vesicles in mammalian cells and its applications. *Nat. Rev. Mol. Cell Biol.* 21, 585–606. <https://doi.org/10.1038/s41580-020-0251-y>
- Ohno, S.-I., Drummen, G.P.C., Kuroda, M., 2016. Focus on Extracellular Vesicles: Development of Extracellular Vesicle-Based Therapeutic Systems. *Int. J. Mol. Sci.* 17, 172. <https://doi.org/10.3390/ijms17020172>
- Palmer, T.D., Takahashi, J., Gage, F.H., 1997. The adult rat hippocampus contains primordial neural stem cells. *Mol. Cell. Neurosci.* 8, 389–404. <https://doi.org/10.1006/mcne.1996.0595>
- Pancholi, S., Tripathi, A., Bhan, A., Acharya, M.M., Pillai, P., 2022. Emerging Concepts on the Role of Extracellular Vesicles and Its Cargo Contents in Glioblastoma-Microglial Crosstalk. *Mol. Neurobiol.* 59, 2822–2837. <https://doi.org/10.1007/s12035-022-02752-3>
- Paolicelli, R.C., Bergamini, G., Rajendran, L., 2019. Cell-to-cell Communication by Extracellular Vesicles: Focus on Microglia. *Neuroscience* 405, 148–157. <https://doi.org/10.1016/j.neuroscience.2018.04.003>
- Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Mito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A., Colone, M., Tatti, M., Sargiacomo, M., Fais, S., 2009. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J. Biol. Chem.* 284, 34211–34222. <https://doi.org/10.1074/jbc.M109.041152>
- Pascucci, L., Scattini, G., 2021. Imaging extracellular vesicles by transmission electron microscopy: Coping with technical hurdles and morphological interpretation. *Biochim. Biophys. Acta Gen. Subj.* 1865, 129648. <https://doi.org/10.1016/j.bbagen.2020.129648>

Pazyra-Murphy, M.F., Hans, A., Courchesne, S.L., Karch, C., Cosker, K.E., Heerssen, H.M., Watson, F.L., Kim, T., Greenberg, M.E., Segal, R.A., 2009. A retrograde neuronal survival response: target-derived neurotrophins regulate MEF2D and bcl-w. *J. Neurosci. Off. J. Soc. Neurosci.* 29, 6700–6709. <https://doi.org/10.1523/JNEUROSCI.0233-09.2009>

Peng, S., Wu, J., Mufson, E.J., Fahnestock, M., 2004. Increased proNGF Levels in Subjects with Mild Cognitive Impairment and Mild Alzheimer Disease. *J. Neuropathol. Exp. Neurol.* 63, 641–649. <https://doi.org/10.1093/jnen/63.6.641>

Peter, C., Waibel, M., Radu, C.G., Yang, L.V., Witte, O.N., Schulze-Osthoff, K., Wesselborg, S., Lauber, K., 2008. Migration to apoptotic “find-me” signals is mediated via the phagocyte receptor G2A. *J. Biol. Chem.* 283, 5296–5305. <https://doi.org/10.1074/jbc.M706586200>

Pinet, S., Bessette, B., Vedrenne, N., Lacroix, A., Richard, L., Jauberteau, M.-O., Battu, S., Lalloué, F., 2016. TrkB-containing exosomes promote the transfer of glioblastoma aggressiveness to YKL-40-inactivated glioblastoma cells. *Oncotarget* 7, 50349–50364. <https://doi.org/10.18632/oncotarget.10387>

Radu, C.G., Yang, L.V., Riedinger, M., Au, M., Witte, O.N., 2004. T cell chemotaxis to lysophosphatidylcholine through the G2A receptor. *Proc. Natl. Acad. Sci. U. S. A.* 101, 245–250. <https://doi.org/10.1073/pnas.2536801100>

Rajendran, L., Bali, J., Barr, M.M., Court, F.A., Krämer-Albers, E.-M., Picou, F., Raposo, G., van der Vos, K.E., van Niel, G., Wang, J., Breakefield, X.O., 2014. Emerging Roles of Extracellular Vesicles in the Nervous System. *J. Neurosci.* 34, 15482–15489. <https://doi.org/10.1523/JNEUROSCI.3258-14.2014>

Ramasubramanian, L., Kumar, P., Wang, A., 2019. Engineering Extracellular Vesicles as Nanotherapeutics for Regenerative Medicine. *Biomolecules* 10, 48. <https://doi.org/10.3390/biom10010048>

Ramirez, M.I., Amorim, M.G., Gadelha, C., Milic, I., Welsh, J.A., Freitas, V.M., Nawaz, M., Akbar, N., Couch, Y., Makin, L., Cooke, F., Vettore, A.L., Batista, P.X., Freezor, R., Pezuk, J.A., Rosa-Fernandes, L., Carreira, A.C.O., Devitt, A., Jacobs, L., Silva, I.T., Coakley, G., Nunes, D.N., Carter, D., Palmisano, G., Dias-Neto, E., 2018. Technical challenges of working with extracellular vesicles. *Nanoscale* 10, 881–906. <https://doi.org/10.1039/c7nr08360b>

Ransohoff, R.M., 2016. How neuroinflammation contributes to neurodegeneration. *Science* 353, 777–783. <https://doi.org/10.1126/science.aag2590>

Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V., Melief, C.J., Geuze, H.J., 1996. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* 183, 1161–1172. <https://doi.org/10.1084/jem.183.3.1161>

Raposo, G., Stoorvogel, W., 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383. <https://doi.org/10.1083/jcb.201211138>

Reichardt, L.F., 2006. Neurotrophin-regulated signalling pathways. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 361, 1545–1564. <https://doi.org/10.1098/rstb.2006.1894>

Rm, S., Dm, L., Td, C., T, P., La, G., Dr, K., 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. *Neuron* 12. [https://doi.org/10.1016/0896-6273\(94\)90223-2](https://doi.org/10.1016/0896-6273(94)90223-2)

- Robbins, P.D., Morelli, A.E., 2014. Regulation of Immune Responses by Extracellular Vesicles. *Nat. Rev. Immunol.* 14, 195–208. <https://doi.org/10.1038/nri3622>
- Rodríguez-Tébar, A., Dechant, G., Götz, R., Barde, Y.A., 1992. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* 11, 917–922. <https://doi.org/10.1002/j.1460-2075.1992.tb05130.x>
- Roux, P.P., Barker, P.A., 2002. Neurotrophin signaling through the p75 neurotrophin receptor. *Prog. Neurobiol.* 67, 203–233. [https://doi.org/10.1016/s0301-0082\(02\)00016-3](https://doi.org/10.1016/s0301-0082(02)00016-3)
- Ruppert, K.A., Nguyen, T.T., Prabhakara, K.S., Toledano Furman, N.E., Srivastava, A.K., Harting, M.T., Cox, C.S., Olson, S.D., 2018. Human Mesenchymal Stromal Cell-Derived Extracellular Vesicles Modify Microglial Response and Improve Clinical Outcomes in Experimental Spinal Cord Injury. *Sci. Rep.* 8, 480. <https://doi.org/10.1038/s41598-017-18867-w>
- Salvi, V., Sozio, F., Sozzani, S., Del Prete, A., 2017. Role of Atypical Chemokine Receptors in Microglial Activation and Polarization. *Front. Aging Neurosci.* 9, 148. <https://doi.org/10.3389/fnagi.2017.00148>
- Sanwlani, R., Gangoda, L., 2021. Role of Extracellular Vesicles in Cell Death and Inflammation. *Cells* 10, 2663. <https://doi.org/10.3390/cells10102663>
- Savina, A., Furlán, M., Vidal, M., Colombo, M.I., 2003. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J. Biol. Chem.* 278, 20083–20090. <https://doi.org/10.1074/jbc.M301642200>
- Schachtrup, C., Ryu, J.K., Mammadzada, K., Khan, A.S., Carlton, P.M., Perez, A., Christian, F., Le Moan, N., Vagena, E., Baeza-Raja, B., Rafalski, V., Chan,

J.P., Nitschke, R., Houslay, M.D., Ellisman, M.H., Wyss-Coray, T., Palop, J.J., Akassoglou, K., 2015. Nuclear pore complex remodeling by p75(NTR) cleavage controls TGF- β signaling and astrocyte functions. *Nat. Neurosci.* 18, 1077–1080. <https://doi.org/10.1038/nn.4054>

Schain, M., Kreisl, W.C., 2017. Neuroinflammation in Neurodegenerative Disorders-a Review. *Curr. Neurol. Neurosci. Rep.* 17, 25. <https://doi.org/10.1007/s11910-017-0733-2>

Schmidt, S.I., Blaabjerg, M., Freude, K., Meyer, M., 2022. RhoA Signaling in Neurodegenerative Diseases. *Cells* 11, 1520. <https://doi.org/10.3390/cells11091520>

Serpe, C., Monaco, L., Relucenti, M., Iovino, L., Familiari, P., Scavizzi, F., Raspa, M., Familiari, G., Civiero, L., D'Agnano, I., Limatola, C., Catalano, M., 2021. Microglia-Derived Small Extracellular Vesicles Reduce Glioma Growth by Modifying Tumor Cell Metabolism and Enhancing Glutamate Clearance through miR-124. *Cells* 10, 2066. <https://doi.org/10.3390/cells10082066>

Shaimardanova, A.A., Solovyeva, V.V., Chulpanova, D.S., James, V., Kitaeva, K.V., Rizvanov, A.A., 2020. Extracellular vesicles in the diagnosis and treatment of central nervous system diseases. *Neural Regen. Res.* 15, 586–596. <https://doi.org/10.4103/1673-5374.266908>

Sheybani, N.D., Batts, A.J., Mathew, A.S., Thim, E.A., Price, R.J., 2020. Focused Ultrasound Hyperthermia Augments Release of Glioma-derived Extracellular Vesicles with Differential Immunomodulatory Capacity. *Theranostics* 10, 7436–7447. <https://doi.org/10.7150/thno.46534>

Shi, J., Zhang, Y., Yao, B., Sun, P., Hao, Y., Piao, H., Zhao, X., 2020. Role of Exosomes in the Progression, Diagnosis, and Treatment of Gliomas. *Med. Sci.*

Monit. Int. Med. J. Exp. Clin. Res. 26, e924023.
<https://doi.org/10.12659/MSM.924023>

Sierra, A., de Castro, F., Del Río-Hortega, J., Rafael Iglesias-Rozas, J., Garrosa, M., Kettenmann, H., 2016. The “Big-Bang” for modern glial biology: Translation and comments on Pío del Río-Hortega 1919 series of papers on microglia. *Glia* 64, 1801–1840. <https://doi.org/10.1002/glia.23046>

Skeldal, S., Sykes, A.M., Glerup, S., Matusica, D., Palstra, N., Autio, H., Boskovic, Z., Madsen, P., Castrén, E., Nykjaer, A., Coulson, E.J., 2012. Mapping of the Interaction Site between Sortilin and the p75 Neurotrophin Receptor Reveals a Regulatory Role for the Sortilin Intracellular Domain in p75 Neurotrophin Receptor Shedding and Apoptosis *. *J. Biol. Chem.* 287, 43798–43809. <https://doi.org/10.1074/jbc.M112.374710>

Skog, J., Würdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T., Carter, B.S., Krichevsky, A.M., Breakefield, X.O., 2008. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476. <https://doi.org/10.1038/ncb1800>

Smith, T.I., Russell, A.E., 2022. Extracellular vesicles in reproduction and pregnancy. *Extracell. Vesicles Circ. Nucleic Acids* 3, 275–300. <https://doi.org/10.20517/evcna.2022.27>

Soligo, M., Albin, M., Bertoli, F.L., Marzano, V., Protto, V., Bracci-Laudiero, L., Minnone, G., De Benedetti, F., Chiaretti, A., Mantuano, E., Manni, L., 2019. Different responses of PC12 cells to different pro-nerve growth factor protein variants. *Neurochem. Int.* 129, 104498. <https://doi.org/10.1016/j.neuint.2019.104498>

Sørensen, M.D., Dahlrot, R.H., Boldt, H.B., Hansen, S., Kristensen, B.W., 2018. Tumour-associated microglia/macrophages predict poor prognosis in high-grade gliomas and correlate with an aggressive tumour subtype. *Neuropathol. Appl. Neurobiol.* 44, 185–206. <https://doi.org/10.1111/nan.12428>

Sowers, J.L., Johnson, K.M., Conrad, C., Patterson, J.T., Sowers, L.C., 2014. The Role of Inflammation in Brain Cancer, in: Aggarwal, B.B., Sung, B., Gupta, S.C. (Eds.), *Inflammation and Cancer, Advances in Experimental Medicine and Biology*. Springer, Basel, pp. 75–105. https://doi.org/10.1007/978-3-0348-0837-8_4

Sproviero, D., La Salvia, S., Giannini, M., Crippa, V., Gagliardi, S., Bernuzzi, S., Diamanti, L., Ceroni, M., Pansarasa, O., Poletti, A., Cereda, C., 2018. Pathological Proteins Are Transported by Extracellular Vesicles of Sporadic Amyotrophic Lateral Sclerosis Patients. *Front. Neurosci.* 12.

Steinbichler, T.B., Dudás, J., Riechelmann, H., Skvortsova, I.-I., 2017. The role of exosomes in cancer metastasis. *Semin. Cancer Biol.* 44, 170–181. <https://doi.org/10.1016/j.semcancer.2017.02.006>

Stetefeld, J., McKenna, S.A., Patel, T.R., 2016. Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys. Rev.* <https://doi.org/10.1007/s12551-016-0218-6>

Stevens, B., Allen, N.J., Vazquez, L.E., Howell, G.R., Christopherson, K.S., Nouri, N., Micheva, K.D., Mehalow, A.K., Huberman, A.D., Stafford, B., Sher, A., Litke, A.M., Lambris, J.D., Smith, S.J., John, S.W.M., Barres, B.A., 2007. The classical complement cascade mediates CNS synapse elimination. *Cell* 131, 1164–1178. <https://doi.org/10.1016/j.cell.2007.10.036>

Stuffers, S., Sem Wegner, C., Stenmark, H., Brech, A., 2009. Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic Cph. Den.* 10, 925–937. <https://doi.org/10.1111/j.1600-0854.2009.00920.x>

Sullivan, R., 2015. Epididymosomes: a heterogeneous population of microvesicles with multiple functions in sperm maturation and storage. *Asian J. Androl.* 17, 726–729. <https://doi.org/10.4103/1008-682X.155255>

Sun, H., Burrola, S., Wu, J., Ding, W.-Q., 2020. Extracellular Vesicles in the Development of Cancer Therapeutics. *Int. J. Mol. Sci.* 21, 6097. <https://doi.org/10.3390/ijms21176097>

Sun, Y., Lim, Y., Li, F., Liu, S., Lu, J.-J., Haberberger, R., Zhong, J.-H., Zhou, X.-F., 2012. ProBDNF Collapses Neurite Outgrowth of Primary Neurons by Activating RhoA. *PLOS ONE* 7, e35883. <https://doi.org/10.1371/journal.pone.0035883>

Sung, B.H., Weaver, A.M., 2017. Exosome secretion promotes chemotaxis of cancer cells. *Cell Adhes. Migr.* 11, 187–195. <https://doi.org/10.1080/19336918.2016.1273307>

Suo, D., Park, J., Harrington, A.W., Zweifel, L.S., Mihalas, S., Deppmann, C.D., 2014. Coronin-1 is a neurotrophin endosomal effector that is required for developmental competition for survival. *Nat. Neurosci.* 17, 36–45. <https://doi.org/10.1038/nn.3593>

Takenouchi, T., Tsukimoto, M., Iwamaru, Y., Sugama, S., Sekiyama, K., Sato, M., Kojima, S., Hashimoto, M., Kitani, H., 2015. Extracellular ATP induces unconventional release of glyceraldehyde-3-phosphate dehydrogenase from microglial cells. *Immunol. Lett.* 167, 116–124. <https://doi.org/10.1016/j.imlet.2015.08.002>

Testa, G., Mainardi, M., Vannini, E., Pancrazi, L., Cattaneo, A., Costa, M., 2022. Disentangling the signaling complexity of nerve growth factor receptors by CRISPR/Cas9. *FASEB J.* 36, e22498. <https://doi.org/10.1096/fj.202101760RR>

Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G.K., Ayre, D.C., Bach, J.M., Bachurski, D., Baharvand, H., Balaj, L., Baldacchino, S., Bauer, N.N., Baxter, A.A., Bebawy, M., Beckham, C., Bedina Zavec, A., Benmoussa, A., Berardi, A.C., Bergese, P., Bielska, E., Blenkiron, C., Bobis-Wozowicz, S., Boilard, E., Boireau, W., Bongiovanni, A., Borràs, F.E., Bosch, S., Boulanger, C.M., Breakefield, X., Breglio, A.M., Brennan, M., Brigstock, D.R., Brisson, A., Broekman, M.L.D., Bromberg, J.F., Bryl-Górecka, P., Buch, S., Buck, A.H., Burger, D., Busatto, S., Buschmann, D., Bussolati, B., Buzás, E.I., Byrd, J.B., Camussi, G., Carter, D.R.F., Caruso, S., Chamley, L.W., Chang, Y.T., Chaudhuri, A.D., Chen, C., Chen, S., Cheng, L., Chin, A.R., Clayton, A., Clerici, S.P., Cocks, A., Cocucci, E., Coffey, R.J., Cordeiro-da-Silva, A., Couch, Y., Coumans, F.A.W., Coyle, B., Crescitelli, R., Criado, M.F., D'Souza-Schorey, C., Das, S., de Candia, P., De Santana, E.F., De Wever, O., del Portillo, H.A., Demaret, T., Deville, S., Devitt, A., Dhondt, B., Di Vizio, D., Dieterich, L.C., Dolo, V., Dominguez Rubio, A.P., Dominici, M., Dourado, M.R., Driedonks, T.A.P., Duarte, F. V., Duncan, H.M., Eichenberger, R.M., Ekström, K., EL Andaloussi, S., Elie-Caille, C., Erdbrügger, U., Falcón-Pérez, J.M., Fatima, F., Fish, J.E., Flores-Bellver, M., Försönits, A., Frelet-Barrand, A., Fricke, F., Fuhrmann, G., Gabrielsson, S., Gámez-Valero, A., Gardiner, C., Gärtner, K., Gaudin, R., Ghossein, Y.S., Giebel, B., Gilbert, C., Gimona, M., Giusti, I., Goberdhan, D.C.I., Görgens, A., Gorski, S.M., Greening, D.W., Gross, J.C., Gualerzi, A., Gupta, G.N., Gustafson, D., Handberg, A., Haraszti, R.A., Harrison, P., Hegyesi, H., Hendrix, A., Hill,

A.F., Hochberg, F.H., Hoffmann, K.F., Holder, B., Holthofer, H., Hosseinkhani, B., Hu, G., Huang, Y., Huber, V., Hunt, S., Ibrahim, A.G.E., Ikezu, T., Inal, J.M., Isin, M., Ivanova, A., Jackson, H.K., Jacobsen, S., Jay, S.M., Jayachandran, M., Jenster, G., Jiang, L., Johnson, S.M., Jones, J.C., Jong, A., Jovanovic-Talisman, T., Jung, S., Kalluri, R., Kano, S. ichi, Kaur, S., Kawamura, Y., Keller, E.T., Khamari, D., Khomyakova, E., Khvorova, A., Kierulf, P., Kim, K.P., Kislinger, T., Klingeborn, M., Klinke, D.J., Kornek, M., Kosanović, M.M., Kovács, Á.F., Krämer-Albers, E.M., Krasemann, S., Krause, M., Kurochkin, I. V., Kusuma, G.D., Kuypers, S., Laitinen, S., Langevin, S.M., Languino, L.R., Lannigan, J., Lässer, C., Laurent, L.C., Lavieu, G., Lázaro-Ibáñez, E., Le Lay, S., Lee, M.S., Lee, Y.X.F., Lemos, D.S., Lenassi, M., Leszczynska, A., Li, I.T.S., Liao, K., Libregts, S.F., Ligeti, E., Lim, R., Lim, S.K., Linē, A., Linnemannstöns, K., Llorente, A., Lombard, C.A., Lorenowicz, M.J., Lörincz, Á.M., Lötvall, J., Lovett, J., Lowry, M.C., Loyer, X., Lu, Q., Lukomska, B., Lunavat, T.R., Maas, S.L.N., Malhi, H., Marcilla, A., Mariani, J., Mariscal, J., Martens-Uzunova, E.S., Martin-Jaular, L., Martinez, M.C., Martins, V.R., Mathieu, M., Mathivanan, S., Maugeri, M., McGinnis, L.K., McVey, M.J., Meckes, D.G., Meehan, K.L., Mertens, I., Minciacchi, V.R., Möller, A., Møller Jørgensen, M., Morales-Kastresana, A., Morhayim, J., Mullier, F., Muraca, M., Musante, L., Mussack, V., Muth, D.C., Myburgh, K.H., Najrana, T., Nawaz, M., Nazarenko, I., Nejsun, P., Neri, C., Neri, T., Nieuwland, R., Nimrichter, L., Nolan, J.P., Nolte-'t Hoen, E.N.M., Noren Hooten, N., O'Driscoll, L., O'Grady, T., O'Loughlen, A., Ochiya, T., Olivier, M., Ortiz, A., Ortiz, L.A., Osteikoetxea, X., Ostegaard, O., Ostrowski, M., Park, J., Pegtel, D.M., Peinado, H., Perut, F., Pfaffl, M.W., Phinney, D.G., Pieters, B.C.H., Pink, R.C., Pisetsky, D.S., Pogge von Strandmann, E., Polakovicova, I., Poon, I.K.H., Powell, B.H., Prada, I., Pulliam, L., Quesenberry, P., Radeghieri, A., Raffai, R.L., Raimondo, S., Rak, J., Ramirez,

M.I., Raposo, G., Rayyan, M.S., Regev-Rudzki, N., Ricklefs, F.L., Robbins, P.D., Roberts, D.D., Rodrigues, S.C., Rohde, E., Rome, S., Rouschop, K.M.A., Rughetti, A., Russell, A.E., Saá, P., Sahoo, S., Salas-Huenuleo, E., Sánchez, C., Saugstad, J.A., Saul, M.J., Schiffelers, R.M., Schneider, R., Schøyen, T.H., Scott, A., Shahaj, E., Sharma, S., Shatnyeva, O., Shekari, F., Shelke, G.V., Shetty, A.K., Shiba, K., Siljander, P.R.M., Silva, A.M., Skowronek, A., Snyder, O.L., Soares, R.P., Sódar, B.W., Soekmadji, C., Sotillo, J., Stahl, P.D., Stoorvogel, W., Stott, S.L., Strasser, E.F., Swift, S., Tahara, H., Tewari, M., Timms, K., Tiwari, S., Tixeira, R., Tkach, M., Toh, W.S., Tomasini, R., Torrecilhas, A.C., Tosar, J.P., Toxavidis, V., Urbanelli, L., Vader, P., van Balkom, B.W.M., van der Grein, S.G., Van Deun, J., van Herwijnen, M.J.C., Van Keuren-Jensen, K., van Niel, G., van Royen, M.E., van Wijnen, A.J., Vasconcelos, M.H., Vechetti, I.J., Veit, T.D., Vella, L.J., Velot, É., Verweij, F.J., Vestad, B., Viñas, J.L., Visnovitz, T., Vukman, K. V., Wahlgren, J., Watson, D.C., Wauben, M.H.M., Weaver, A., Webber, J.P., Weber, V., Wehman, A.M., Weiss, D.J., Welsh, J.A., Wendt, S., Wheelock, A.M., Wiener, Z., Witte, L., Wolfram, J., Xagorari, A., Xander, P., Xu, J., Yan, X., Yáñez-Mó, M., Yin, H., Yuana, Y., Zappulli, V., Zarubova, J., Žekas, V., Zhang, J. ye, Zhao, Z., Zheng, L., Zheutlin, A.R., Zickler, A.M., Zimmermann, P., Zivkovic, A.M., Zocco, D., Zuba-Surma, E.K., 2018a. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 7. <https://doi.org/10.1080/20013078.2018.1535750>

Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G.K., Ayre, D.C., Bach, J.-M., Bachurski, D., Baharvand, H., Balaj, L., Baldacchino, S., Bauer, N.N., Baxter, A.A., Bebawy, M., Beckham, C., Bedina Zavec, A.,

Benmoussa, A., Berardi, A.C., Bergese, P., Bielska, E., Blenkiron, C., Bobis-Wozowicz, S., Boilard, E., Boireau, W., Bongiovanni, A., Borràs, F.E., Bosch, S., Boulanger, C.M., Breakefield, X., Breglio, A.M., Brennan, M.Á., Brigstock, D.R., Brisson, A., Broekman, M.L., Bromberg, J.F., Bryl-Górecka, P., Buch, S., Buck, A.H., Burger, D., Busatto, S., Buschmann, D., Bussolati, B., Buzás, E.I., Byrd, J.B., Camussi, G., Carter, D.R., Caruso, S., Chamley, L.W., Chang, Y.-T., Chen, C., Chen, S., Cheng, L., Chin, A.R., Clayton, A., Clerici, S.P., Cocks, A., Cocucci, E., Coffey, R.J., Cordeiro-da-Silva, A., Couch, Y., Coumans, F.A., Coyle, B., Crescitelli, R., Criado, M.F., D'Souza-Schorey, C., Das, S., Datta Chaudhuri, A., de Candia, P., De Santana, E.F., De Wever, O., del Portillo, H.A., Demaret, T., Deville, S., Devitt, A., Dhondt, B., Di Vizio, D., Dieterich, L.C., Dolo, V., Dominguez Rubio, A.P., Dominici, M., Dourado, M.R., Driedonks, T.A., Duarte, F.V., Duncan, H.M., Eichenberger, R.M., Ekström, K., EL Andaloussi, S., Elie-Caille, C., Erdbrügger, U., Falcón-Pérez, J.M., Fatima, F., Fish, J.E., Flores-Bellver, M., Försonits, A., Frelet-Barrand, A., Fricke, F., Fuhrmann, G., Gabrielsson, S., Gámez-Valero, A., Gardiner, C., Gärtner, K., Gaudin, R., Ghossein, Y.S., Giebel, B., Gilbert, C., Gimona, M., Giusti, I., Goberdhan, D.C., Görgens, A., Gorski, S.M., Greening, D.W., Gross, J.C., Gualerzi, A., Gupta, G.N., Gustafson, D., Handberg, A., Haraszti, R.A., Harrison, P., Hegyesi, H., Hendrix, A., Hill, A.F., Hochberg, F.H., Hoffmann, K.F., Holder, B., Holthofer, H., Hosseinkhani, B., Hu, G., Huang, Y., Huber, V., Hunt, S., Ibrahim, A.G.-E., Ikezu, T., Inal, J.M., Isin, M., Ivanova, A., Jackson, H.K., Jacobsen, S., Jay, S.M., Jayachandran, M., Jenster, G., Jiang, L., Johnson, S.M., Jones, J.C., Jong, A., Jovanovic-Talisman, T., Jung, S., Kalluri, R., Kano, S., Kaur, S., Kawamura, Y., Keller, E.T., Khamari, D., Khomyakova, E., Khvorova, A., Kierulf, P., Kim, K.P., Kislinger, T., Klingeborn, M., Klink, D.J., Kornek, M., Kosanović, M.M., Kovács, Á.F., Krämer-Albers, E.-M., Krasemann, S., Krause, M., Kurochkin,

I.V., Kusuma, G.D., Kuypers, S., Laitinen, S., Langevin, S.M., Languino, L.R., Lannigan, J., Lässer, C., Laurent, L.C., Lavieu, G., Lázaro-Ibáñez, E., Le Lay, S., Lee, M.-S., Lee, Y.X.F., Lemos, D.S., Lenassi, M., Leszczynska, A., Li, I.T., Liao, K., Libregts, S.F., Ligeti, E., Lim, R., Lim, S.K., Linē, A., Linnemannstöns, K., Llorente, A., Lombard, C.A., Lorenowicz, M.J., Lörincz, Á.M., Lötvall, J., Lovett, J., Lowry, M.C., Loyer, X., Lu, Q., Lukomska, B., Lunavat, T.R., Maas, S.L., Malhi, H., Marcilla, A., Mariani, J., Mariscal, J., Martens-Uzunova, E.S., Martin-Jaular, L., Martinez, M.C., Martins, V.R., Mathieu, M., Mathivanan, S., Maugeri, M., McGinnis, L.K., McVey, M.J., Meckes, D.G., Meehan, K.L., Mertens, I., Minciacchi, V.R., Möller, A., Møller Jørgensen, M., Morales-Kastresana, A., Morhayim, J., Mullier, F., Muraca, M., Musante, L., Mussack, V., Muth, D.C., Myburgh, K.H., Najrana, T., Nawaz, M., Nazarenko, I., Nejsun, P., Neri, C., Neri, T., Nieuwland, R., Nimrichter, L., Nolan, J.P., Nolte-'t Hoen, E.N., Noren Hooten, N., O'Driscoll, L., O'Grady, T., O'Loghlen, A., Ochiya, T., Olivier, M., Ortiz, A., Ortiz, L.A., Osteikoetxea, X., Østergaard, O., Ostrowski, M., Park, J., Pegtel, D.M., Peinado, H., Perut, F., Pfaffl, M.W., Phinney, D.G., Pieters, B.C., Pink, R.C., Pisetsky, D.S., Pogge von Strandmann, E., Polakovicova, I., Poon, I.K., Powell, B.H., Prada, I., Pulliam, L., Quesenberry, P., Radeghieri, A., Raffai, R.L., Raimondo, S., Rak, J., Ramirez, M.I., Raposo, G., Rayyan, M.S., Regev-Rudzki, N., Ricklefs, F.L., Robbins, P.D., Roberts, D.D., Rodrigues, S.C., Rohde, E., Rome, S., Rouschop, K.M., Ruggetti, A., Russell, A.E., Saá, P., Sahoo, S., Salas-Huenuleo, E., Sánchez, C., Saugstad, J.A., Saul, M.J., Schiffelers, R.M., Schneider, R., Schøyen, T.H., Scott, A., Shahaj, E., Sharma, S., Shatnyeva, O., Shekari, F., Shelke, G.V., Shetty, A.K., Shiba, K., Siljander, P.R.-M., Silva, A.M., Skowronek, A., Snyder, O.L., Soares, R.P., Sódar, B.W., Soekmadji, C., Sotillo, J., Stahl, P.D., Stoorvogel, W., Stott, S.L., Strasser, E.F., Swift, S., Tahara, H., Tewari, M., Timms, K., Tiwari, S., Tixeira, R.,

Tkach, M., Toh, W.S., Tomasini, R., Torrecilhas, A.C., Tosar, J.P., Toxavidis, V., Urbanelli, L., Vader, P., van Balkom, B.W., van der Grein, S.G., Van Deun, J., van Herwijnen, M.J., Van Keuren-Jensen, K., van Niel, G., van Royen, M.E., van Wijnen, A.J., Vasconcelos, M.H., Vechetti, I.J., Veit, T.D., Vella, L.J., Velot, É., Verweij, F.J., Vestad, B., Viñas, J.L., Visnovitz, T., Vukman, K.V., Wahlgren, J., Watson, D.C., Wauben, M.H., Weaver, A., Webber, J.P., Weber, V., Wehman, A.M., Weiss, D.J., Welsh, J.A., Wendt, S., Wheelock, A.M., Wiener, Z., Witte, L., Wolfram, J., Xagorari, A., Xander, P., Xu, J., Yan, X., Yáñez-Mó, M., Yin, H., Yuana, Y., Zappulli, V., Zarubova, J., Žekas, V., Zhang, J., Zhao, Z., Zheng, L., Zheutlin, A.R., Zickler, A.M., Zimmermann, P., Zivkovic, A.M., Zocco, D., Zuba-Surma, E.K., 2018b. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 7, 1535750. <https://doi.org/10.1080/20013078.2018.1535750>

Thomas, L., Florio, T., Perez-Castro, C., 2020. Extracellular Vesicles Loaded miRNAs as Potential Modulators Shared Between Glioblastoma, and Parkinson's and Alzheimer's Diseases. *Front. Cell. Neurosci.* 14, 590034. <https://doi.org/10.3389/fncel.2020.590034>

Tian, T., Zhu, Y.-L., Zhou, Y.-Y., Liang, G.-F., Wang, Y.-Y., Hu, F.-H., Xiao, Z.-D., 2014. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J. Biol. Chem.* 289, 22258–22267. <https://doi.org/10.1074/jbc.M114.588046>

Timmerman, R., Burm, S.M., Bajramovic, J.J., 2018. An Overview of in vitro Methods to Study Microglia. *Front. Cell. Neurosci.* 12, 242. <https://doi.org/10.3389/fncel.2018.00242>

- Trotta, T., Panaro, M.A., Cianiulli, A., Mori, G., Di Benedetto, A., Porro, C., 2018. Microglia-derived extracellular vesicles in Alzheimer's Disease: A double-edged sword. *Biochem. Pharmacol.* 148, 184–192. <https://doi.org/10.1016/j.bcp.2017.12.020>
- Turola, E., Furlan, R., Bianco, F., Matteoli, M., Verderio, C., 2012. Microglial microvesicle secretion and intercellular signaling. *Front. Physiol.* 3, 149. <https://doi.org/10.3389/fphys.2012.00149>
- Underwood, C.K., Reid, K., May, L.M., Bartlett, P.F., Coulson, E.J., 2008. Palmitoylation of the C-terminal fragment of p75(NTR) regulates death signaling and is required for subsequent cleavage by gamma-secretase. *Mol. Cell. Neurosci.* 37, 346–358. <https://doi.org/10.1016/j.mcn.2007.10.005>
- Vaegter, C.B., Jansen, P., Fjorback, A.W., Glerup, S., Skeldal, S., Kjolby, M., Richner, M., Erdmann, B., Nyengaard, J.R., Tessarollo, L., Lewin, G.R., Willnow, T.E., Chao, M.V., Nykjaer, A., 2011. Sortilin associates with Trk receptors to enhance anterograde transport and neurotrophin signaling. *Nat. Neurosci.* 14, 54–61. <https://doi.org/10.1038/nn.2689>
- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., Lötvall, J.O., 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. <https://doi.org/10.1038/ncb1596>
- Van den Broek, B., Pintelon, I., Hamad, I., Kessels, S., Haidar, M., Hellings, N., Hendriks, J.J.A., Kleinewietfeld, M., Brône, B., Timmerman, V., Timmermans, J.-P., Somers, V., Michiels, L., Irobi, J., 2020. Microglial derived extracellular vesicles activate autophagy and mediate multi-target signaling to maintain cellular homeostasis. *J. Extracell. Vesicles* 10, e12022. <https://doi.org/10.1002/jev2.12022>

- van Koppen, A., Joles, J.A., van Balkom, B.W.M., Lim, S.K., de Kleijn, D., Giles, R.H., Verhaar, M.C., 2012. Human embryonic mesenchymal stem cell-derived conditioned medium rescues kidney function in rats with established chronic kidney disease. *PloS One* 7, e38746. <https://doi.org/10.1371/journal.pone.0038746>
- Vandendriessche, C., Bruggeman, A., Van Cauwenberghe, C., Vandenbroucke, R.E., 2020. Extracellular Vesicles in Alzheimer's and Parkinson's Disease: Small Entities with Large Consequences. *Cells* 9, 2485. <https://doi.org/10.3390/cells9112485>
- Vaz, A.R., Pinto, S., Ezequiel, C., Cunha, C., Carvalho, L.A., Moreira, R., Brites, D., 2019. Phenotypic Effects of Wild-Type and Mutant SOD1 Expression in N9 Murine Microglia at Steady State, Inflammatory and Immunomodulatory Conditions. *Front. Cell. Neurosci.* 13, 109. <https://doi.org/10.3389/fncel.2019.00109>
- Verbeke, S., Meignan, S., Lagadec, C., Germain, E., Hondermarck, H., Adriaenssens, E., Le Bourhis, X., 2010. Overexpression of p75(NTR) increases survival of breast cancer cells through p21(waf1). *Cell. Signal.* 22, 1864–1873. <https://doi.org/10.1016/j.cellsig.2010.07.014>
- Verderio, C., Muzio, L., Turola, E., Bergami, A., Novellino, L., Ruffini, F., Riganti, L., Corradini, I., Francolini, M., Garzetti, L., Maiorino, C., Servida, F., Vercelli, A., Rocca, M., Libera, D.D., Martinelli, V., Comi, G., Martino, G., Matteoli, M., Furlan, R., 2012. Myeloid microvesicles are a marker and therapeutic target for neuroinflammation. *Ann. Neurol.* 72, 610–624. <https://doi.org/10.1002/ana.23627>

Verkhatsky, A., Ho, M.S., Zorec, R., Parpura, V., 2019. The Concept of Neuroglia. *Adv. Exp. Med. Biol.* 1175, 1–13. https://doi.org/10.1007/978-981-13-9913-8_1

Vilar, M., Mira, H., 2016. Regulation of Neurogenesis by Neurotrophins during Adulthood: Expected and Unexpected Roles. *Front. Neurosci.* 10, 26. <https://doi.org/10.3389/fnins.2016.00026>

Villarroya-Beltri, C., Baixauli, F., Mittelbrunn, M., Fernández-Delgado, I., Torralba, D., Moreno-Gonzalo, O., Baldanta, S., Enrich, C., Guerra, S., Sánchez-Madrid, F., 2016. ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins. *Nat. Commun.* 7, 13588. <https://doi.org/10.1038/ncomms13588>

Villarroya-Beltri, C., Guerra, S., Sánchez-Madrid, F., 2017. ISGylation - a key to lock the cell gates for preventing the spread of threats. *J. Cell Sci.* 130, 2961–2969. <https://doi.org/10.1242/jcs.205468>

Wake, H., Moorhouse, A.J., Nabekura, J., 2011. Functions of microglia in the central nervous system--beyond the immune response. *Neuron Glia Biol.* 7, 47–53. <https://doi.org/10.1017/S1740925X12000063>

Wang, K.C., Kim, J.A., Sivasankaran, R., Segal, R., He, Z., 2002. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* 420, 74–78. <https://doi.org/10.1038/nature01176>

Wehrman, T., He, X., Raab, B., Dukipatti, A., Blau, H., Garcia, K.C., 2007. Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron* 53, 25–38. <https://doi.org/10.1016/j.neuron.2006.09.034>

Weskamp, G., Schlöndorff, J., Lum, L., Becherer, J.D., Kim, T.-W., Saftig, P., Hartmann, D., Murphy, G., Blobel, C.P., 2004. Evidence for a critical role of the tumor necrosis factor alpha convertase (TACE) in ectodomain shedding of the p75 neurotrophin receptor (p75NTR). *J. Biol. Chem.* 279, 4241–4249. <https://doi.org/10.1074/jbc.M307974200>

Wiklander, O.P.B., Brennan, M.Á., Lötvall, J., Breakefield, X.O., El Andaloussi, S., 2019. Advances in therapeutic applications of extracellular vesicles. *Sci. Transl. Med.* 11, eaav8521. <https://doi.org/10.1126/scitranslmed.aav8521>

Wolf, S.A., Boddeke, H.W.G.M., Kettenmann, H., 2017. Microglia in Physiology and Disease. *Annu. Rev. Physiol.* 79, 619–643. <https://doi.org/10.1146/annurev-physiol-022516-034406>

Xiang, J., Wan, C., Guo, R., Guo, D., 2016. Is Hydrogen Peroxide a Suitable Apoptosis Inducer for All Cell Types? *BioMed Res. Int.* 2016. <https://doi.org/10.1155/2016/7343965>

Xu, J., Liao, K., Zhou, W., 2018. Exosomes Regulate the Transformation of Cancer Cells in Cancer Stem Cell Homeostasis. *Stem Cells Int.* 2018, e4837370. <https://doi.org/10.1155/2018/4837370>

Yaar, M., Zhai, S., Fine, R.E., Eisenhauer, P.B., Arble, B.L., Stewart, K.B., Gilchrest, B.A., 2002. Amyloid beta binds trimers as well as monomers of the 75-kDa neurotrophin receptor and activates receptor signaling. *J. Biol. Chem.* 277, 7720–7725. <https://doi.org/10.1074/jbc.M110929200>

Yamashita, T., Tohyama, M., 2003. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat. Neurosci.* 6, 461–467. <https://doi.org/10.1038/nn1045>

Yáñez-Mó, M., Siljander, P.R.-M., Andreu, Z., Zavec, A.B., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J.M., Ghobrial, I.M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N.H.H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Krämer-Albers, E.-M., Laitinen, S., Lässer, C., Lener, T., Ligeti, E., Linē, A., Lipps, G., Llorente, A., Lötvall, J., Manček-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E.N.M., Nyman, T.A., O'Driscoll, L., Olivan, M., Oliveira, C., Pállinger, É., Del Portillo, H.A., Reventós, J., Rigau, M., Rohde, E., Sammar, M., Sánchez-Madrid, F., Santarém, N., Schallmoser, K., Ostendorf, M.S., Stoorvogel, W., Stukelj, R., Van der Grein, S.G., Vasconcelos, M.H., Wauben, M.H.M., De Wever, O., 2015. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* 4, 27066. <https://doi.org/10.3402/jev.v4.27066>

Yang, F., Liao, X., Tian, Y., Li, G., 2017. Exosome separation using microfluidic systems: size-based, immunoaffinity-based and dynamic methodologies. *Biotechnol. J.* 12. <https://doi.org/10.1002/biot.201600699>

Yang, L.V., Radu, C.G., Wang, L., Riedinger, M., Witte, O.N., 2005. Gi-independent macrophage chemotaxis to lysophosphatidylcholine via the immunoregulatory GPCR G2A. *Blood* 105, 1127–1134. <https://doi.org/10.1182/blood-2004-05-1916>

Yang, Y., Boza-Serrano, A., Dunning, C.J.R., Clausen, B.H., Lambertsen, K.L., Deierborg, T., 2018. Inflammation leads to distinct populations of extracellular vesicles from microglia. *J. Neuroinflammation* 15, 168. <https://doi.org/10.1186/s12974-018-1204-7>

Yang, Z., Shi, J., Xie, J., Wang, Y., Sun, J., Liu, T., Zhao, Y., Zhao, X., Wang, X., Ma, Y., Malkoc, V., Chiang, C., Deng, W., Chen, Y., Fu, Y., Kwak, K.J.,

Fan, Y., Kang, C., Yin, C., Rhee, J., Bertani, P., Otero, J., Lu, W., Yun, K., Lee, A.S., Jiang, W., Teng, L., Kim, B.Y.S., Lee, L.J., 2020. Large-scale generation of functional mRNA-encapsulating exosomes via cellular nanoporation. *Nat. Biomed. Eng.* 4, 69–83. <https://doi.org/10.1038/s41551-019-0485-1>

Ye, Z., Zhang, T., He, W., Jin, H., Liu, C., Yang, Z., Ren, J., 2018. Methotrexate-Loaded Extracellular Vesicles Functionalized with Therapeutic and Targeted Peptides for the Treatment of Glioblastoma Multiforme. *ACS Appl. Mater. Interfaces* 10, 12341–12350. <https://doi.org/10.1021/acsami.7b18135>

Yekula, Anuroop, Yekula, Anudeep, Muralidharan, K., Kang, K., Carter, B.S., Balaj, L., 2020. Extracellular Vesicles in Glioblastoma Tumor Microenvironment. *Front. Immunol.* 10.

Yuyama, K., Sun, H., Usuki, S., Sakai, S., Hanamatsu, H., Mioka, T., Kimura, N., Okada, M., Tahara, H., Furukawa, J., Fujitani, N., Shinohara, Y., Igarashi, Y., 2015. A potential function for neuronal exosomes: Sequestering intracerebral amyloid- β peptide. *FEBS Lett.* 589, 84–88. <https://doi.org/10.1016/j.febslet.2014.11.027>

Zanin, J.P., Abercrombie, E., Friedman, W.J., 2016. Proneurotrophin-3 promotes cell cycle withdrawal of developing cerebellar granule cell progenitors via the p75 neurotrophin receptor. *eLife* 5, e16654. <https://doi.org/10.7554/eLife.16654>

Zendrini, A., Guerra, G., Sagini, K., Vagner, T., Di Vizio, D., Bergese, P., 2022. On the surface-to-bulk partition of proteins in extracellular vesicles. *Colloids Surf. B Biointerfaces* 218, 112728. <https://doi.org/10.1016/j.colsurfb.2022.112728>

Zendrini, A., Paolini, L., Busatto, S., Radeghieri, A., Romano, M., Wauben, M.H.M., van Herwijnen, M.J.C., Nejsun, P., Borup, A., Ridolfi, A., Montis, C., Bergese, P., 2020. Augmented COlorimetric NANoplasmonic (CONAN) Method for Grading Purity and Determine Concentration of EV Microliter Volume Solutions. *Front. Bioeng. Biotechnol.* 7, 452. <https://doi.org/10.3389/fbioe.2019.00452>

Zendrini, A., Paolini, L., Busatto, S., Radeghieri, A., Romano, M., Wauben, M.H.M., van Herwijnen, M.J.C., Nejsun, P., Borup, A., Ridolfi, A., Montis, C., Bergese, P., 2019. Augmented COlorimetric NANoplasmonic (CONAN) Method for Grading Purity and Determine Concentration of EV Microliter Volume Solutions. *Front. Bioeng. Biotechnol.* 7, 452. <https://doi.org/10.3389/fbioe.2019.00452>

Zhang, X.-H., Tee, L.Y., Wang, X.-G., Huang, Q.-S., Yang, S.-H., 2015. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol. Ther. - Nucleic Acids* 4, e264. <https://doi.org/10.1038/mtna.2015.37>

Zhang, Y., Feng, Y., Justus, C.R., Jiang, W., Li, Z., Lu, J.Q., Brock, R.S., McPeck, M.K., Weidner, D.A., Yang, L.V., Hu, X.-H., 2012. Comparative study of 3D morphology and functions on genetically engineered mouse melanoma cells. *Integr. Biol. Quant. Biosci. Nano Macro* 4, 1428–1436. <https://doi.org/10.1039/c2ib20153d>

Zhang, Y., Liu, Y., Liu, H., Tang, W.H., 2019. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci.* 9, 19. <https://doi.org/10.1186/s13578-019-0282-2>

Zhang, Y., Moheban, D.B., Conway, B.R., Bhattacharyya, A., Segal, R.A., 2000. Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. *J. Neurosci. Off.*

J. Soc. Neurosci. 20, 5671–5678. <https://doi.org/10.1523/JNEUROSCI.20-15-05671.2000>

Zhu, L., Kalimuthu, S., Gangadaran, P., Oh, J.M., Lee, H.W., Baek, S.H., Jeong, S.Y., Lee, S.-W., Lee, J., Ahn, B.-C., 2017. Exosomes Derived From Natural Killer Cells Exert Therapeutic Effect in Melanoma. *Theranostics* 7, 2732–2745. <https://doi.org/10.7150/thno.18752>

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., Amigorena, S., 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med.* 4, 594–600. <https://doi.org/10.1038/nm0598-594>

Publications Lists

Fluorolabeling of the PPTase-Related Chemical Tags: Comparative Study of Different Membrane Receptors and Different Fluorophores in the Labeling Reactions

Rosy Amodeo, Domenica Convertino, Mariantonietta Calvello, **Lorenzo Ceccarelli**, Fulvio Bonsignore, Cosetta Ravelli, Antonino Cattaneo, Claudia Martini, Stefano Luin, Stefania Mitola, Giovanni Signore, Laura Marchetti;
Front Mol Biosci. 2020 Aug 7;7:195. doi: [10.3389/fmolb.2020.00195](https://doi.org/10.3389/fmolb.2020.00195). eCollection 2020

Microglia extracellular vesicles: focus on molecular composition and biological function

Lorenzo Ceccarelli, Chiara Giacomelli, Laura Marchetti, Claudia Martini;
Biochem Soc Trans 2021; BST20210202. doi: <https://doi.org/10.1042/BST20210202>

Advances in microglia cellular models: focus on extracellular vesicle production

Lorenzo Ceccarelli, Laura Marchetti, Chiara Giacomelli, Claudia Martini;
Biochem Soc Trans 2021; BST20210203. doi: <https://doi.org/10.1042/BST20210203>

The Mediterranean Diet Positively Affects Resting Metabolic Rate and Salivary Microbiota in Human Subjects: A Comparison with the Vegan Regimen

Lorenzo Ceccarelli, Simona Daniele, Giorgia Scarfò, Jonathan Fusi, Elisa Zappelli, Denise Biagini, Tommaso Lomonaco, Fabio Di Francesco, Ferdinando Franzoni, Claudia Martini;
Biology 2021, 10, 1292. doi: <https://doi.org/10.3390/biology10121292>

Human Microglia Extracellular Vesicles Derived from Different Microglia Cell Lines: Similarities and Differences

Lorenzo Ceccarelli, Laura Marchetti, Milena Rizzo, Aldo Moscardini, Valentina Cappello, Eleonora Da Pozzo, Miriam Romano, Chiara Giacomelli, Paolo Bergese, and Claudia Martini;
ACS Omega 2022, 7, 27, 23127–23137. doi: <https://doi.org/10.1021/acsomega.2c00816>

A Specific Combination of Nutraceutical Ingredients exerts cytoprotective effects in human cholinergic neurons

Elisa Zappelli; Simona Daniele; Matteo Vergassola; **Lorenzo Ceccarelli**; Elisa Chelucci; Giorgina Mangano; Lucia Durando; Lorella Ragni; Claudia Martini.
PharmaNutrition 22 (2022) 100317. doi: <https://doi.org/10.1016/j.phanu.2022.100317>

α -Glycerol-phosphoryl-ethanolamine protects human hippocampal neurons from ageing-induced cellular alterations

Elisa Zappelli; Simona Daniele; **Lorenzo Ceccarelli**; Matteo Vergassola; Lorella Ragni; Giorgina Mangano; Claudia Martini.
Eur J Neurosci. 2022;56:4514–4528. doi : <https://doi.org/10.1111/ejn.15783>

Endometrial Dysbiosis Is Related to Inflammatory Factors in Women with Repeated Implantation Failure: A Pilot Study

*Vito Cela; Simona Daniele; Maria Elena Rosa Obino; Maria Ruggiero; Elisa Zappelli; **Lorenzo Ceccarelli**; Francesca Papini; Ilaria Marzi; Giorgia Scarfò; Fulvia Tosi; Ferdinando Franzoni; Claudia Martini and Paolo Giovanni Artini.*

J. Clin. Med. 2022, 11, 2481. doi: <https://doi.org/10.3390/jcm11092481>