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Role of glial extracellular vesicles in neuronal pathology in an *in vitro* model of stroke

Dissertation

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1 Content

2 Aim of the study

Cerebral ischemia, caused by an occlusion of a major brain artery, is still one of the leading causes of death and disability worldwide (Murray et al. 2012). In high-income countries low physical activity, higher intake of sugar and fat, caused by industrial produced food, lead to the rising number of patients with adiposity, high blood pressure, high blood cholesterol and diabetes mellitus type II. These developments result in rising incidents of atherosclerosis which is one of the main causes of ischemic stroke (Herrington et al. 2016). The occluded artery cannot supply the affected brain region with sufficient glucose and oxygen which leads to neuronal death by necrosis (Dirnagl et al. 1999, Puig et al. 2018). At present, the therapeutic options are limited, and many patients cannot benefit from them due to their time limitation (Powers et al. 2018).

One of the determining factors for the neuronal fate is the immune response. Activation of microglia, the immune resident cells of the central nervous system (CNS), together with infiltration of immune cells from the periphery is seen as the cause of the infarct growth (Dirnagl et al. 1999). Microglia are activated by damage associated molecular patterns (DAMPs) released by dying cells. The purpose of activated microglia is to restore neuronal function and clean damaged areas. However, it can also lead to an inflammatory reaction harmful for neuronal cells (Xiong et al. 2016). Additionally, astrocytes, the most abundant cells in the CNS, with various homeostatic and immunocompetent functions, can change properties during neuroinflammation (Becerra-Calixto and Cardona-Gomez 2017). Thus, as a reaction to ischemia and cell death, astrocytes become active and proliferate in a process known as astrogliosis, contributing to the repair process and scar formation (Sofroniew and Vinters 2010, Zamanian et al. 2012, Becerra-Calixto and Cardona-Gomez 2017). But astrocytes can also play detrimental roles by upregulating the proinflammatory transcriptional factor NF-κB which activates the transcription of proinflammatory cytokines such as Interleukin 6 (IL-6) (Colombo and Farina 2016). Interestingly, in 2017, Liddelow and colleagues highlighted the importance of microglia-astrocyte communication, showing that microglia *per se* do not have the capacity to kill neurons but they activate astrocytes which are then the executors of the neuronal death (Liddelow et al. 2017). Thus, the study of how

glial cells react in stroke but also how they communicate is important to increase the knowledge of stroke pathophysiology.

At present, cell communication between brain cells through extracellular vesicles (EVs) is an intensive field of research (Harding et al. 2013). EVs are lipid bilayer enclosed vesicles that are released by almost every cell and carry molecules such as lipids, proteins, and extracellular RNA (Valadi et al. 2007, Fruhbeis et al. 2013). Emerging data shows that this type of vesicles can exert important inter- and intracellular communication after stroke (Puig et al. 2018). The aim of the present study was to examine the effect of glial EVs on neurons subjected to oxygen-glucose deprivation (OGD) as an *in vitro* model of stroke. Our goal was to understand better the communication axis between microglia and astrocyte through EVs in the post-ischemic neuronal survival.

3 Introduction

3.1 Ischemic stroke

An elderly adipose person (patient X) with sudden weakness and numbness in the left arm and leg, together with a sudden difficulty of speaking is delivered to a common emergency department in a high-income city. Those symptoms, combined with known risk factors such as high age and obesity, are indicators for an ischemic stroke (Hankey 2017). Immediately after arriving at the hospital neurological examinations, blood tests, cranial computed tomography, and magnetic resonance imaging are implemented, and stroke diagnosis is confirmed. The therapeutic options are limited to intravenous systemic thrombolysis with recombinant tissue plasminogen activator (rtPA) or to interventional thrombectomy (Ramee and White 2014). The main criteria for the selection of the currently available therapeutic options is the time window between initial symptoms and delivery at the hospital (Ramee and White 2014). In this case, the symptoms of patient X most likely started more than 8 hours ago. Because the intravenous rtPA therapy is only applicable in a time window of 4,5 hours patient X can only benefit from mechanical thrombectomy (Hankey 2017, Andrews et al. 2019). Even though many studies indicate positive outcomes after mechanical thrombectomy, there is still a three-fold higher risk for these patients to suffer recurrent strokes, vasospasms, or subarachnoid haemorrhage (Emprechtinger et al. 2017, Andrews et al. 2019). Unfortunately, patient X did not benefit from the therapy and is impaired by disability.

Patient X is one of many examples why stroke is one of the leading causes of death and disability worldwide. Due to increased aging of the population, the incidence of stroke is rising, and therapeutic options are still limited (Lackland et al. 2014). Therefore, the scientific community is committed to intensify the understanding of molecular mechanisms in stroke to develop new therapeutic approaches.

3.2 Pathophysiology of postischemic inflammation

The majority of strokes have an ischemic origin which is defined as a lack of oxygen and glucose supply due to the blockage of a main arterial vessel by a thrombus or an embolus (Zhang and Chopp 2016). In the extremely sensitive neuronal cells, the resulting lack of oxygen and glucose eventually leads to cellular damage, causing necrotic or apoptotic cell death. In the core of the infarct zone, cells are irreversibly injured. Cells in the area surrounding the infarct zone (penumbra) are electrically silent but metabolically active and they can either die, after a shorter/longer period of time, or survive (Broughton et al. 2009). Cell damage occurring in the penumbra is characterised by an intense secondary inflammatory response (Dirnagl et al. 1999).

At a molecular lever, the blockage of a main arterial vessel in the brain, by a thrombus or embolus, leading to an inefficient supply of oxygen and glucose, impairs the energy production in form of adenosine triphosphate (ATP). This leads to the failure of the ATP dependent Na^{2+}/K^{+} ATPase. Consequently, the ionic imbalance of increasing Na^{2+} influx and K^{+} efflux leads to depolarisation. Due to the intracellular accumulation of Na^{2+} water follows the osmotic gradient and accumulates intracellularly leading to oedema. (Dirnagl et al. 1999). The loss of the electrochemical ion gradient impairs glutamate transport, decreasing the glutamate reuptake and increasing the extracellular glutamate concentration (Yi and Hazell 2006). This leads to excitotoxicity, a neurotoxic process triggered by increased extracellular glutamate concentration that overstimulates glutamate receptors causing enhanced Ca^{2+} influx to the neurons (Dong et al. 2009). The increased intracellular Ca^{2+} concentration results in augmented activation of catabolic enzymes eventually ending in apoptotic or necrotic cell death. Additionally, there is a production of reactive oxygen species (ROS) which can damage DNA and RNA together with detrimental oxidation of proteins (Dong et al. 2009).

Damaged cells release DAMPs, which bind to pattern recognition receptors (PRRs) causing further expression of pro-inflammatory genes and immune activation (Vidale et al. 2017). PRRs are mainly expressed in innate immune cells either as transmembrane (e.g. Toll like receptors), or cytoplasmic receptors (e.g.

NOD like receptors) causing transcription of inflammatory mediators by signalling cascades (Takeuchi and Akira 2010). In this case the inflammatory cascade is not initiated by external pathogens (pathogen associated molecular pattern PAMP) but by the endogenous released DAMPs (Xiong et al. 2016). Due to the absence of external microorganism this process is called sterile inflammation, the endogenous reaction of tissue damage to generate reparation (Chen and Nunez 2010). The acute neuroinflammatory reaction is initially observed in microglia and astrocytes, which express specific receptors to monitor their environment. A rapid reaction by generating proinflammatory cytokines activates the inflammatory cascade to protect and defend the neuronal cells, promoting phagocytosis and neurogenesis (Di Napoli and Shah 2011). However, besides defence and reparation, inflammation has long been uncovered as an integral part of the pathophysiology in stroke, due to the close relation between repairing and destruction of tissue. In sterile inflammation the reaction of endogenous inflammatory cells often leads to further cell destruction, for example by neutrophilic invasion and increased production of ROS as described above (Dong et al. 2009, Chen and Nunez 2010). However, the molecular mechanisms in the core region and penumbra are highly complex and only partially understood. Fig. 1 gives an overview of the pathophysiological post-ischemic inflammation (Dirnagl et al. 1999).

To gain a better understanding of the post-ischemic inflammatory reaction, it is also important to study the interaction between the different cerebral cells. Recently, it has been discovered that glial cells and neurons are communicating through EVs (Zhang and Chopp 2016, Puig et al. 2018).

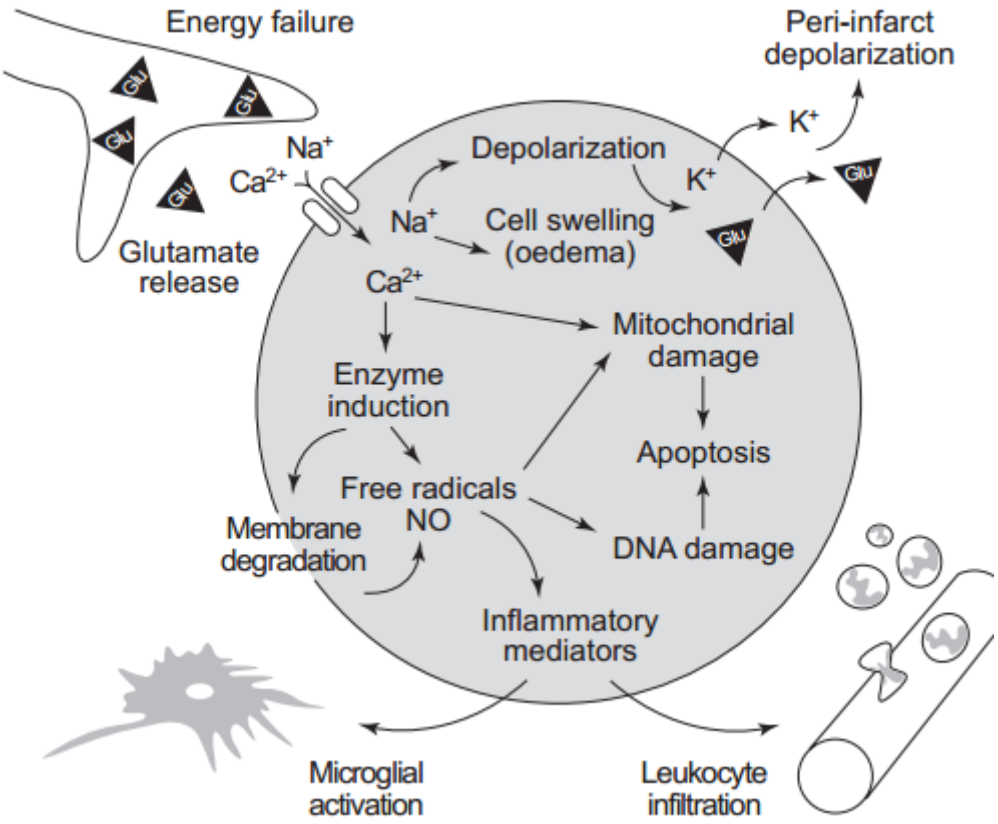


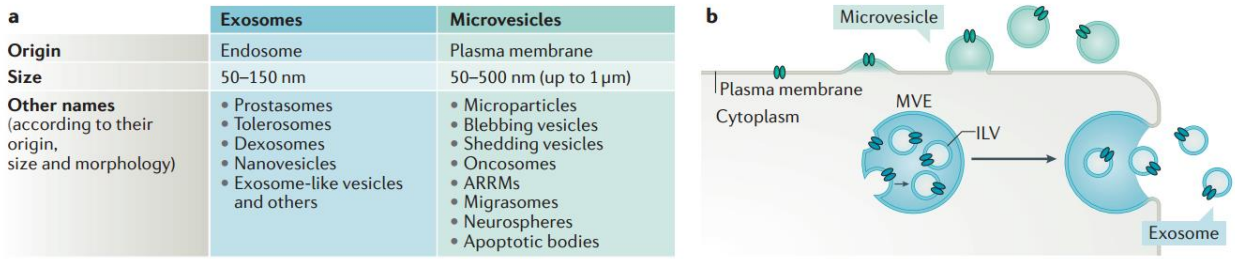
Fig. 1 Simplified overview of pathophysiological mechanisms in the focally ischemic brain.

The lack of energy develops to depolarization of neurons. This activates glutamate receptors resulting in increases of intracellular levels of Ca^{2+}, Na^{2+} and Cl^{2+} whereas, K^{+} is released extracellularly. Following the osmotic gradient generated by Na^{2+}, water shifts into the intracellular space and results in cell swelling. The increased intracellular Ca^{2+} level activates several enzymes such as lipases, endonucleases, and proteases. Apoptosis is induced by free radicals, harming membranes, DNA and mitochondria. Free radicals trigger the expression of proinflammatory genes inducing the synthesis of transcription factors for inflammatory mediators such as platelet activating factor, TNF α, and IL 1β- activating microglia and leading to leukocyte infiltration due to upregulation of endothelial adhesion molecules (Dirnagl et al. 1999).

3.3 Extracellular vesicles (EVs)

At present, an intensive field of research is on cell communication between cells through EVs (Harding et al. 2013). All types of brain cells can release EVs. EVs can be categorized in microvesicles, and exosomes, which vary in size, origin, morphology, and cargo content (Fruhbeis et al. 2013, van Niel et al. 2018). Exosomes are small extracellular vesicles (50-150 nm in size) derived from intraluminal vesicles (ILVs) generated in the endocytic pathway and contained in multivesicular endosomes (MVE). When the MVE fuses with the plasma membrane, ILVs are released as exosomes. In contrast, microvesicles (150 – 1000 nm in size) are shed from the plasma membrane (van Niel et al. 2018).

Apoptotic bodies, a subtype of microvesicles are also released from the plasma membrane but only from cells undergoing apoptosis. They can have a size between 1 to 5 µm (Gyorgy et al. 2011). However, in the lasts years, it has been recognized that this categorization is not precise as exosomes and microvesicles can present similar appearance, size, and composition as shown in Fig. 2 (van Niel et al. 2018). For the sake of simplicity, it is recommended to use the term EVs (Witwer et al. 2017). EV-cargo consist of proteins, RNA, and metabolites which can have a dramatic effect on the recipient cell. The cargo reflects the state of the releasing cells and can vary depending on surrounding conditions such as in inflammation (Zhang and Chopp 2016, Tkach et al. 2018, van Niel et al. 2018). It is well described that EVs can have an immunomodulatory effect in inflammatory processes, such as in post-stroke inflammation as described in the next paragraph (Robbins and Morelli 2014, Paolicelli et al. 2018, Wang et al. 2018).



a

	Exosomes	Microvesicles
Origin	Endosome	Plasma membrane
Size	50–150 nm	50–500 nm (up to 1 µm)
Other names (according to their origin, size and morphology)	• Prostasomes • Tolerosomes • Dexosomes • Nanovesicles • Exosome-like vesicles and others	• Microparticles • Blebbing vesicles • Shedding vesicles • Oncosomes • ARRMs • Migrasomes • Neurospheres • Apoptotic bodies

Fig. 2 Main features of extracellular vesicles.

***a** | Extracellular vesicles consist of a diverse population of released membrane vesicles. They vary in size, origin, morphology, and cargo content. Recently, EVs are classified in two distinct classes: exosomes and microvesicles.*
***b** | Microvesicles are secreted by budding of the plasma membrane. When intraluminal vesicles (ILV) formed in the lumen of multivesicular endosomes (MVE) are secreted to the extracellular space by MVEs fusion with the plasma membrane, are then referred to as exosomes (van Niel et al. 2018).*

3.3.1 The role of microglia and microglial-derived EVs in post-ischemic inflammation

Microglia constitute 10 to 15 % of the non-neuronal cells in the brain (Gomez-Nicola and Perry 2015). They have been discovered in the 19th century by Pío del Río-Hortega and were initially regarded as cells that maintain the structure and stability of the brain (Sierra et al. 2016, Wolf et al. 2017). Since then, many other functions have been attributed to microglia, mainly as the brain resident

macrophages reacting to injury, but also as synapsis modulators by performing synaptic pruning (Gomez-Nicola and Perry 2015). Microglia are constantly monitoring the microenvironment to react to CNS homeostasis disturbances, and therefore, they are one of the first cells to react after stroke (Wolf et al. 2017, Bachiller et al. 2018). It is broadly referred to that microglia can have two phenotypes, the pro- / and the anti-inflammatory phenotypes M1 and M2 respectively (Bachiller et al. 2018). In the case of infiltration by pathogenic agents, microglia develop rapidly to a phagocytic state (M1), initiating an immune response (Kabba et al. 2017). M1 microglia releases pro-inflammatory cytokines such as tumour necrosis factor (TNF) α, IL-1β, and ROS to fight pathogens (Bachiller et al. 2018). When microglia are in the M2 anti-inflammatory phenotype state, they release growth factors, TNF β, and IL-10 and contribute to brain repair processes (Schmieder et al. 2012, Ansari 2015). However, recent studies have shown that M1/M2 phenotypes do not correctly represent the complex reaction that microglia presents regarding certain environmental stimuli (Bachiller et al. 2018). For example, in a mouse model of Alzheimer's disease, it was observed that microglia are reacting at a transcriptional level depending on environmental stimuli such as apolipoproteins and can evolve to dysfunctional microglia. Thus, apolipoprotein E (APOE) induces a transcriptional pathway by binding a receptor expressed on myeloid cells (TREM2) such as on microglia. The TREM2-APOE pathway can shift microglial function into neurodegenerative microglia in neurodegenerative diseases, contributing then to neuronal loss (Krasemann et al. 2017). Hence depending on local environmental stimuli and surrounded proteins, microglia can react distinctly and lose their tolerogenic function (Hickman et al. 2013, Krasemann et al. 2017).

In stroke, neurons at the penumbra can release "eat me", "find me" or "help me" signals leading to phagocytosis or neuroprotection (Li et al. 2020). As mentioned above, microglia express PRRs which in ischemic events bind to DAMPs (such as ATP, S100 or high mobility group box 1 (HMGB1)) released by damaged cells (Vidale et al. 2017). This initialises a complex cascade of inflammatory reactions comprising transcriptional changes in microglia leading to cytokine expression and release of TNFα and IL-6, which in turn recruit inflammatory cells such as neutrophils and lymphocytes (Hickey et al. 1997, Fielding et al. 2008, Yang et al.

2018). Interestingly, TNFα and IL-6 were also identified as being released in EVs (Drago et al. 2017). Hence, microglia can interact with neighbouring cells not only by direct cell to cell contact, aggregation, or adherence but additionally by releasing EVs.

As EVs have also immunomodulatory properties, several studies are focusing on the effect of microglia derived EVs in ischemic neuroinflammation (Eldh et al. 2010, Drago et al. 2017, Paolicelli et al. 2018). It is now known that the secretion of EVs derived from microglia increases during inflammation (Antonucci et al. 2012). Thus, ischemia-induced cell damage leads to release of intracellular ATP to the extracellular space as a DAMP, binding to P2X7 receptors expressed on microglia. This leads to the activation of caspase-1 which cuts pro-IL-1β, leading to the formation of IL-1β, increased expression of pro-inflammatory genes, and enhanced secretion of EVs. These EVs can contain cleaved IL-1β, which stimulates neuroinflammation (Fruhbeis et al. 2013, Budnik et al. 2016, Drago et al. 2017, Chang et al. 2018, Paolicelli et al. 2018). EVs released by microglia are taken up by astrocytes which then secrete further pro-inflammatory cytokines such as IL-6, IL1β and TNFα (Drago et al. 2017). However, IL-1β can also participate in the post-ischemic brain tissue reparation (Hewett et al. 2012). Hence, it was observed that IL-1β can inhibit the blood-brain barrier (BBB) permeability and promote the secretion of neurotrophic growth factors (Herx et al. 2000, Mason et al. 2001). It was also demonstrated that microglia-derived EVs which are stimulated by increased extracellular ATP concentration additionally contain proteins such as IL-10, Thrombopentin 1 and 4, promoting repairing processes such as synaptogenesis and neurite outgrowth (Fruhbeis et al. 2013). Thus, it is very important to study the spatio-temporal release of microglia EVs as they present not only detrimental but also beneficial effects in the post-ischemic inflammation. (Arber and Caroni 1995, Eroglu et al. 2009, Drago et al. 2017). These observations, regarding the influences of microglia-derived EVs in the ischemic neuroinflammatory process, indicate that further investigations need to be conducted to fully understand the complexity of post-ischemic neuroinflammation.

3.3.2 The role of astrocytes and astrocyte-derived EVs in post-ischemic inflammation

Astrocytes are the most abundant glial cells in the brain (Colombo and Farina 2016). They play a crucial role in BBB formation by assisting the endothelial cells in keeping their biochemical homeostasis. For example, by regulating the transport of water, vasomodulators, and neurotransmitters (Escartin et al. 2019). Furthermore, they support neuronal homeostasis by storing and providing glucose to neurons. Because they highly express potassium channels, they can maintain the extracellular ion homeostasis by scavenging extracellular potassium derived from neuronal activity (Chen and Swanson 2003, Colombo and Farina 2016, Becerra-Calixto and Cardona-Gomez 2017). After injury, inflammation, or ischemia, astrocytes become reactive in a process known as astrogliosis (Li et al. 2008). The external stimuli (ischemia, trauma, inflammation) alter astrocytic morphology by increasing molecular expression of glial fibrillary acidic proteins (GFAP) and vimentin -which are crucial components of the cytoskeleton- and therefore promote astrocytic scar formation and reparation of damage associated lesions (Sofroniew and Vinters 2010, Becerra-Calixto and Cardona-Gomez 2017). Additionally, gene profiling of ischemia-activated astrocytes demonstrated the secretion of proinflammatory cytokines such as IL-6, CLCF1, and neurogenerative proteins like thrombospondins (Eroglu et al. 2009, Zamanian et al. 2012). Nevertheless, reactive astrocytes can develop anti- and proinflammatory characteristics (Sofroniew and Vinters 2010). Depending on the location, point in time, origin and genetic profiling, astrocytes react diversely and heterogeneously (Sofroniew 2014). For example, when Hamby *et. al*. injected TNF-β1, LPS and interferon γ (INF) *in vivo* into the cortex of mice and *in vitro* into astrocyte cell cultures they detected the gene expression and calcium signalling of astrocytes. They could conclude that reactive astrocytes behave diversely and heterogeneously to those stimuli, because the final results of levels and patterns of the expression of chemokines, cytokines, GFAP were very different, even though same conditions were applied. Furthermore, the expression of chemokines, etc. varied in time course experiments, suggesting a temporal difference in reactive astrogliosis (Sofroniew and Vinters 2010, Hamby et al. 2012). It is described that astrocytes react initially with pro-inflammatory severe reactive astrogliosis in the

core, subsequently in the penumbra anti-inflammatory reaction protects the surrounding healthy tissue and inhibits an over inflammatory reaction (Sofroniew 2009, Sofroniew and Vinters 2010).

Importantly, astrocytes, as microglia, express P2X7 receptors which bind to the rising extracellular ATP concentration derived from cell damage in stroke (Bianco et al. 2009). As in microglia, also astrocytes secrete EVs, containing IL1-β which, as outlined above, has pro- and anti-inflammatory roles (Mason et al. 2001). Additionally, it has been shown that EVs derived from astrocytes subjected to an *in vitro* model of ischemia had a neuroprotective effect to neurons, mediated through prion protein (PrP) and synapsin I (Wang et al. 2011, Guitart et al. 2016). However, the molecular mechanisms leading to promotion of neurogenesis and angiogenesis by these proteins is not well understood. Interestingly, astrocyte-derived EVs can also transport whole organelles such as mitochondria. These mitochondria were shown to be functional, and they were able to rescue ischemic neurons *in vitro* and *in vivo* (Hayakawa et al. 2016).

3.3.3 Communication axis microglia – astrocytes in post-ischemic inflammation

Astrocytes and microglia, as glial residential cells in the CNS react rapidly after cerebral ischemia. Both can be activated by certain stimuli such as increased extracellular ATP levels and released DAMPs (Iadecola and Anrather 2011). However, they do not react independently in post-ischemic inflammation but on a highly interactive base (Jha et al. 2019). Liddelow *et. al*. demonstrated that astrocytes become toxic to neurons only in the presence of reactive microglia, suggesting that a communication axis between microglia and astrocytes is necessary to activate astrocytes, and that microglia *per se* are not harmful to the surrounding neurons. Thus, they showed that following inflammatory stimulation, microglia secrete IL-1α, TNF and complement subcomponent 1q (C1Q), inducing an A1 phenotype (toxic) on astrocytes. In microglia knockout mice, the A1 phenotype was abolished, suggesting that microglia – astrocytes communication and microglial activation of astrocytes are mandatory for the reactive astrogliosis in

cerebral inflammation (Liddelow et al. 2017). Furthermore, it was identified that hypoxically and lipopolysaccharide (LPS) stressed astrocytes communicate with microglia through lipocalin 2 (lpc 2), contributing to the inflammatory cascade (Jang et al. 2013, Wang et al. 2015, Jha et al. 2019). Microglia and astrocytes can release several proteins such as inflammatory cytokines and organelles by shedding EVs (Budnik et al. 2016, Hayakawa et al. 2016, Jha et al. 2019). For example, Yang *et al*. performed microglial stimulation by LPS and demonstrated secretion of EVs containing 49 different proteins such as TNF indicating that the microglia astrocyte crosstalk occurs through EVs (Yang et al. 2018). The possibility of studying the role of nano-sized vesicles in the neuroinflammatory process occurring after stroke only emerged due to technical developments in recent years (Harding et al. 2013). Developing a better and deeper understanding of the molecular mechanisms in post-stroke inflammation is a promising field in order to enhance therapeutic options and develop new targets (Chen and Chopp 2018). The aim of the present work is, therefore, to investigate, the communication axis between microglia-astrocytes through EVs and evaluate how this affects neuronal survival by using an *in vitro* model of stroke.

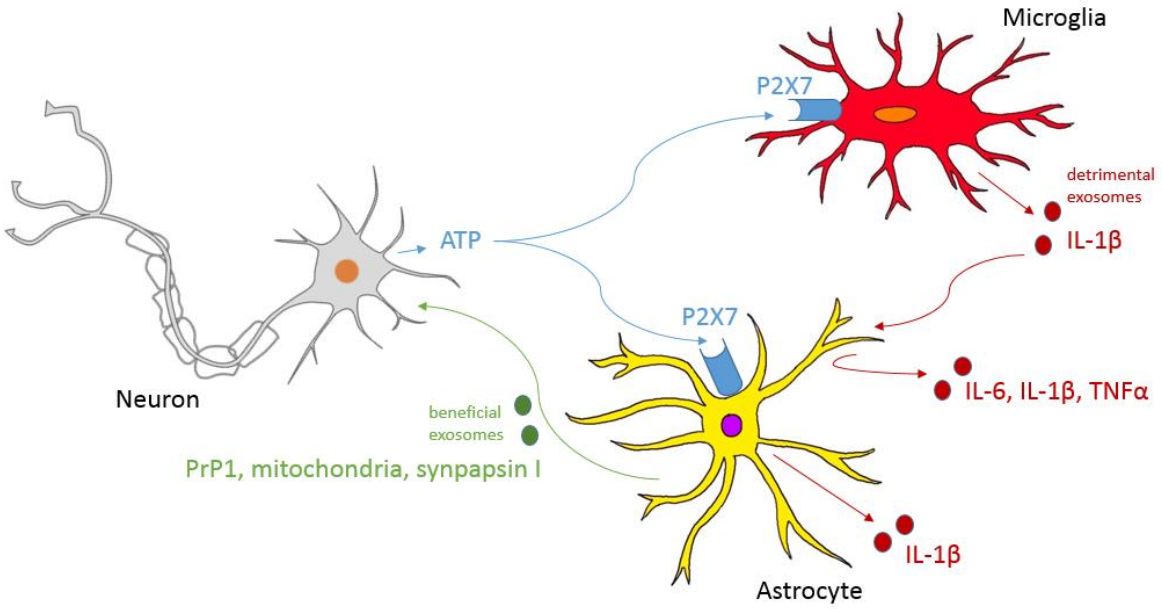


Fig. 3 EV-secretion after stroke and its influences.

Hypoxia and hypoglycaemia induce neuronal cell stress and death. Stressed and damaged neurons release ATP which binds to P2X7 receptors on microglia and astrocytes. P2X7 activation triggers EV secretion of EVs containing inflammatory signals such as IL-1β. Microglia derived EVs secreted after ATP stimulation are taken up by astrocytes and increase secretion of EVs containing inflammatory signals as well (IL-6, TNFα).However, ischemic cell stress in astrocytes induces secretion of EVs containing proteins and enzymes which are beneficial for neuronal survival (such as PrP1, mitochondria, and synapsin I).

Cell figures extracted from [online] URL: https://openclipart.org/detail/204598/simple-neuron, https://www.neuroscientificallychallenged.com/glossary/microglia/,https://www.neuroscientificallychallenged.com/glossary/astrocytes/ [stand: 22.11.2018, 15:15].

4 Material and Methods

4.1 Animals

All animal experiments were approved by the local animal care committee (Behörde für Lebensmittelsicherheit und Veterinärwesen) and in accordance with the guidelines of the animal facility of the University Medical Center Hamburg-Eppendorf. C57BL/6 mice were used in all experiments.

4.2 Cell cultures

4.2.1 Primary mixed cell culture of astrocytes and microglia

To generate cell cultures, C57BL/6 P0-P2 (0-2 postnatal days) were decapitated, and the brain carefully dissected and immediately immersed in HBSS containing 1% HEPES buffer (Gibco #14025-050 and Gibco #15603). The cerebellum, bulbi, and meninges were removed, and the remaining tissue was chopped. The suspension was then transferred into a 15 ml falcon and centrifuged at 310x*g* for 5 min at room temperature (RT) (Eppendorf 5804R centrifuge). The supernatant was discarded, and the pellet was washed with HBSS/1 % HEPES buffer and centrifuged again (310x*g*, 5 min, RT). The final pellet was then resuspended in 6 ml digestion solution containing HBSS buffer, 0,5 mg/ml papain (Sigma #P4762-100mg) and 10 µg/ml DNase (Sigma #DN25) and incubated in a water bath for 30 min at 37°C while shaking. Afterwards 6 ml of basal medium eagle (BME) + 10% heat-inactivated fetal bovine serum (FBS) + 0,1 % Gentamicin was added in order to stop the digestion (Gibco #41010-026, PAN Biotech #P40-47500, Gibco #15750-060). The cell suspension was centrifuged one more time (310x*g*, 5 min, RT) and then resuspended in 5 ml BME medium. To obtain a single cell solution, the tissue was pipetted up and down with Pasteur pipettes of decreasing diameters and filtered with a 70 µm cell strainer. The final cell suspension was

diluted in 5 ml BME medium. 20 µl of the final cell suspension was mixed with Trypan blue (Sigma, #T8154) and viable cells were counted with Invitrogen Countess II automated cell counter (ThermoFisher #AMAQAX1000). Around $3*10^6$ cells were plated in cell culture Petri dishes (10 cm), around 185.000 cells in 12 well plates and around 20.000 cells in 96-well plates for further use. Cells were fed twice a week with BME medium. After two weeks, cells were confluent and used for the experiments.

4.2.2 Primary astrocytes cell culture

Primary mixed glial cells were prepared as described above and seeded into T75 flasks. On day 14, when the cells reached confluency, the medium was removed, and cells were washed twice with phosphate buffered saline (PBS) (Gibco#14190-094). Then, cells were incubated with 2 ml trypsin-EDTA (Gibco #25300-054) at 37 °C until all cells were detached. To the final cell suspension, 5 ml of BME medium was added, transferred into a 15 ml falcon, and centrifuged at 300x*g* for 5 min at RT. The remaining pellet was resuspended in BME medium. 0,1 % of anti-mouse CD11b antibody (BD Biosciences #553308) was added and the falcon was incubated on ice for 20 min. Afterwards, BME medium was added to the cell suspension and washed twice (300x*g*, 5 min, RT). The remaining pellet was then resuspended in BME medium containing sheep anti-rat IgG Dynabeads (Invitrogen #11035) and incubated at 4 °C for 30 min. CD11b positive cells (microglia, attached to the magnetic beads by CD11b antibody) were separated from CD11b negative cells (astrocytes, flow-through) by application of a magnetic field (Thermo Fisher, Invitrogen Dyna bead separation). The astrocyte enriched cell suspension was quantified and plated on cell culture Petri dishes (10 cm), T75 flasks and 12 and 48-well plates. To confirm the astrocyte enrichment, fluorescence-activated cell sorting (FACS) analysis was performed.

4.2.3 Primary neuronal cell culture

Primary neuronal cell culture was prepared as described before (Beaudoin et al. 2012). Coverslips (Marienfeld #0111530) in 12-well plates and 48 well plates were previously coated with 0,1 mg/ml Poly-L-lysine (Sigma #P9155-5mg) in borate buffer (Boric Acid, pH with NaOH) overnight and washed with PBS three times next day. Wildtype C57BL/6 pups (max. 2 days postnatal) were decapitated, the brain was carefully removed and put in a solution of PBS/10 mM glucose. The cerebellum, bulbi, and meninges were removed, the hippocampus was dissected, and transferred to a 15 ml falcon containing PBS/10 mM glucose solution. When the tissue had settled at the bottom of the falcon, the solution was removed and 5 ml of digestion solution containing PBS/10 mM Glucose with 0,5 mg/ml papain and 60 µg/ml DNAse was added and incubated for 45 min in a water bath at 37 °C. Afterwards, the digestion solution was removed, and the tissue washed with plating medium (minimum essential medium (MEM) Gibco #5100-046, 20mM glucose, 10 % horse serum Gibco #16050122), four times. The final cell suspension was pipetted up and down with a 1000 µl pipet to obtain a single-cell solution. The final cell suspension was quantified as previously described for the mixed glia culture and plated with plating medium. After 5h, the plating medium was changed to Neurobasal A (Gibco #10888022) containing 0,1 % Gentamicin, 1x Glutamax (Gibco #35050-038) and 2 % B27 serum (Gibco #17304-044).

4.3 *In vitro* stroke model

4.3.1 Oxygen-glucose deprivation (OGD)

OGD was performed on day 18 after plating for primary astrocytes and mixed glial cell cultures, and on day 12 for primary neuronal cell cultures as previously described (Wang et al. 2012).

4.3.1.1 OGD with primary astrocyte and mixed glial cell culture

Cells were washed twice with warm PBS, incubated with Dulbecco's modified eagle medium (DMEM) without glucose (Thermo Fisher #11966025), and transferred into an anaerobic chamber filled with a gas mixture of 95 % N_2, 5 % CO2 at 37 °C (Fig. 4). After 2h of incubation, cells were reintroduced to the regular

atmospheric oxygen level for an additional 48h in BME medium containing 10 % of FBS and Gentamicin. Cell viability was then measured with MTT assay (as described below).

4.3.1.2 OGD with primary neuronal cell culture

Cells were washed twice with 37°C warm PBS and incubated with Neurobasal medium without glucose (Gibco #A24775-01) and transferred into an anaerobic chamber filled with a gas mixture of 95 % N_2, 5 % CO2 at 37 °C. After 45 min incubation, cells were reintroduced to the regular atmospheric oxygen level for an additional 48h in Neurobasal A medium containing 0,1 % Gentamicin, 1x Glutamax and 2 % B27 serum. Cell viability was assessed by MTT assay and propidium iodide staining (as described below).

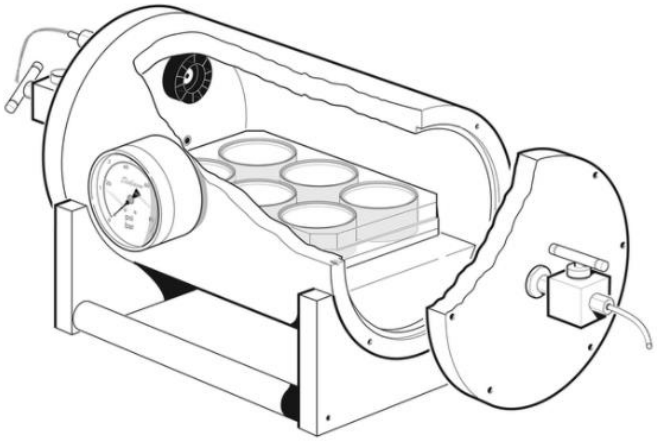


Fig. 4 Hypoxic chamber *(Banks et al. 2010)*

4.4 Cell characterization

4.4.1 Fluorescence Activated Cell Sorter analysis (FACS)

FACS analysis was performed as previously described (Gelderblom et al. 2009). Briefly, cells were trypsinized as described above. The pellet after centrifugation was resuspended with FACS buffer (0.5 % bovine serum albumin (BSA, Sigma #A7906), 0.02 % sodium azide in PBS and centrifuged at 350x*g* for 5 min at RT. After three washes, the pellet was suspended in 800 µl FACS buffer. 194 µl of the cell suspension was transferred into a FACS tube and 2 µl FC block (bioxcell

#CUSTOM24G2) was added. After 10 min, anti CD45 (BV421) (Biolegend #103134) and anti GLAST (PE) (Miltenyi Biotech #13095821)) antibodies were added in a dilution of 1:100 and incubated on ice for 30 min. The cell suspension was then centrifuged twice (350x *g*, 5 min, RT). The final pellet was diluted in 400 µl FACS buffer. Cells were analysed on a BD LSR Fortessa with FlowJo software. Up to 50.000 events per tube were acquired. Statistical analysis was performed by using unpaired t-test (Prism; Graph Pad). Data is shown as mean and standard error of the mean (SEM) of 8 independent experiments.

4.5 Immunocytochemistry

Immunocytochemistry was performed as previously described (Wang et al. 2012). Briefly, cells were plated on poly-L-lysine coated coverslips. When cells reached confluency (day 18 to 20), they were fixed with 4 % paraformaldehyde for 10 min at RT. After 3x 5 min washes with PBS, coverslips were incubated in PBS with 0, 1 % bovine serum albumin (BSA) and 0.3 % Triton X-100 for 1 h in a wet chamber to permeabilize the cells. Primary antibodies diluted in PBS containing 0, 1 % BSA were applied for 1 h at RT in a wet chamber. To label the astrocytes, rabbit anti-glial fibrillary acidic protein (GFAP) antibody (DAKO #Z0334) was used at a concentration of 1:800. A mouse anti-CD11b (Biolegend #101201) was used to label microglia at a concentration of 1:100. After 3x 5 min washes with PBS, cells were incubated with secondary antibody Alexa Fluor donkey anti-mouse 555 (Life Technologies, A31572) diluted in 0, 1 % BSA for 30 min at room temperature. After further washings in PBS, coverslips were mounted with mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Roth #416250531) onto glass slides. Finally, staining was assessed with the Leica SP8 confocal microscope. Further image analysis was performed by using LAS X software.

4.6 Cell viability assays

4.6.1 Propidium iodide (PI) nuclei acid staining

The PI staining was used to assess neuronal cell death after OGD treatments. PI is a commonly used fluorescent reagent which binds non-specifically to nucleic acids such as DNA and RNA. It is not able to infiltrate cells with an intact cell membrane and, therefore, only detectable in necrotic and dead cells (Niu et al. 2015). The PI staining was done after the OGD treatment, on day 14. Briefly the PI staining solution in the final concentration of 5µg/ml (immunochemistry technologies #638) was added to the cell media and incubated for 2h at 37°C. Afterwards, the medium was removed, and cells were washed three times with 37°C warm PBS. Then the cells were fixed with 4 % paraformaldehyde for 10 min followed by five more washes with PBS. Coverslips were mounted with mounting media containing DAPI onto glass slides, images taken with the LEICA TCS SP8 microscope and further analysed with the LAS X software.

4.6.2 Cell counting

Images were taken of primary neurons treated with 45 min OGD and incubated with EVs of several origins and conditions as depicted in Fig. 17. As a control, untreated primary neuronal cell cultures incubated with different EVs were used. For each condition, three overview pictures (magnification 40x) were taken, and propidium iodide-stained cells (nuclei and cytosol) were counted in relation to the total cell number. Each experiment was repeated three times and cell counting was performed in a blind fashion by three independent investigators.

4.6.3 MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega CellTiter 96 Non-Radioactive cell proliferation assay (#G400)) was used according to the instructions of the supplier. Viability was assessed 48h after OGD treatment. Briefly, cells were incubated for 4h with Dye Solution (0,1 µl of dye solution per µl medium) containing the tetrazolium salt which is converted to a

formazan product by living cells. Then Stop Solution in the same amount as the medium was added to solubilize the formazan product. Media containing the formazan product was transferred to 96 well plates and the absorbance measured at 570 nm using a 4BioRad microplate reader.

4.7 Extracellular vesicles

4.7.1 EV isolation from primary astrocyte and mixed glial cell cultures

Conditioned media, depleted from EVs present in the FBS was prepared as described (Thery, 2006). Briefly, BME containing 20 % FBS was centrifuged at 100.000x*g* for 18 h at 4°C (Beckman ultracentrifuge optima L-100 XP). After centrifugation, the supernatant was collected and filtered with a 0,22 µm filter (GE Healthcare). The conditioned media was then diluted 1:1 with (EV-free) BME to get 10% FBS. As shown in Fig. 5, either OGD treated or untreated cells were incubated at day 18 with EV-depleted conditioned media for 48 h. The media was collected and centrifuged at 300x*g* at RT to discard dead cells and cell debris. The supernatant was further filtered with a 0,22 µm filter. The remaining supernatant was then centrifuged with differential ultracentrifugation at 100,000xg for 70 min (Beckman Coulter Optima L-100 XP Ultracentrifuge. The final pellet was resuspended in PBS and stored at -80°C. Vesicles were quantified with a nanoparticle tracking device (NanoSight, Malvern Instruments) and further characterized by Western Blot.

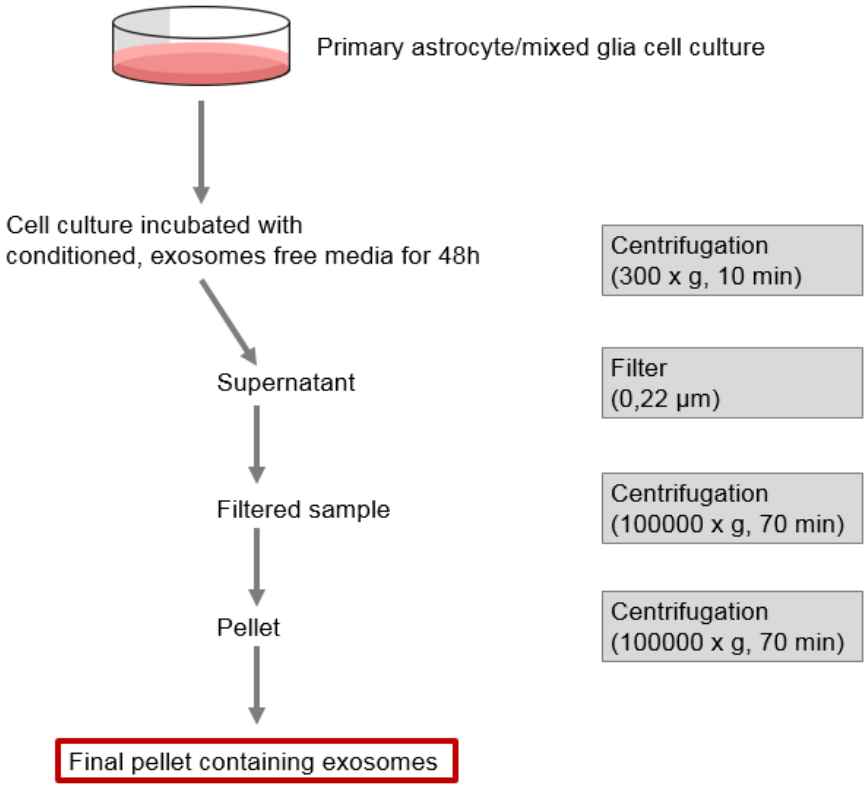


Fig. 5 Overview of the steps to isolate EVs secreted by primary cell cultures.

4.8 Western Blot

To characterize the EVs, 35 µl of EVs resuspension were incubated with 2x RIPA buffer (100 mM Tris-HCl, 300 mM NaCl, 2 % NP40, 1 % Na-Deoxycholate, 0,2 % sodium dodecyl sulfate (SDS)) containing protease inhibitors (Roche,# 11697498001) for 10 min on ice. Afterwards, samples were mixed with sample buffer (4x, Thermofisher # NP0007) and reducing agent (10x, Thermo Fisher # NP0009). The lysate was then heated 70°C for 10 min. 45 µl of the sample was loaded in a 10%Bis-Tris Gel 1,5 mm x 10 well (Thermo Fisher #NO0315BOX) together with a protein ladder (Thermo Fisher #26616) and a cell lysate from cell culture from which EVs were isolated for comparison. The chamber was filled with SDS running buffer (Thermo Fisher # NP0002) and 500 µl antioxidant (Thermo Fisher # NP0005). The gel ran for 1 h at 150 V. Afterwards, the proteins were transferred to a nitrocellulose membrane in a wet chamber in 10x sandwich buffer (Thermo Fisher #NP0006-1) with 10x methanol at 400 mA for 1h. The membrane was then incubated with blocking buffer (1x Rotiblock (Roth #A151.4) in TBST:

100 mM tris base, 1,4 M NaCl, pH7,4, 1 % Tween-20) to block unspecific union of the antibody. Membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-Alix (1:500, Milipore #ABC40), mouse anti-Flotilin (1:1000, BD Bioscience #610820), mouse anti-GM130 (1:1000, BD Bioscience #610822). Next day, membranes were washed three times for 5 min with TBST and then incubated with the appropriate HRP-conjugated secondary antibodies (Cell Signalling, 1:1000) in blocking buffer for 45 min followed by six washes for 5 min with TBST. Membranes were developed using the SuperSignal West Femto trial kit (Thermo Fisher #34095) with the Chemidoc xRS imaging system (BioRad). Further analysis was done with Quantity One 1 D Analysis software (BioRad).

5 Results

The aim of the present work was to assess the consequences of the communication of astrocytes and microglia through EVs in the survival of neurons after ischemia in an *in vitro* model of stroke by using primary cell cultures. For this, we have first established several methods.

The first part (5.1) includes the successful establishment of mixed glial and enriched astrocyte cell cultures by characterisation with FACS analysis (Fig.6 + 8) and immunocytochemistry (Fig. 7). Part two (5.2), summarizes successful EV isolation and EV characterisations (FIG 9 -11). In the third part (5.3), the establishment of primary neuronal cell cultures could be confirmed (Fig. 12 + 13). The OGD treatment was set by morphology, and MTT assay (Fig. 14 – 16). The experiments in the last part (5.4) illustrate the potential of the cell-to-cell communication between astrocytes, microglia, and neurons after stroke. Primary neuronal cell cultures were treated with OGD and incubated with EVs from mixed glia cell cultures or astrocyte cell cultures (Fig. 17). Their resulting cell viability was assessed (Fig. 18 + 19).

5.1 Primary mixed glial and astrocyte cell cultures

5.1.1 Primary mixed glial cell cultures are consistently populated by around 65% of astrocytes and 19% of microglia

To assess the amount of microglia and astrocytes present in the cultures, we performed FACS analysis on different incubation days (from 11 to 45). As shown in Fig. 6 we obtained about 65 ± 2.16 % SEM GLAST+ astrocytes and around 19 ± 3% SEM CD45 microglia (P<0.0001, Fig. 6 n=8). The remaining 16% constituted oligodendrocytes, fibroblasts, and other cell types, such as progenitor cells. We also observed that the different population frequencies remained stable until our last observation (day 45). Further characterisation was performed by immunocytochemistry as shown in Figure 7 a, b. As a result, a stable and reliable mixed glial cell culture protocol was prepared providing a reliable ground for further experiments.

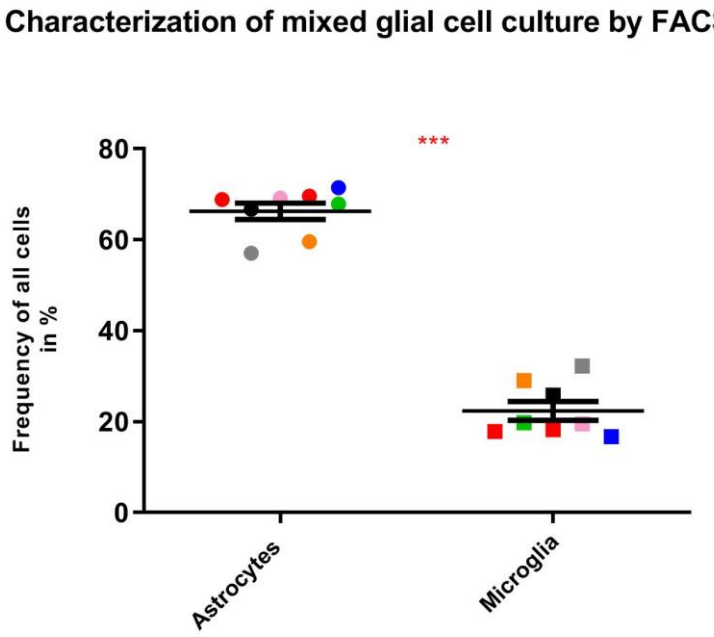


Fig. 6 Characterization of mixed glial cell culture by FACS.

Astrocytes were immunostained with GLAST (PE) and microglia with CD45 (BV421). Measurements were done on day 11 (2 times red), day 14 blue, day 15 green, day 18 pink, day 34 orange, day 35 black and day 45 grey without any significant change in cell population.

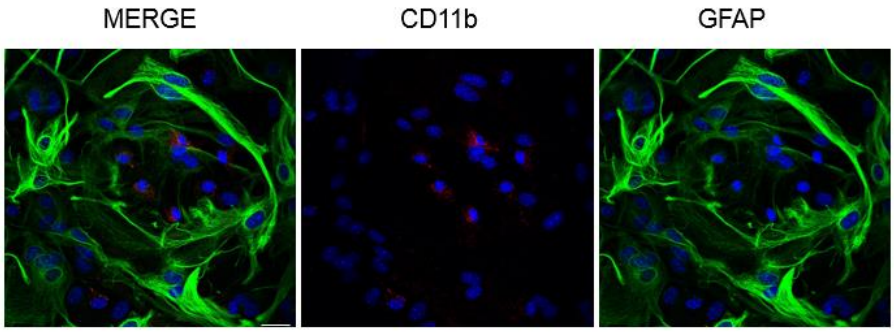


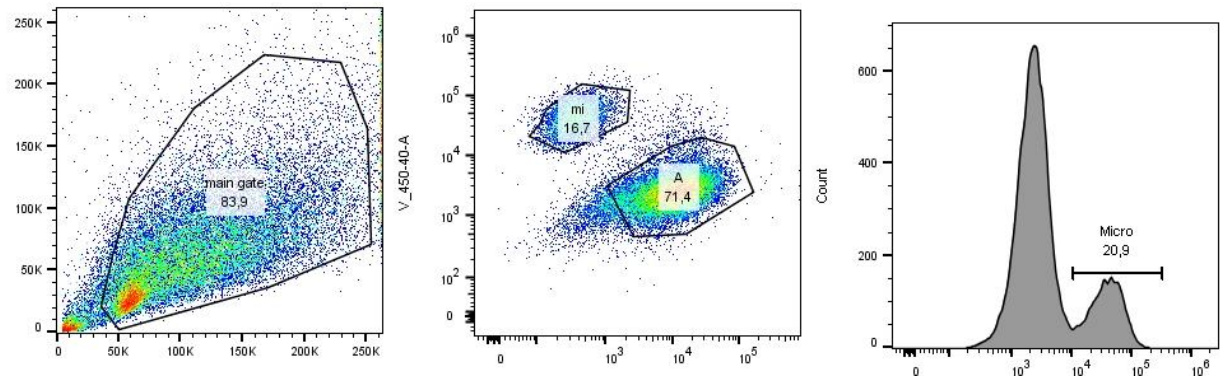
Fig. 7 Immunocytochemistry of mixed glial cells.

Confocal images of immunofluorescent staining of CD11b (in red) and GFAP (in green)). Nuclei are stained DAPI (blue).
Scale bar: 25µm.

5.1.2 Primary astrocyte cell culture consists of 98% astrocytes

To study the potential communication axis between microglia and astrocytes, we generated a pure astrocyte cell culture. A potential activation of astrocytes, due to microglial-derived EVs could be excluded because of the absence of microglia. To obtain a pure astrocyte culture, microglia were depleted from primary mixed cell cultures on day 14. The depletion was performed by magnetic separation with CD11b antibodies exclusively recognising microglia. FACS analysis was conducted before and after depletion with antibodies for CD45 a well-known marker for microglia, and GLAST, a glutamate transporter expressed exclusively on astrocytes (Cosenza-Nashat et al. 2006, Zhang et al. 2019). We observed that after depletion only GLAST + astrocytes were detectable (Fig. 8 a, b). Hence, further experiments were done with a pure astrocyte cell culture.

A | before depletion



B | after depletion

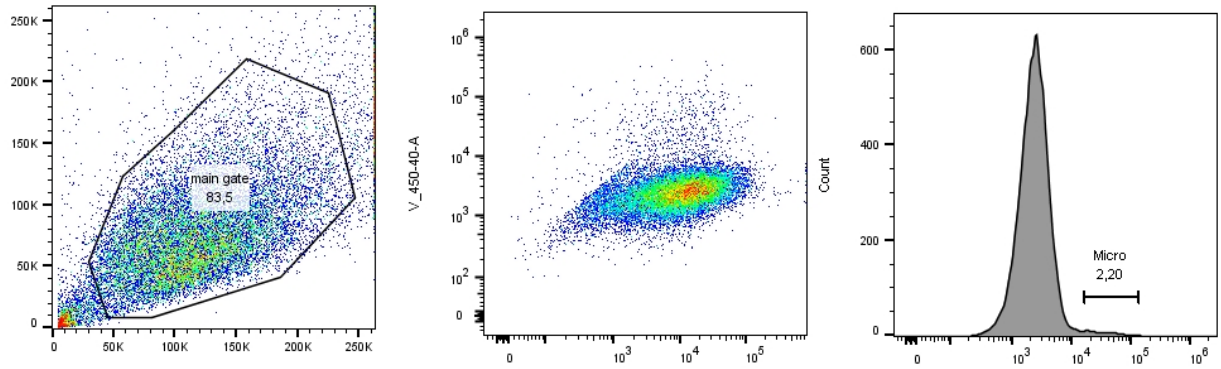


***Fig. 8 FACS analysis and gating strategy for mixed glial cell culture**.*

Cells were immunostained with CD45 (BV421) for microglia and GLAST (PE) for astrocytes.
***A** | the histogram shows a presence of 20,9% CD45 positive cells.*
***B** | shows the remaining cells after the microglia depletion, which are mainly GLAST positive.*
The histogram in B shows a decrease in CD45 positive cells to only 2,2%.

5.2 Cell culture derived EVs

5.2.1 Isolated EVs have an average diameter of 50 – 150 nm

Following the protocol previously described, EVs were isolated from conditioned, EV-free media and analysed with the Nanoparticle tracking analysis (NTA). Figure 9 shows the quantification and size of isolated EVs. Since the sample was diluted 15-times, the concentration value provided by the software was multiplied by 15. EVs were analysed under light scattering mode. EV size was heterogeneous ranging from 30 to 480 nm, with a mean size of 153.2 nm, and mode of 124.5 nm. The mean concentration was 9.23 × 10^8 particles/ml (*n*=38).

Those results prove to fit with previously published data of the *International Society for Extracellular Vesicles, ISEV* (Thery et al. 2018). Additionally, the isolated EVs were visualised by electron microscopy which confirmed the size and integrity of our isolated EVs (Fig. 10).

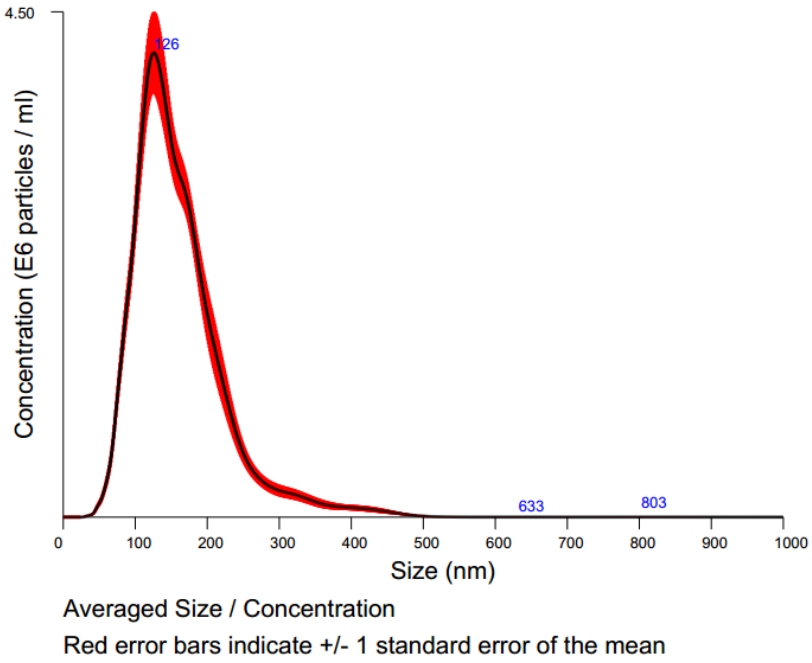


Fig. 9 Quantification and sizing of EVs

using the NanoSight particle counter. X-axis represents particle size and the y-axis represents the mode peak particle concentration. The total particle concentration is represented by the areas under the curves Values are reported in the main text.

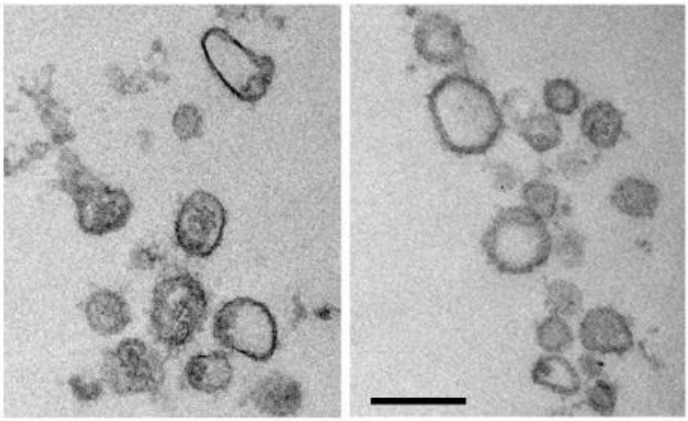


Fig. 10 Transmission electron microscopy

of EVs isolated from conditioned media of primary mixed glial cell cultures. Scale bar 200 nm.

5.2.2 Western Blot characterisation show that isolated EVs contain Alix and Flotillin I and Prion Protein

EV samples were characterized with known markers of EVs by western blot (Thery et al. 2006). The known markers of EVs such as the cytosolic protein Alix (96 kDa), the membrane-bound flotillin I (49 kDa) and prion protein (30 kDa) were detected in the EV-enriched sample (Fig. 11).

According to the minimal information for studies of extracellular vesicles (MISEV) guidelines, Alix is a commonly accepted cytosolic marker for EVs (Thery et al. 2018). Endosomal sorting complexes required for transport (ESCRT) machinery are involved in the formation of the MVEs as illustrated in Figure 2 (Hurley 2010, van Niel et al. 2018). The ESCRT complexes consist of several proteins, where Alix plays crucial role as a homodimer accessory protein, by targeting the ESCRT machinery (Hurley 2010). Furthermore, MISEV guidelines approved Flotillin I as a general accepted membrane associated marker for EVs (Thery et al. 2018). Several studies indicate that Flotillin I plays role in the EV formation process and even influences EV-cargo (Meister and Tikkanen 2014, Phuyal et al. 2014).

The Golgi protein GM130 is commonly used as a negative marker and should not be present in EVs (Weide et al. 2001, Thery et al. 2018). The sample was negative for GM130, showing absence of contamination (Tkach et al. 2018).

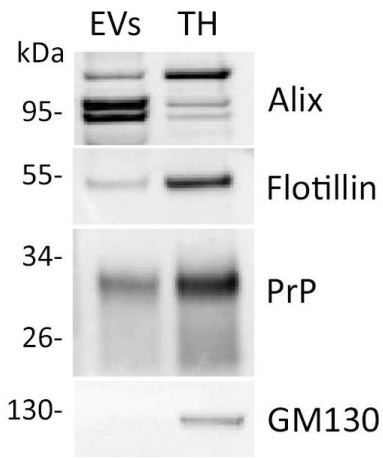


Fig. 11 Characterization of conditioned media isolated EVS.

Western blot s of secreted EVs isolated from conditioned media of mixed glia culture developed with the with the EV markers Alix, Flotilin and PrP is shown. TH is cell lysate.

5.3 Primary neuronal cell culture and the *in vitro* model of stroke by oxygen-glucose deprivation (OGD)

5.3.1 Determining the right density and OGD treatment time for primary neuronal cell cultures

It is known that neuronal cell density is an important factor to consider ensuring viable cells that can create a neuronal network and enough synaptic contacts (Previtera et al. 2010, Biffi et al. 2013). Therefore, we established a protocol for primary neuronal cell cultures. As shown in Figure 12, we could observe that by increasing the density, there was a visible improvement on the healthy appearance of primary neurons. To ensure the maturity of the primary neuronal cell culture, cells were stained with known markers of matured primary neurons on 10 days *in vitro* (NeuN) (Fig. 13)

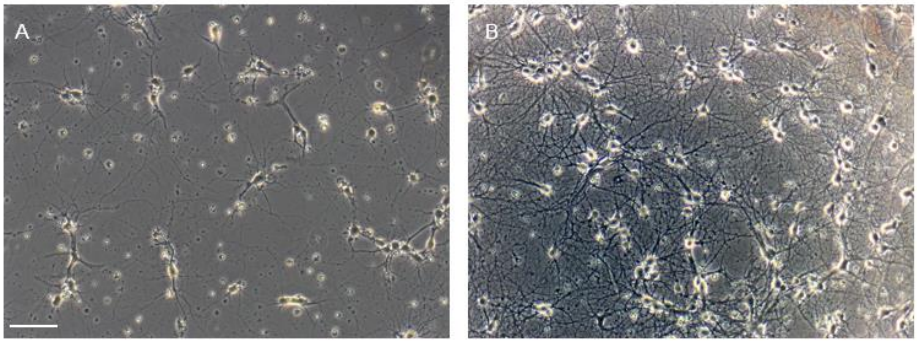


Fig. 12 Morphological overview of primary neuronal cell culture

A *| Primary neuronal cell culture day 12, 12 well plate, 80 000 cells per well.*
B *| Primary neuronal cell culture day 12, 12 well plate, 180 000 cells per well Magnification 20x. Scale bar 50 µm*

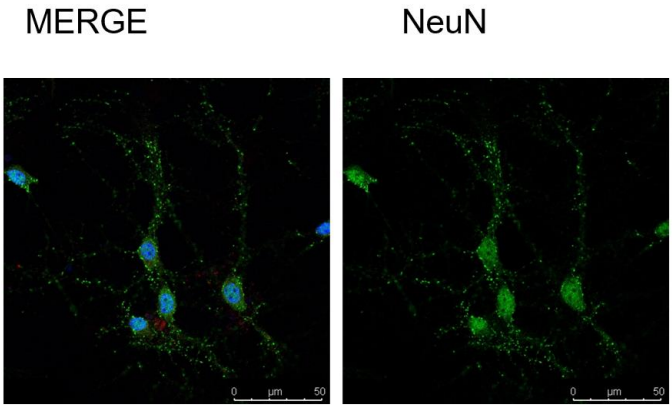


Fig. 13 Immunocytochemistry of primary neurons.

Immunofluorescent staining of NeuN (in green). Nuclei are stained DAPI (blue). Scale bar: 50µm.

5.3.2 Establishment of OGD conditions to mimic ischemic cell death

OGD is a method that allows to mimic ischemic stroke in an *in vitro* model with primary cell cultures (Tasca et al. 2015). Using this method, it was important to determine the time of OGD treatment in which primary neuronal cell cultures are stressed but not lethally affected, i.e., about 50% of survival. Hence, we established the correct time-point of OGD where around 50% of neurons survive. Initially, OGD exposure of 30 min was applied. To our surprise, and as it is shown in Figure 14, treated cells with lower density were benefiting from the 30 min OGD treatment in comparison to untreated control. The morphology of the treated primary cell culture in this case was characterised by an enlargement of axons and neurite branches connecting neurons. Because we aimed to stress the primary neuronal cell cultures with OGD treatment the time was then extended to 1h. Figure 15 shows a clear increase of survival after 30 min OGD (cell viability 430.5 ± 40. 5 % SEM) in comparison to untreated (cell viability set to 100%) and 1h in treated cell cultures (cell viability 68. 5 ± 24. 4 % SEM). This was a decisive factor for the final OGD treatment time of 45 min, for the sake of stressing the primary neuronal cell cultures and to simultaneously prevent increased cell death. Hence, primary neuronal cell cultures (three replicates in 48 well-plates) with a density of 45.000 cells per well and coverslips in 12 well plates with a density of 180.000 cells per well were seeded and cells subjected to 45 min OGD treatment on day 9.

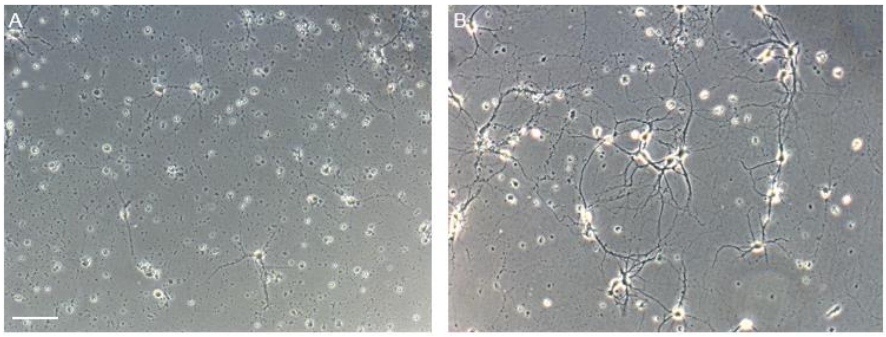
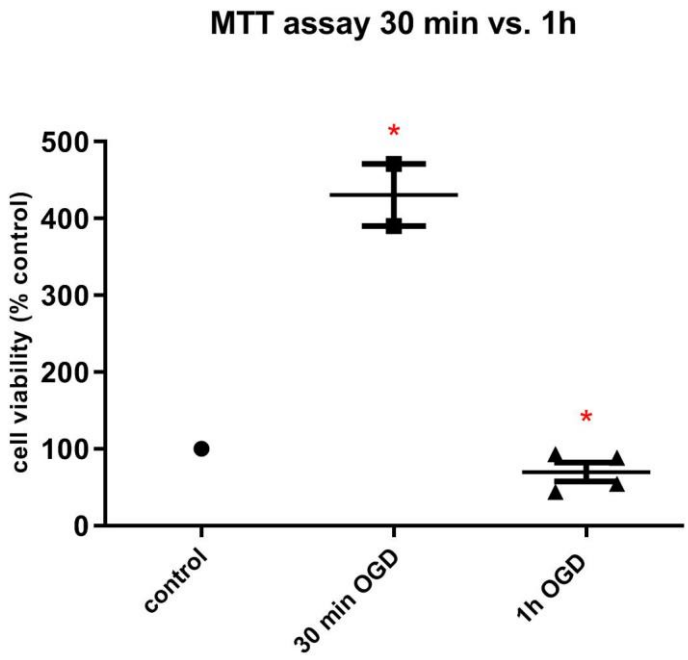


Fig. 14 Morphological overview of primary neuronal cell culture.

A *| primary neuronal cell culture day 14*
B *| primary neuronal cell culture day 14 after 30 min OGD on day 12. Magnification 20x. Scale bar 50 µm.*



Fig, 15 Graphical representation of the cell viability (MTT assay)

*of primary neuronal cell culture treated with 30 min or 1h OGD. Measurements were done 48h after treatment. The results are normalized with an unexposed control. (**P<0.01) set to 100%.*

5.3.3 Two hours of OGD results in 67% viability in mixed glial cell cultures

The aim of this experiment was to establish the appropriate time where astrocytes and mixed glial cell cultures suffer from stress but not detrimental lack of oxygen and glucose, thus mimicking the penumbra. Hence, we wanted to establish the time-point where about half of the cells survived. We set up several time points of OGD duration (1h, 2h, 6h and 8h) and cell viability was assessed. We chose to treat mixed glial and astrocyte cell cultures with 2h OGD because the resulting viability of treated cells measured with MTT assay was 67.91 ±15,75 % SEM (Fig. 16).

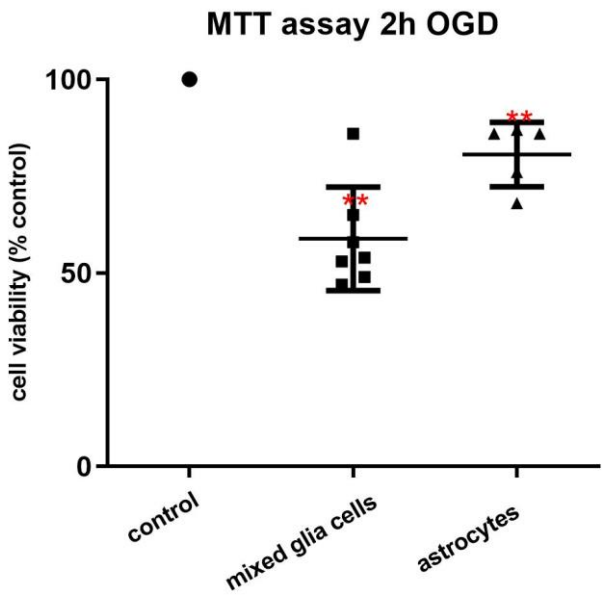


Fig. 16 Graphical representation of the percentage of cell viability (MTT assay)

*of mixed glial cell culture (n=7) and astrocyte cell culture (n=5) treated with 2h OGD on day 9. Measurements were done 48h after OGD treatment. The results are normalized with an unexposed control. (**P<0.01) set to 100%.*

5.4 Primary neuronal cell cultures under ischemic stress incubated with glial EVs

5.4.1 EVs isolated from primary astrocyte and mixed glial cell cultures under ischemic stress show increased neuroprotection to neurons subjected to OGD

Immediately after OGD, primary neurons were incubated with 6.25 x 10^5 EVs per 48-well and 2.5 x 10^6 EVs per 12-well with either, treated (2h OGD) or untreated EVs derived from astrocyte cell culture (A treated, A untreated), or with EVs derived from mixed glial cell culture, (M treated, M untreated) as shown in the scheme of Fig. 17. Cell viability was measured 48h after EV-incubation. Figure 18 gives an overview of the MTT assay results in which untreated viable neurons are defined as control with 100% viability. There were no statistically significant differences between the treatments, however, neurons subjected to OGD and incubated with treated EVs indicate a tendency towards higher cell viability.

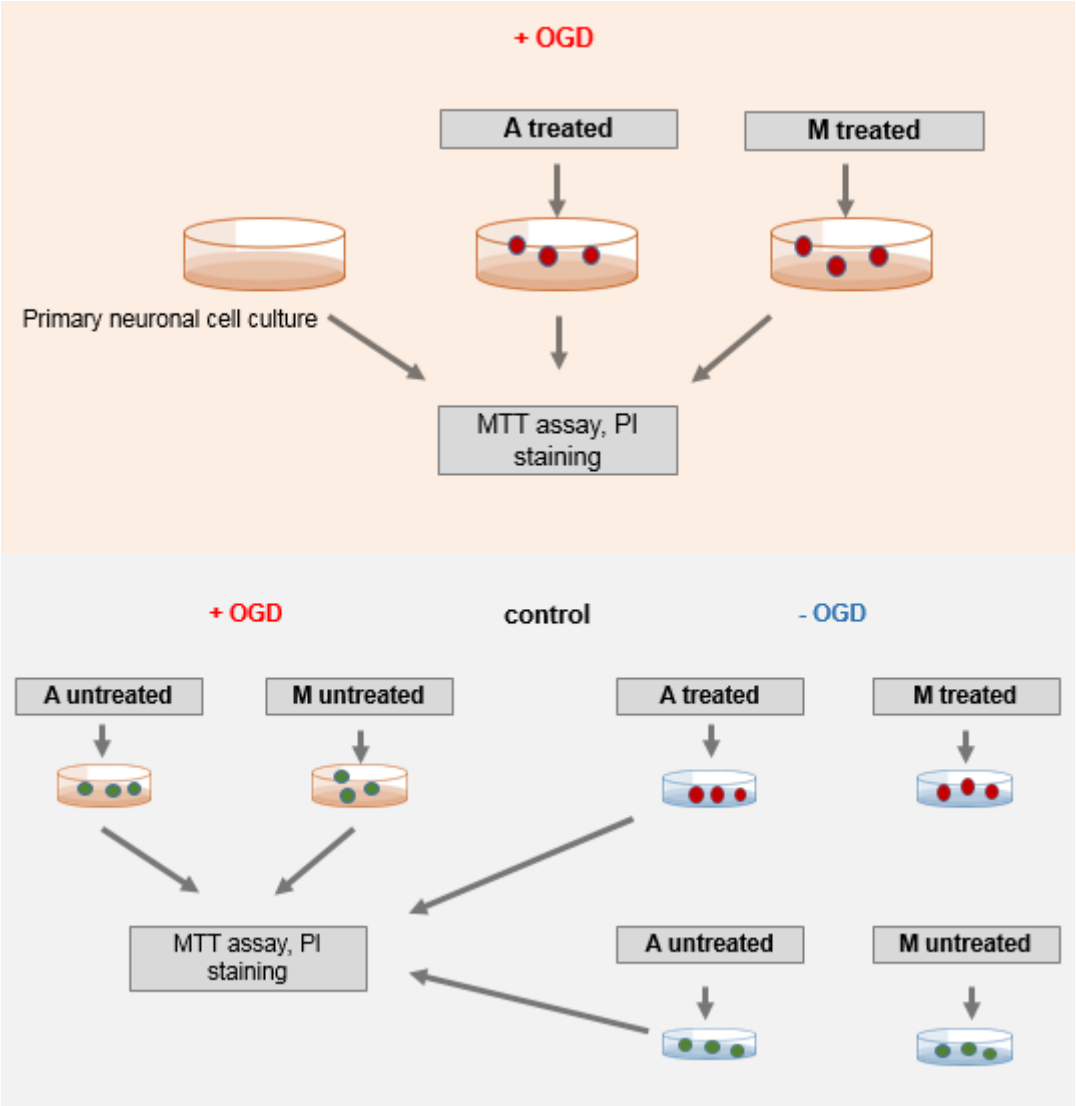


Fig. 17 Schematic overview of primary neuronal cell culture treatment

Primary neuronal cell cultures were treated with 45 min OGD and directly incubated with a certain amount of EVs derived from OGD treated astrocytes (A treated) or mixed glia (M treated) (6,25 x 10^5 EVs per 96-well, 2,5 x 10^6 EVs per 12-well). Cell viability was measured 48 h later with MTT assay and propidium iodide staining. As a control, OGD treated neurons were incubated with EVs derived from untreated astrocytes (A untreated) or mixed glia (M untreated) and untreated neurons were incubated with OGD treated and untreated EVs derived from astrocytes or mixed glia. Controls can be found on the supplement data.

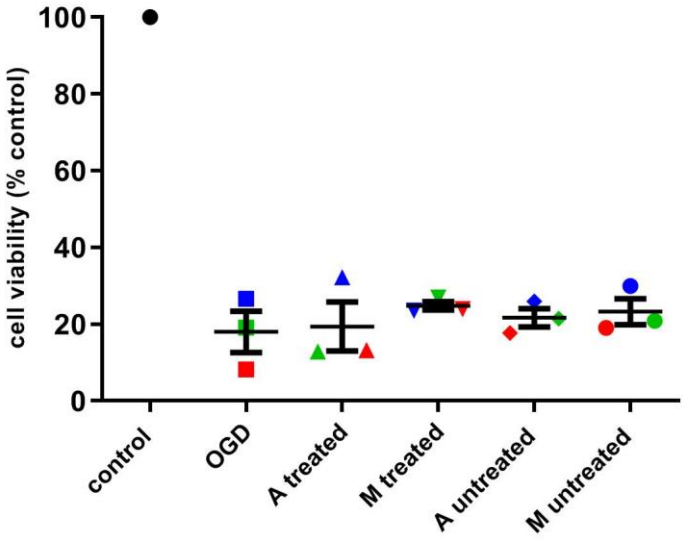


Fig. 18 Graphical representation of cell viability (MTT assay) of OGD-subjected primary neuronal cell culture treated or not with EVs.

Primary neuronal cell culture treated with 45 min OGD were incubated with 6,25 x 10^5 astrocyte EVs either treated with 2h OGD (A treated) or untreated (A untreated) and EVs from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 12. (n=3). p>0,05. Each color represents one distinct neuronal cell culture. Measurements were done 48h after OGD treatment. The results are normalized with an unexposed control set to 100%.

Additionally, OGD-subjected primary neuronal cell cultures treated with EVs, plated on coverslips were stained with propidium iodide. Figure 18 shows the values obtained from the cell viability assessment indicating decreased cell death when neurons were incubated with EVs (independently of the treatment and cell origin) although differences are not statistically significant (p=0,512).

In the supplement data (Suppl. Fig 1-5), graphs of the total mean between the three countings and each single graph can be found.

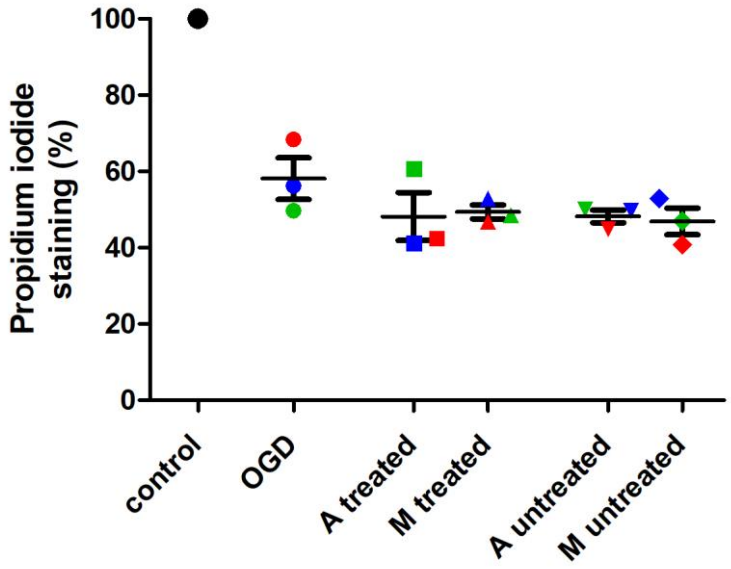


Fig. 19 Graphical representation of cell viability (Propidium iodide staining) of primary neuronal cell culture subjected to OGD, incubated with EVs from either treated or non-treated astrocytes or mixed cultures.

Primary neuronal cell culture was incubated with 2,5 x 10^6 EVs per well derived from astrocyte cell culture either treated with 2h OGD (A treated) or untreated (A untreated) and derived from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 18. (n=3). p>0,05. Each color represents one distinct neuronal cell culture. Propidium iodide staining was done 48h after OGD treatment. Images were taken with Leica TCA SP8 X confocal microscopy.

6 Discussion

In ischemic stroke, the blockage of a main cerebral artery, causing lack of oxygen and glucose initiates a sterile inflammatory process which persists even after reperfusion (Dirnagl et al. 1999). The sterile post-ischemic inflammation causes secondary cell damage, which is involved in the final clinical outcome (Iadecola and Anrather 2011). Main players on the cerebral inflammation are resident glial cells such as astrocytes and microglia, but also infiltrating immune cells (Dirnagl et al. 1999, Gelderblom et al. 2009, Jin et al. 2013). In the activated sterile neuroinflammatory cascades after stroke, microglia proliferate rapidly after stroke, as presented in Figure 20 A (Gelderblom et al. 2009).

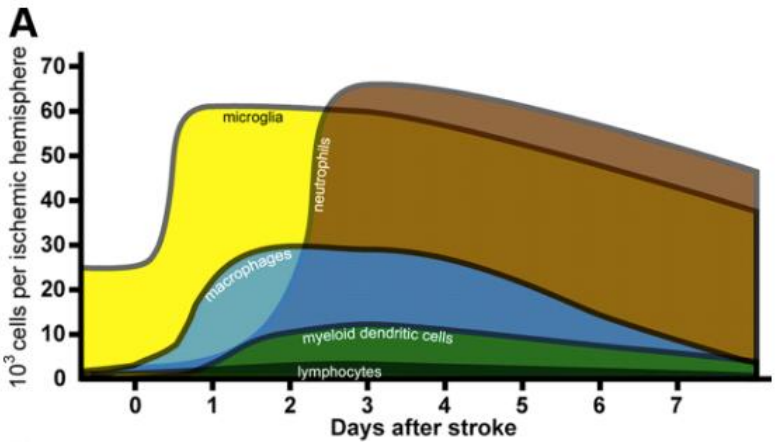


Fig. 20 A |Overview of transitory dynamics of post-ischemic inflammation

Numbers of cells per hemisphere found ipsilesional to the infarct. Curves are extrapolated from data obtained for sham conditions, 12 hours, 1-, 3-, and 7-days post-reperfusion. (Gelderblom et al. 2009)

These resident glial cells are contributing and initialising the post-ischemic inflammation through a highly complex cell communication axis (Iadecola and Anrather 2011). Known cell to cell communication and intercellular signalling are direct cell to cell contact, paracrine secretion, and GAP junctions (Lodish et al. 2000, Mittelbrunn and Sanchez-Madrid 2012, Fruhbeis et al. 2013). Liddelow *et al*. demonstrated intercellular communication between microglia and astrocytes through molecules such as IL-1α, TNF and complement subcomponent 1q (C1q). They showed that LPS stressed microglia did not harm neurons. However, through activation of astrocytes neuronal harm was observed. Hence, the communication

axis between microglia and astrocytes seems important for the astrocytic response and plays a crucial part in the inflammatory reaction (Liddelow et al. 2017). Based on these findings the present a study that aimed to investigate whether the communication between microglia and astrocytes through EVs had a role in the survival of neurons after ischemia. With this purpose, we have performed several *in vitro* experiments.

Firstly, we have established a protocol to generate mixed glia cell cultures stably populated with 65% of astrocytes and 19 % of microglia. We have completed a protocol to generate a highly enriched astrocyte cell culture. Next, we established a working protocol to treat glial and neuronal cell culture with an *in vitro* model of stroke to stress the cells mimicking the penumbra area. Our aim was to establish the best neuronal culture conditions to obtain a reliable result after OGD. For this, we followed previously described protocols and plated cells at different densities (Beaudoin et al. 2012). As previously described, primary neurons need a certain density to generate enough cytokines and growth factors to stimulate cell growth (Previtera et al. 2010). Therefore, we adjusted the right density of cells (Fig. 12). Primary neurons were initially exposed to 30 min OGD, because several studies indicate that a time frame between 30 to 60 min OGD-exposure decreases neuronal survival up to 50 % (Gidday 2006, Gao et al. 2015). Unexpectedly, primary neuronal cell cultures showed a clear increase of survival and appeared stronger after 30 min OGD in comparison to untreated cells and 1h treated cell cultures (Fig. 14). Due to the lower density used first, the morphology of primary neurons was characterised by few and small neurite branches and axons. We hypothesized that exposure to 30 min OGD might have served as a stimulus to alter pro survival gene activation and protein synthesis (Gidday 2006). We hypothesize that a mechanism known as ischemic preconditioning might have occurred in these neuronal cell cultures, leading to stronger primary neuronal cell cultures in comparison to untreated control (Gidday 2006). Since our aim was to stress primary neuronal cell cultures, the final experiments were performed with a higher density and with an exposure time of 45 min OGD.

We established a protocol to successfully isolate EVs, confirmed by western blot, electron microscopy and NTA.

Our cell viability measurements by PI staining and MTT assay showed a tendency to a higher viability in the neuronal cell cultures incubated with EVs.

Several studies have confirmed communication between glial cells through molecules i.e., cytokines, microRNA, and the transportation of these molecules through EVs (Fruhbeis et al. 2013, Hayakawa et al. 2016, Yang et al. 2021). Hayakawa *et al*. demonstrated the astrocytic secretion of vesicles containing mitochondria. In here, neuronal cell cultures were treated with OGD and incubated with conditioned astrocytic media containing mitochondria-loaded vesicles. Interestingly, an increase in neuronal cell viability was detected in neurons which took up the mitochondria-loaded vesicles in comparison to OGD-treated neurons incubated with media depleted from those vesicles. Further, in an *in vivo* model of stroke (transient focal ischemia in mice), mice were injected with astrocytic media containing vesicles with labelled mitochondria. They showed that labelled mitochondria were taken up by neurons in the penumbra, thus proposing the transfer of mitochondria from astrocytes to neurons, leading to neuronal rescue in stroke (Hayakawa et al. 2016). Additionally, a recently published paper shows that OGD-treated primary neurons secrete EVs containing microRNA-98. These EVs are taken up by microglia which prevents microglial phagocytosis of salvageable neurons. This suggests a bidirectional communications axis between neurons and glia cells. (Yang et al. 2021). In other studies, in our lab, we have also demonstrated that brain derived EVs are taken up by primary neuronal cell cultures (Fig. 21.) (Brenna et al. 2020). One of the reasons that could account for the lack of significance in our experiments is the limited number of neurons counted. Thus, increasing the number of experiments and the number of neurons assessed might increase significance.

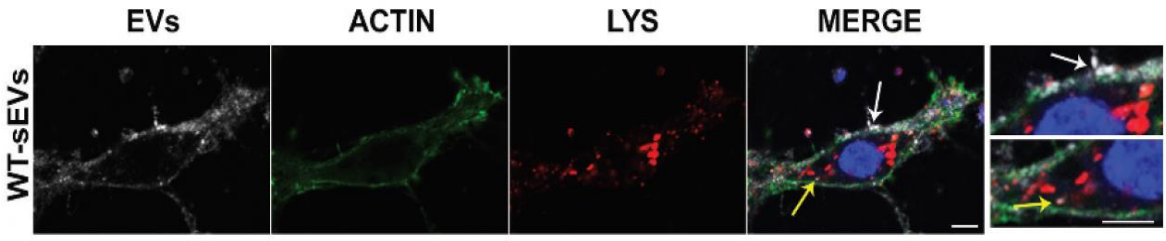


Fig. 21 Brain-derived small EVs are taken up by primary neurons.

Confocal microscopy images of primary neurons from WT mice (labelled with phalloidin in green) incubated for 3h with sEVs labelled with mCLING (white). Lysosomes were labelled with LAMP-1 (red) and nuclei stained with DAPI (blue). White arrows show sEVs in the plasma membrane and yellow arrows indicate colocalization with LAMP-1.
Scale bar is 5 µm (Brenna et al. 2020).

Of note, by comparing all assessed neuronal viability, measured by PI staining, in the single research-assessment summarized in Fig. 19, we could observe a tendency of enhanced neuronal viability of OGD-treated neurons incubated with EVs derived from OGD-treated astrocytes (if we do not consider the outlier). This is interesting as it suggests a neuroprotective effect from the astrocytes towards neurons rather than a detrimental effect. This effect is abolished when astrocytes are cultivated in the presence of microglia, as then, the mixed EVs (from microglia and from astrocytes) have negative effect on the neuronal viability. Hence, it could be that microglia have a detrimental effect on astrocytes as shown by Liddelow *et al*. (Liddelow et al. 2017). Thus, our results show that EVs derived from astrocytes transport a rather neuroprotective cargo and only in the presence of microglia, astrocytic EVs decrease neuronal viability after ischemia.

A neuroprotective effect from astrocyte-derived EVs on primary neuronal cell cultures subjected to ischemia has been already observed (Wang et al. 2011, Guitart et al. 2016, Hayakawa et al. 2016). In an *in vitro* study Guitart *et al*. demonstrated that astrocyte-derived EVs enhance the cell viability of primary neuronal cells stressed by OGD and that the neuroprotective effect was related to the presence of PrP. They treated neuronal cell cultures with OGD and incubated them with hypoxia-stressed astrocyte-derived EVs, isolated from either wild-type astrocytes or from PrP-deficient astrocytes. They observed a higher neuronal survival in the group incubated with wild-type astrocyte-derived EVs. These results indicate that astrocytes transport protective PrP through EVs. The authors suggest that PrP changes conformation under ischemic stress, leading to the loss of binding to clusterin/apolipoprotein J (heat shock protein associated with apoptosis). The bonding of clusterin/apolipoprotein J to PrP is necessary to transport them via EVs to neurons. By the inability of clusterin/apolipoprotein J to bind to PrP they cannot be transported to neurons and therefore they cannot induce apoptosis. (Han et al. 2001, Guitart et al. 2016). Despite the inhibition of bonding PrP to cluster/apolipoprotein J, enhanced levels of S3 and P0 ribosomal proteins, apolipoprotein E and laminin receptors were observed in the astrocyte-derived EVs. These are playing roles in neural repair and neuroprotection (Beffert et al. 2006, Ahn et al. 2013, Guitart et al. 2016). Interestingly, we confirmed the presence of PrP in our isolated EVs (Fig. 11) which could account for the neuroprotective tendencies of astrocyte-derived EVs. However, the molecular

mechanism whereby PrP and PrP-related proteins exert a protective role in ischemia is not fully understood.

An additional experiment by Wang *et al.* also showed beneficial effects of astrocyte-derived EVs. In their study, primary neuronal cell cultures were subjected to OGD, followed by incubation with EVs isolated from astrocyte-enriched cell cultures. They could show that these EVs contained synapsin I. When primary neuronal cell cultures grown with substrate coated synapsin I were submitted to oxidative stress (by the presence of hydrogen peroxide), they observed higher cell viability rate in comparison to control groups coated with only poly-l-lysin. They demonstrated that synapsin I, as an oligomannose-binding lectin, binds to NCAM (neuronal cell adhesion molecule, involved in neurite outgrowth). When comparing primary hippocampal neurons and NCAM-deficient primary hippocampal neurons both incubated with synapsin I, they showed enhanced neurite outgrowth only in the NCAM-expressing primary neurons. Hence, under oxidative stress, glial cells secrete EVs containing synapsin I which bind in an oligomannose-dependent interaction to NCAM on neurons and promote neurite outgrowth (Wang et al. 2011).

Furthermore, astrocyte-derived vesicles transport whole organelles such as mitochondria. It was demonstrated that in stroke, this could be a mechanism that leads to neuronal recovery by supporting cell viability (Hayakawa et al. 2016). In another study Pascua-Meastro *et al.* demonstrated the transport of Apolipoprotein D (ApoD) by astrocytes through EVs, which is neuroprotective. They treated primary neurons with paraquat treatment, triggering an oxidative insult, and incubated them with EVs isolated from astrocytic media containing ApoD, or with EV-depleted media. They observed higher neuronal viability by neuronal uptake of ApoD-loaded astrocytic EVs (Pascua-Maestro et al. 2018). They argue that ApoD protects neurons from oxidative-stressed-triggered membrane permeabilization by reducing free-radical generating lipid hydroperoxides through modification of gene expression (Bajo-Graneras et al. 2011, Pascua-Maestro et al. 2018).

These studies indicate a beneficial and protective role of astrocyte-derived EVs in ischemia. The exact processes by which these EVs influence neuronal reaction to ischemia are not understood yet and must be investigated further. Even though, these studies demonstrated beneficial and protective roles of EVs, their influences

are much more complex and multifaceted and probably the spatio-temporal pattern also play an important role. For example, the transfer of cleaved IL-1β can have pro- and anti-inflammatory effects in recipient cells (Hewett et al. 2012). IL-1β is a proinflammatory cytokine, that activates further acute-phase proteins, contributing to inflammation (Dinarello 2005). Measuring the viability of primary neurons co-cultured with astrocytes and treated with IL-1β showed higher neurotoxicity in comparison to pure primary neurons incubated with IL-1β. This indicates that IL-1β promotes neurotoxicity in the presence of astrocytes. which would induce neurotoxicity by caspase activation (Thornton et al. 2006). On the other hand, in an *in vivo* model with IL-1β$^{-/-}$ mice the presence of IL-1β appears to be a necessary factor to produce insulin-like growth factor 1 (IGF1), which promotes proliferation (Mason et al. 2001). Several studies indicate the bidirectional protective and harmful effect that IL1-β can have in ischemia, still leaving open the exact mechanism on which these effects might depend on (Hewett et al. 2012).

Related to the crosstalk between microglia and astrocytes, Drago *et al*. demonstrated in their *in vitro* study a bidirectional influence between these two types of glia. They isolated EVs secreted by primary microglia stimulated with ATP and incubated them with primary astrocytic cultures. By q-PCR they were able to detect an increase of IL-6, IL-1β and IL-10 in astrocytes being exposed to ATP-stimulated microglial EVs (Drago et al. 2017). These cytokines are known to have both, pro and anti-inflammatory properties (Mason et al. 2001, Hewett et al. 2012, Drago et al. 2017). Drago *et al*. observed an increase of metabolic enzymes in ATP-stimulated microglial EVs suggesting that those enzymes might act as metabolic sources for the recipient cell such as astrocytes which might be beneficial for neuronal fate. However, they insist on intensifying further studies detecting the impact on astrocytic metabolism (Drago et al. 2017).
Additionally, it has been shown that astrocytes can activate distant microglia. For example, Lehrmann *et al*. analysed the activation of microglial cells by morphological transformation and upregulation of the constitutive microglial/macrophage markers (OX42 Ab - *complement type-3 receptor*, OX18 Ab - *MHC I marker*, OX1 Ab - *leukocyte marker*, ED1 Ab - *lysosomal macrophage marker*, OX6 Ab - *MHC II marker*) in immunocytochemistry in a rodent TMCAO

(transient middle cerebral artery occlusion) model. Here even microglia located far distant from the infarct zone and penumbra in undamaged tissue were activated (Lehrmann et al. 1997). Those distant activation may be explained by astrocytes activating distant microglia via waves of intracellular Ca^{2+} elevation (Schipke et al. 2002, Liu et al. 2011). Schipke *et al*. induced Ca^{2+} waves in mice brain slices. Reacting astrocytes were labelled with enhanced green fluorescence protein (EGFP) and red-shifted fluorescent Ca^{2+} Orange, allowing simultaneously imaging of astrocytes and intracellular Ca^{2+}. When Ca^{2+} waves were induced in mice brain slices the Ca^{2+} Orange labelling showed reaction of Ca^{2+} waves in non-astrocytic glial cells in distant areas with an average speed of 13.9 +/. 1.8 µm/s (*n*=26). They could confirm microglial reaction over hundreds of micrometres away from the original stimulation by recording microglial membrane currents (Schipke et al. 2002). Astrocytic Ca^{2+} waves are initialised by increased extracellular ATP which is known to be elevated in stroke (Dirnagl et al. 1999, Verderio and Matteoli 2001, Liu et al. 2011).

EVs are an intense focus of study not only as communication tools but also for their therapeutic potential in stroke (Chen and Chopp 2018, Li et al. 2018). Many studies have been focusing on the beneficial effects of mesenchymal stem cells (MSCs) because they promote cell proliferation, repair processes and regeneration by releasing trophic growth factors and anti-inflammatory factors (Li et al. 2018). It could be shown that MSCs have beneficial effects in stroke by reducing the infarct volume, promoting neuronal plasticity and recovery (Xin et al. 2013a, Xin et al. 2014, Puig et al. 2018). But, in fact, MSCs perform their beneficial and promising effects through EVs containing certain growth factors, cytokines, microRNA and mRNA (Xin et al. 2013a, Xin et al. 2013b, Xin et al. 2014, Chang et al. 2018). MSC-derived EVs are secreted in large amounts and, therefore, can be isolated easily (Li et al. 2018). Initial studies showed beneficial anti-inflammatory effects such as reduced cerebral injury, neurite remodelling and functional recovery by intravenous injection of MSC-derived EVs in rodent models (Xin et al. 2013a, Chang et al. 2018, Li et al. 2018). The fact that EVs can transport mRNA and microRNA indicates that gene regulatory processes are initialised in the recipient cells (Chen and Chopp 2018).

Another potential therapeutic approach is to modify the cargo of EVs, for example, by loading them with regulatory microRNAs which has beneficial therapeutic effects in stroke, such as miR-124 (Xin et al. 2014, Chang et al. 2018, Li et al. 2018). MiR-124 is one of the most abundant microRNA in neurons and several *in vivo* studies illustrated a correlation between infarct size and serum concentration of miR-124 in patients (Lagos-Quintana et al. 2002, Weng et al. 2011, Ji et al. 2016). MiR-124 has anti-inflammatory and regenerative functions, such as promoting neuronal restoration and angiogenesis. Injecting miR-124 in mice after treating them with a well-established stroke model, resulted in reduced infarct size, enhanced functional recovery and increased angiogenesis (Sun et al. 2015). Based on these results, a clinical trial is established where stroke patients are treated intravenously with MSC-derived EVs containing miR-124 (Oraee-Yazdani 2017).

Focussing on our result, we observed a tendency towards higher viability in neurons incubated with EVs after ischemia. Nevertheless, limited validity is caused by the small number of experiments. Thus, additional experiments are necessary to understand glial EVs influences in OGD treated neurons. Besides MTT assay and propidium iodide staining, neuronal electrical activity can be measured, and further characterisation of signalling pathways activated in neurons together with a full picture of EVs cargo (e.g., proteins, mRNA, lipids) will contribute to increase the understanding of our results.

All in all, EVs, as a field of research has developed remarkably. Since its founding in 2011 the International Society for Extracellular Vesicles (ISEV) connects researchers all over the world working with EVs at its annual meeting, workshops and other events, together with the establishment of a dedicated journal, the Journal of Extracellular Vesicles (Harding et al. 2013). However, the research field of EVs is still in its infancy and improvements of quality controls, standardized isolation methods, appropriate storage guidelines and dose-response influences need to be advanced (Chen and Chopp 2018). Further studies are required to enhance understanding of the role of EVs in stroke to be able to establish new therapeutic targets.

7 Summary

Clinical outcome after ischemic stroke is strongly influenced by the intensity of the post-ischemic inflammation occurring in the penumbra (Iadecola and Anrather 2011). Therapeutic options for treating stroke are still limited and have not improved in the last decades (Ramee and White 2014). Enhancing the understanding of the detailed molecular mechanism occurring during the post-ischemic neuroinflammation is crucial to find new therapeutic targets (Iadecola and Anrather 2011). This study aimed to understand the cell-to-cell communication between astrocytes, microglia, and neurons through EVs in post-ischemic reaction. EVs can be secreted by every cell in the body and function as an effective transport for proteins, lipids, and gene modulatory cargos such as mRNA and microRNA (van Niel et al. 2018). Several studies confirmed the immunomodulatory effects of brain endogenous EVs in stroke (Puig et al. 2018). We established mixed glial cell cultures and pure astrocyte cell cultures and subjected them or not to an *in vitro* model of stroke (OGD). We isolated EVs from these cultures and incubate them with primary neuronal cultures subjected or not to OGD. The neuronal viability was assessed by MTT assay and propidium iodide staining 48 h after glial EV- incubation. Although, no significant differences were observed in all the groups studied, a modest neuronal survival was observed for neuronal cultures incubated with astrocyte-derived EVs treated with OGD without the presence of microglia, which was inhibiting this effect.

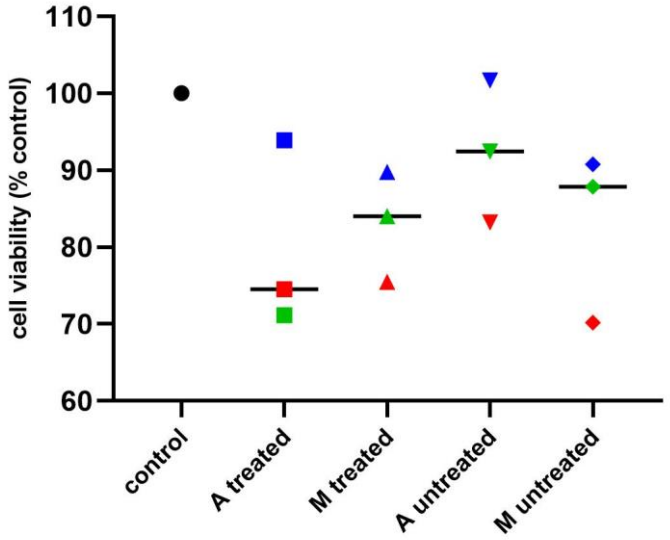
8 Zusammenfassung

Der Therapieerfolg nach einem ischämischen Schlaganfall wird stark von der Intensität der post-ischämischen Entzündung in der Penumbra beeinflusst (Iadecola und Anrather 2011). Die therapeutischen Möglichkeiten zur Behandlung des Schlaganfalls sind immer noch begrenzt und haben sich in den letzten Jahrzehnten nicht verbessert (Ramee und White 2014). Ein besseres Verständnis der detaillierten molekularen Mechanismen, die während der post-ischämischen Neuroinflammation ablaufen, ist entscheidend, um neue therapeutische Ziele zu finden (Iadecola und Anrather 2011). Ziel dieser Studie war es, die Zell-zu-Zell-Kommunikation zwischen Astrozyten, Mikroglia und Neuronen durch EVs in der post-ischämischen Phase zu verstehen. EVs können von jeder Zelle im Körper sezerniert werden und fungieren als hocheffektive Transportmittel für Proteine, Lipide und genmodulierende Cargos wie mRNA und microRNA (van Niel et al. 2018). Mehrere Studien bestätigten die immunmodulatorischen Effekte von hirneigenen EVs bei Schlaganfall (Puig et al. 2018). Wir etablierten Gliazellkulturen und reine Astrozytenzellkulturen und unterzogen sie einem *In-vitro*-Modell des Schlaganfalls (OGD). Wir isolierten EVs aus diesen Kulturen und inkubierten sie mit primären Neuronenzellkulturen, die einem OGD unterzogen wurden oder auch nicht. Die Lebensfähigkeit der Zellen wurde 48 Stunden nach der Inkubation mit EVs mittels MTT-Assay und Propidiumjodid-Färbung bestimmt. Obwohl in allen untersuchten Gruppen keine signifikanten Unterschiede festgestellt wurden, wurde eine geringfügigere Überlebensrate bei neuronalen Zellkulturen beobachtet, die ausschließlich mit, aus Astrozyten gewonnenen, EVs inkubiert wurden.

9 Abbreviations

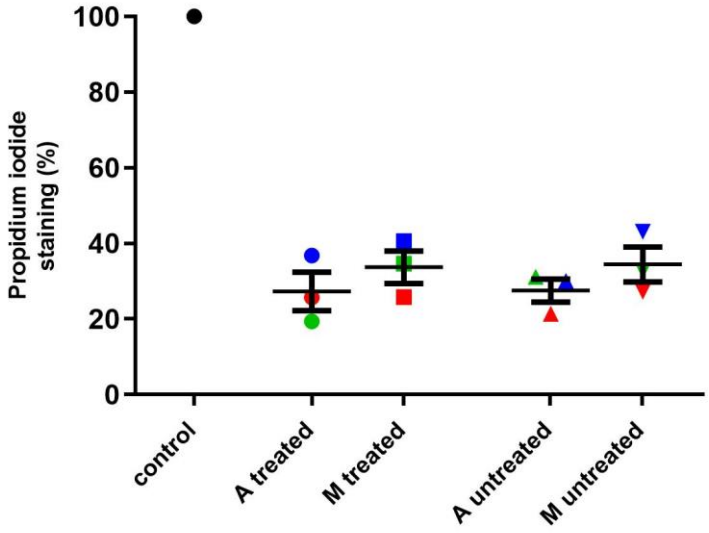
APOE	apolipoprotein E
ATP	adenosine triphosphate
BBB	blood brain barrier
BME	basal medium eagle
BSA	bovine serum albumin
C1Q	complement subcomponent 1q
CNS	central nervous system
DAMPs	damage associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
EGFP	enhanced green fluorescence protein
ESCRT	endosomal sorting complexes required for transport machinery
EVs	extracellular vesicles
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter (astrocyte marker)
IL	interleukin
ILVs	intraluminal vesicles
INF	interferon
ISEV	international society of extracellular vesicles
lpc 2	lipocalin 2
LPS	lipopolysaccharide
MEM	minimum essential medium
MISEV	minimal information for studies of extracellular vesicles
MSCs	mesenchymal stem cells
MVE	multivesicular endosome
NCAM	neuronal cell adhesion molecule
NTA	nanoparticle tracking analysis
OGD	oxygen-glucose deprivation
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PI	propidium iodide
PrP	prion protein
PRRs	pattern recognition receptors
ROS	reactive oxygen species
RT	room temperature
rtPA	recombinant tissue plasminogen activator
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TMCAO	transient middle cerebral artery occlusion
TNF	tumor necrosis factor

10 Supplementary data



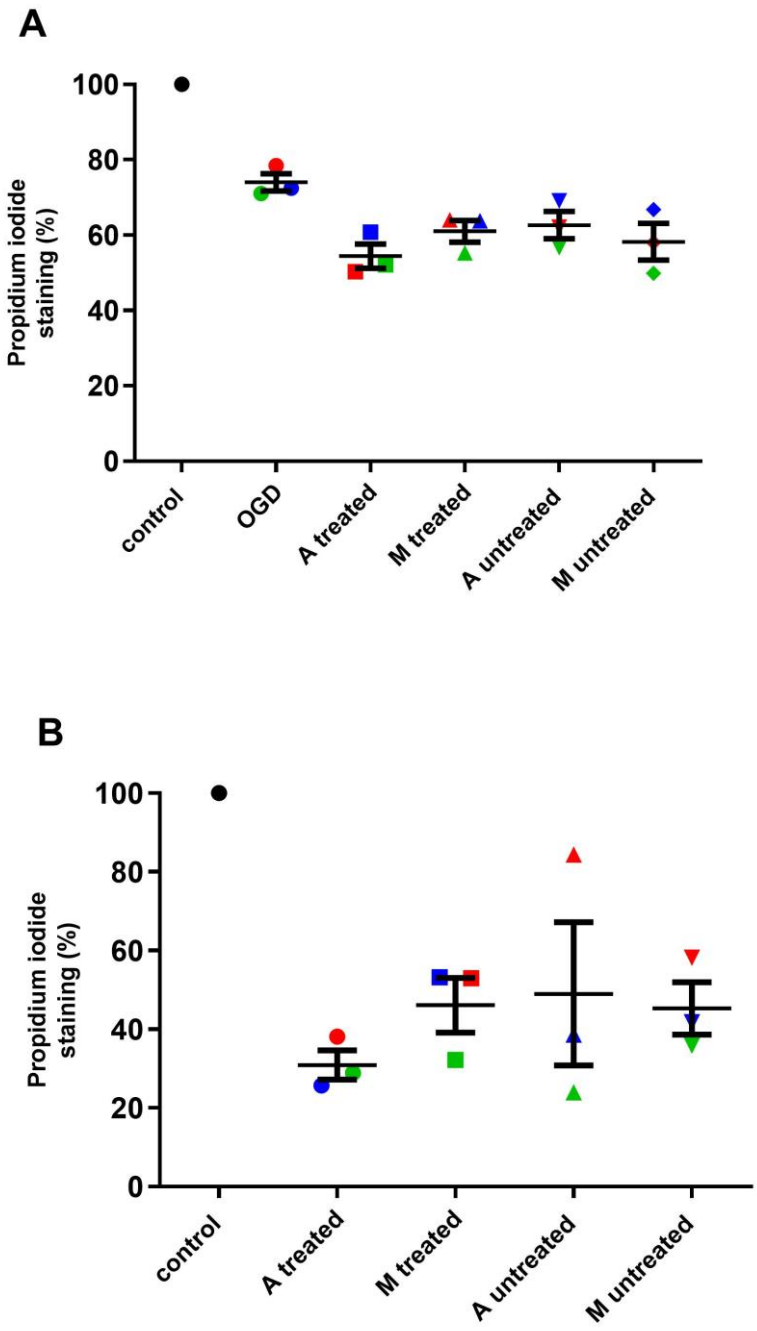
Suppl. Fig. 1 Graphical representation of cell viability control group (MTT assay) of primary neuronal cell culture treated or not with EVs.

Primary neuronal cell culture was incubated with 6,25 x 10^5 astrocyte EVs either treated with 2h OGD (A treated) or untreated (A untreated) and EVs from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 12. (n=3). p>0,05. Each color represents one distinct neuronal cell culture. Measurements were done 48h after EV incubation. The results are normalized with an unexposed control set to 100%.



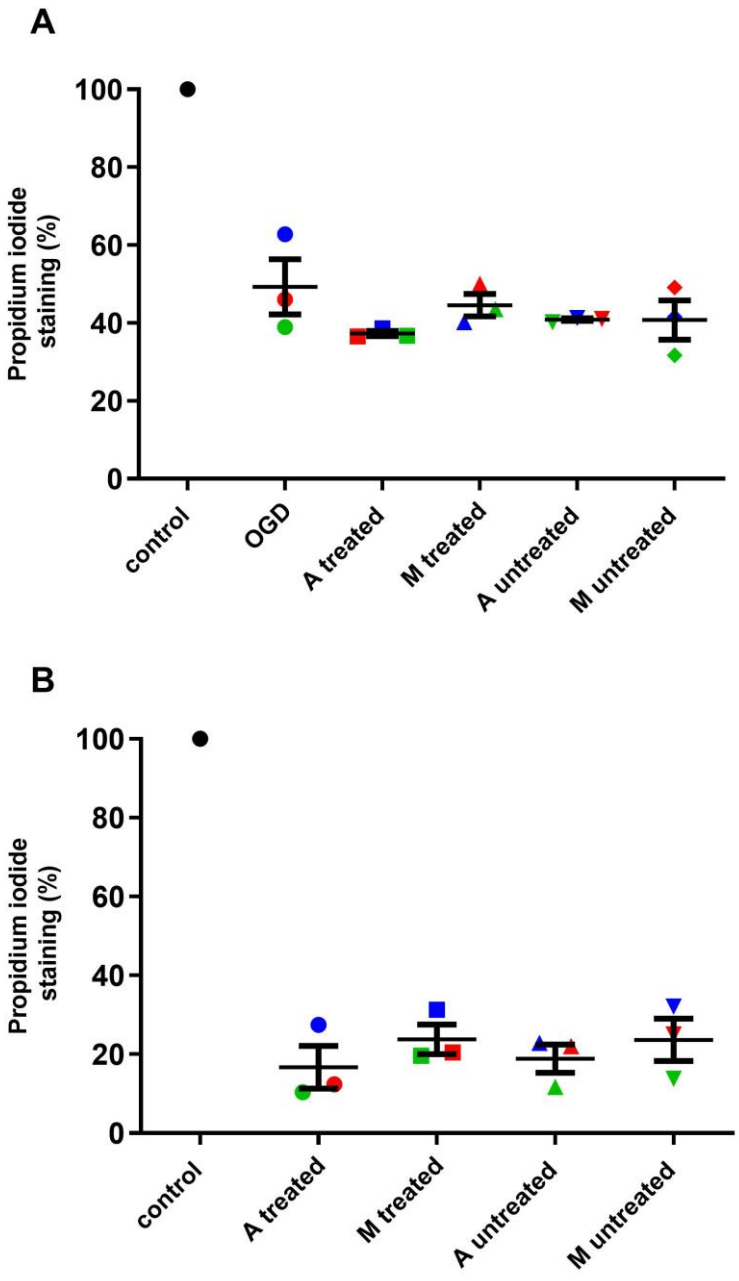
Suppl. Fig. 2 Graphical representation of cell viability control group (Propidium iodide staining) of primary neuronal cell culture incubated with EVs.

Primary neuronal cell culture was incubated with 2,5 x 10^6 EVs per well derived from astrocyte cell culture either treated with 2h OGD (A treated) or untreated (A untreated) and derived from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 18. (n=3). p>0,05. Each color represents one distinct neuronal cell culture. Propidium iodide staining was done 48h after EV incubation. Images were taken with Leica TCA SP8 X confocal microscopy.



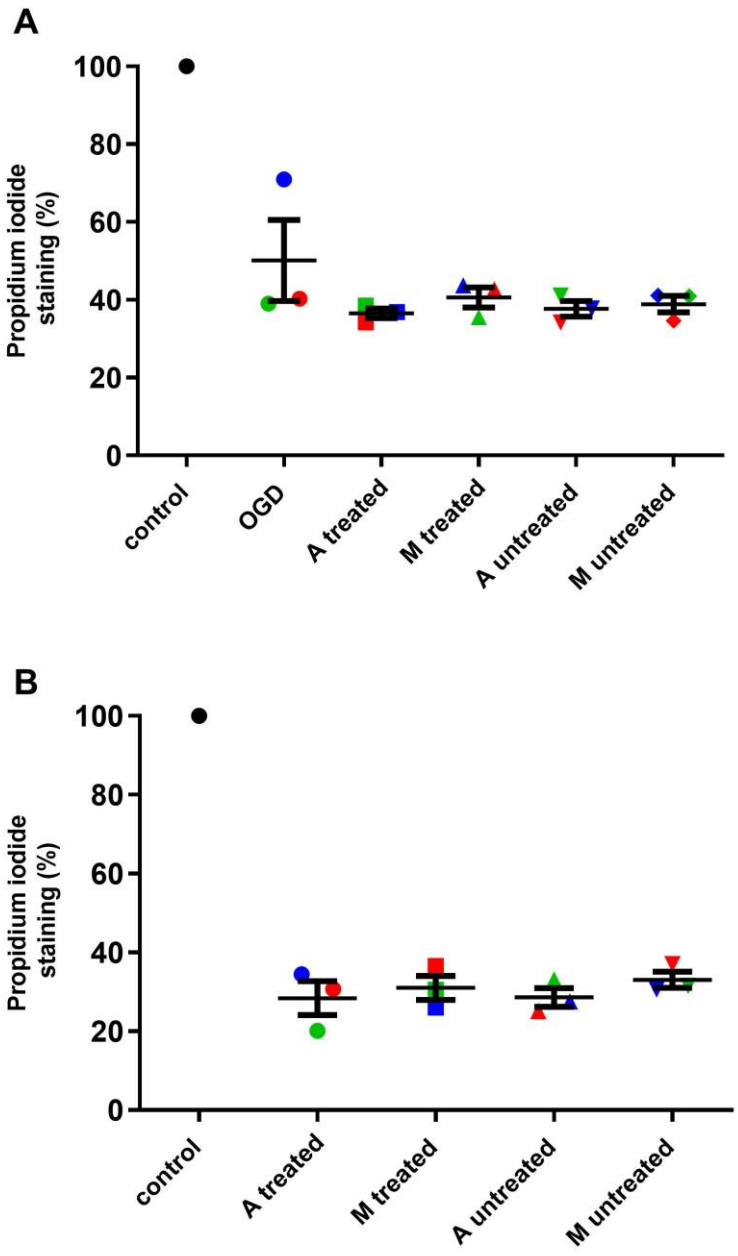
Suppl. Fig 3 Graphical representation of cell viability (Propidium iodide staining) of primary neuronal cell culture by Person 1

A *I primary neuronal cell culture were subjected to OGD incubated with 2,5 x 10^6 EVs per well derived from astrocyte cell culture either treated with 2h OGD (A treated) or untreated (A untreated) and derived from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 18. (n=3). p>0,05.*
B *| control group with primary neuronal cell cultures not subjected to OGD.*
Each color represents one distinct neuronal cell culture. Propidium iodide staining was done 48h after OGD treatment. Images were taken with Leica TCA SP8 X confocal microscopy.



Suppl. Fig. 4 Graphical representation of cell viability (Propidium iodide staining) of primary neuronal cell culture by Person 2

A *| primary neuronal cell culture were subjected to OGD incubated with 2,5 x 10^6 EVs per well derived from astrocyte cell culture either treated with 2h OGD (A treated) or untreated (A untreated) and derived from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 18. (n=3). p>0,05.*
B *| control group with primary neuronal cell cultures not subjected to OGD.*
Each color represents one distinct neuronal cell culture. Propidium iodide staining was done 48h after OGD treatment. Images were taken with Leica TCA SP8 X confocal microscopy.



Suppl. Fig. 5 Graphical representation of cell viability (Propidium iodide staining) of primary neuronal cell culture by Person 3

A *| primary neuronal cell culture were subjected to OGD incubated with 2,5 x 10^6 EVs per well derived from astrocyte cell culture either treated with 2h OGD (A treated) or untreated (A untreated) and derived from mixed glial cell culture either treated with 2h OGD (M treated) or untreated (M untreated) on day 18. (n=3). p>0,05.*
B *| control group with primary neuronal cell cultures not subjected to OGD.*
Each color represents one distinct neuronal cell culture. Propidium iodide staining was done 48h after OGD treatment. Images were taken with Leica TCA SP8 X confocal microscopy.

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13 Lebenslauf

Der Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

14 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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