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The role of Extracellular Vesicles during CNS development

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ABSTRACT

With a diverse set of neuronal and glial cell populations, Central Nervous System (CNS) has one of the most complex structures in the body. Intercellular communication is therefore highly important to coordinate cell-to-cell interactions. Besides electrical and chemical messengers, CNS cells also benefit from another communication route, what is known as extracellular vesicles, to harmonize their interactions. Extracellular Vesicles (EVs) and their subtype exosomes are membranous particles secreted by cells and contain information packaged in the form of biomolecules such as small fragments of DNA, lipids, miRNAs, mRNAs, and proteins. They are able to efficiently drive changes upon their arrival to recipient cells. EVs actively participate in all stages of CNS development by stimulating neural cell proliferation, differentiation, synaptic formation, and mediating reciprocal interactions between neurons and oligodendrocyte for myelination process. The aim of the present review is to enlighten the presence and contribution of EVs at each CNS developmental milestone.

1. Introduction

Extracellular Vesicles (EVs) were initially thought to function as a disposal mechanism carrying cellular waste into extracellular space. They are nowadays established as one of the lines of communication between cells.

The name “extracellular vesicle” is a general term used to describe three subtypes of vesicles: (i) Microvesicles (ii) Exosomes and (iii) Apoptotic bodies. It is important to mention that the terms EVs and exosomes are used interchangeably in literature. There is indeed a high inconsistency within the field regarding the nomenclature used to describe extracellular vesicles. A wide range of terminologies are used in literature while no unambiguous definition based on subtype specific markers or subtype specific isolation methods is available for the

Abbreviations: AsrgI1, Asparaginase-like protein 1; BMPs, Bone Morphogenetic Proteins; CAM kinase II, Calmodulin-dependent protein kinase II; CT-1, Cardiotrophin-1; CNS, Central Nervous System; CME, Clathrin-Mediated Endocytosis; C3, Complement component 3; CFB, Complement Factor B; ESCRT, Endosomal Sorting Complexes Required for Transport; EE, Environmental Enrichment; EGFR, Epidermal Growth Factor Receptor; EAAT2, Excitatory Amino Acid Transporter 2; EVs, Extracellular Vesicles; FBS, Fetal Bovine Serum; FGF, Fibroblast Growth Factor; FGF2, Fibroblast Growth Factor 2; GABA, Gamma Aminobutyric Acid; GFAP, Glial Fibrillary Acidic Protein; GLT1, Glutamate Synthase (NADH); IGFBP6, Insulin-like Growth Factor Binding Protein 6; ITGB4, Integrin Subunit Beta 4; IPCs, Intermediate Progenitor Cells; ICAM, Intracellular Adhesion Molecule; ILVs, Intraluminal Vesicles; LAMP1, Lysosomal-Associated Membrane Protein 1; miRNA, microRNA; MVBs, Multivesicular Bodies; MAG, Myelin Assocciated Glycoprotein; MBP, Myelin Basic Protein; MOG, Myelin Oligodendrocyte Glycoprotein; PLP, Myelin Proteolipid Protein; NGF, Nerve Growth Factor; NPCs, Neural Progenitor Cells; NSCs, Neural Stem Cells; NECs, Neuroepithelial Cells; NSMAF, Neutral Sphingomyelinase Activation Associated Factor; NMDA, N-Methyl-D-Aspartate; IFN-γ, Interferon-gamma; OPCs, Oligodendrocyte Progenitor Cells; OSVZ, Outer Subventricular Zone; PEDF, Pigment Endothelium-Derived Factor; RGCs, Radial Glial cells; oRGsouter, Radial Glia cells; RA, Retinoic Acid; SNPs, Short Neuronal Precursors; SNAREs, Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptors; SHH, Sonic Hedgehog; SVZ, Subventricular Zone; TGFB1, Transforming Growth Factor-beta 1; VEGF, Vascular Endothelial Growth Factor; VEGFR2, Vascular Endothelial Growth Factor Receptor 2; VZ, Ventricular Zone; Wnt, Wingless-type MMTV integration site family; AMPA, α-Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid.

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subtypes. (Mora et al., 2016; Yanez-Mo et al., 2015). For example, the two terms EVs and exosomes are used interchangeably, and a selection for a term seems to be based on authors' preference (Witwer & Thery, 2019). The International Society for Extracellular Vesicles (ISEV) has proposed a consensus nomenclature in which EVs is a preferred generic term to describe lipid bilayer particles that are released by cells and unable to replicate due to the lack of functional nucleus (Thery et al., 2018). In the present review we follow these guidelines, however, when discussing individual studies we adopt the terms used by the authors of the study.

The subtypes of EVs are distinguished based on their size, origin of formation, content, function, and mechanism of release (Doyle & Wang, 2019). With a size ranging from 30nm-to 100 nm, exosomes are the smallest vesicles followed by microvesicles (100 nm-1000 nm) and apoptotic bodies (1000-5000 nm)(Maia et al., 2018). It is important to mention that different methods have been proposed to determine diameters and thus there is an overlap between the subclasses of EVs with respect to size (Borges et al., 2013; Cocucci & Meldolesi, 2015; EL Andaloussi et al., 2013). Therefore, to further characterize EV subpopulations, researchers usually use methods such as Nano Tracking Analysis (NTA) in combination with electron microscopy, flow cytometry or Western blotting of the known EVs markers.

The different EV subpopulations arise from different biogenetic pathways. Apoptotic bodies result from a process that orchestrates the demise of the cell and that produces membrane-encapsulated cellular fragments that can contain organelles, proteins, DNA and RNA. Apoptotic bodies are not homogenous and have a broad size distribution (Battistelli & Falcieri, 2020; Xu et al., 2019) Microvesicles are formed by direct outward budding of the plasma membrane and can be released into the extracellular space by "pinching off" from the cell membrane. This process requires cytoskeletal proteins such as actin, microtubules, and molecular motors such as dynein, kinesis and myosin as well as a collaboration between SNARES, Rab GTPases and tethering factors (Cai et al., 2007; Doyle & Wang, 2019; Tricarico et al., 2017).

As opposed to microvesicles and apoptotic bodies, which have relatively simple biogenetic pathways, exosomes are generated by a complex multistep process that starts with the formation of early endosomes that evolve into late endosomes and multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). Early endosomes are formed by the invagination of the plasma membrane and gradually mature into late endosomes (Doyle & Wang, 2019). Maturation from early to late endosome is mainly mediated by the small GTPases Rab5 and Rab7. In this process, which is known as Rab conversion, the depletion of Rab 5, the marker of early endosome, is accompanied by the recruitment of Rab7 on late endosomes (Poteryaev et al., 2010; Rink et al., 2005). ILVs are formed by inward budding of the limiting membranes of late endosomes (Piper & Katzmann, 2007). Fusion of the limiting membrane of MVBs with the plasma membrane leads to the release of ILVs into the extracellular space. ILVs in the extracellular space are termed exosomes (Caruso Bavisotto et al., 2019; Minciacchi et al., 2015; van Niel et al., 2006; Zhang et al., 2019a). The precise mechanisms of cargo sorting into ILVs have not been resolved yet but are dependent on the biogenetic pathway of ILVs.

ILVs can be produced by the well-studied endosomal sorting complexes required for transport (ESCRT) dependent pathway. This protein machinery consists of four complexes (ESCRT-0,-I,-II,-III) which are sequentially recruited on the late endosome membrane (Colombo et al., 2013; Henne et al., 2011). The two protein subunits of ESCRT-0, Hrs and STAM1/2 (Vps27 and Hse1 in yeast) arrange the recruitment of ubiquitinated cargo to the limiting membrane of the endosome where the inward budding starts (Henne et al., 2011; Piper & Katzmann, 2007). ESCRT-0 then activates the TSG101 containing ESCRT-I complex (Colombo et al., 2013). Inhibition of the ESCRT-0 and I proteins Hrs, STAM1 and TSG101 results in the reduction of exosome secretion (Colombo et al., 2013). The ESCRT-I complex binds and recruits ESCRT-II subunits to the endosome membrane, thereby initiating inward budding (Andreu & Yanez-Mo, 2014; Colombo et al., 2013). Subsequently, ESCRT-II nucleates ESCRT-III complex assembly and polymer formation. Finally, The ATPase Vps4 joins the ESCRT-III polymer and induces its disassembly from the membrane facilitating vesicle scission to form the ILVs (Wollert et al., 2009). In addition to orchestrating ILV formation the ESCRT complexes also determine cargo sorting into the ILVs (Henne et al., 2011; Minciacchi et al., 2015).

The findings that depletion of key ESCRT subunits did not result in full inhibition of MVB formation and exosome secretion pointed to the existence of ESCRT-independent mechanisms of ILV formation (Stuffers et al., 2009; Zhang et al., 2019a). It was shown that members of the tetraspanin protein family such as CD9, CD63, CD81, and CD82, and lipid rafts play pivotal roles (Andreu & Yanez-Mo, 2014; van Niel et al., 2011). ILV formation of the ESCRT-independent pathway requires the production of ceramide by neutral sphingomyelinase 2 (nSMase2) (Trajkovic et al., 2008). Ceramide can induce coalescence of lipid-raft microdomains of the endosomal membranes that promotes domain-induced inward budding giving rise to cargo-loaded ILVs (Trajkovic et al., 2008).

nSMase2 also controls the loading of the ILVs with compounds such as RNAs by its downstream factor NSMAF/FAN (neutral sphingomyelinase associated factor) that is involved in the recruitment of RNA binding proteins for ILV cargo selection (Leidal et al., 2020).

Once formed, MVBs can either fuse with lysosomes for lysosomal degradation or with the plasma membrane to release their content in the form of exosomes into the extracellular space (Piper & Katzmann, 2007). Factors that define the fate of MVBs remain to be resolved. Members of the Rab family including Rab2b, Rab9a, Rab27a, Rab 27b, Rab35, have been shown to be involved in trafficking and docking of MVBs to the plasma membrane (for more information on the role of Rab family in EVs please see references (Blanc & Vidal, 2018; Eitan et al., 2016). It has been suggested that cholesterol enrichment of the MVB membranes stimulates fusion of MVBs with the plasma membrane (Doyle & Wang, 2019; Mobius et al., 2002; Mobius et al., 2003). Posttranslational modifications may also determine the fate of MVBs. A recent study by Villarroya-Beltri et al. showed that ISGylation of TSG101 stimulates lysosomal degradation of MVBs and reduces exosome secretion (Villarroya-Beltri et al., 2016).

The different biogenetic routes also result in different cargo composition of exosomes. Some proteins are only found in exosomes produced by the ESCRT-dependent pathway while others require the ESCRT-independent route for their secretion via exosomes. For example loading of ILVs with the epidermal growth factor (EGFR) requires Hrs and STAM1 of ESCRT complexes (Bache et al., 2003; Raiborg et al., 2002; Urbe et al., 2003). This was further confirmed by Stuffers et al who showed that depletion of ESCRT components inhibit EGFR sorting into ILVs (Stuffers et al., 2009). Inhibition of nSMase2, which is a key player in the ESCRT-independent route of ILV formation (see above) significantly reduces CD82 mediated exosome release of β-catenin suggesting that β-catenin secretion requires the ESCRT-independent pathway (Chairoungdua et al., 2010). Another example was given by Theos et al. indicating that the sorting of melanosomal protein Pmel17 (Pmel17) into ILVs is insensitive to Hrs depletion and therefore Pmel17 sorting is arranged independently of the ESCRT machinery (Theos et al., 2006).

Once released, EVs can target cells in their vicinity as well as reaching those located at far distances. In general, there are three strategies described for EVs targeting and uptake. One strategy is to simply fuse with the plasma membrane of the target cells after which the EV's content is released into the cytoplasm. Membrane fusion is thought to be mediated by several proteins amongst which are soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). The assembly of the four complementary SNARE motifs mediates a tight connection between the two lipid membranes to fuse (Jahn & Scheller, 2006; Kwok et al., 2021). The other route involves ligand-receptor interaction in which EVs dock with membrane exposed ligands to

receptors on the plasma membrane of the recipient cells and trigger cascades of intracellular signaling events (Fu et al., 2020). In the majority of cases, however, internalization of EVs by endocytosis seems to be the most important route. The underlying mechanisms arranging EV endocytosis are not yet clear but several mechanisms have been suggested (for more in depth information the reader is referred to the recently published review on EVs transportation and uptake by (Kwok et al., 2021). These include clathrin-mediated endocytosis (CME). In this model clathrin protein together with other components such as clathrin-adaptor proteins and scaffold proteins form clathrin coated endocytotic vesicles (Kaksonen & Roux, 2018). It is noteworthy to mention here that CME is the major pathway in synaptic vesicle protein internalization (Saheki & De Camilli, 2012). Additionallly, CME actively participates in synaptic cargo retrieval from the plasma membrane after physiological stimuli (Granseth et al., 2006; Nicholson-Fish et al., 2015). Other mechanisms include phagocytosis, micropinocytosis, and caveolin-dependent endocytosis, a process through which cave-like structures known as caveolar vesicles are formed and pinched off from the plasma membrane into the cytosol (Kwok et al., 2021). Several proteins have been identified to play a role in EV uptake by recipient cells. These include lectins, integrins, intracellular adhesion molecules (ICAMs), and proteoglycans (Murphy et al., 2019; van Niel et al., 2018; Zhang et al., 2019a; Zoller, 2009). Tetraspanins, a family of transmembrane proteins, also participate in the interaction between EVs and recipient cells. In addition to their roles in ESCRT-independent ILV biogenesis and cargo sorting, Tetraspanons also have a role in determining the recipient cell for exosome uptake (Jankovicova et al., 2020; van den Boorn et al., 2013). Notably, the distinct tissue-homing behavior of EVs is due to tetraspanin-enriched-microdomains (TEM) in which tetraspanins interact with integrins and determine target selection (van den Boorn et al., 2013).

The content loaded into EVs is generally a collection of biomolecules obtained from the parental cell. However, there are a few common protein markers detectable in almost all EVs/exosomes. These include classic exosome markers such as tetraspanin proteins CD9, CD63, and CD81,CD82 as well as HSP70, HSP90β, ALIX, TSG101 and Flotillin proteins (Doyle & Wang, 2019; Kowal et al., 2016). A large amount of studies has been performed to identify and characterize EV content by Multi-omics approaches such as next generation sequencing and mass spectrometry. The outcome is collected in three online data repositories: Vesiclepedia, EVpedia, and ExoCarta (Kalra et al., 2012; Keerthikumar et al., 2016; Kim et al., 2013). It is thought that in regards to CNS, miRNAs are the standout elements among the other components of EVs (Batiz et al., 2016; Luarte et al., 2016b). They are involved in regulating synaptic plasticity as well as neuroprotection (Micci et al., 2019; Properzi et al., 2015). Moreover, each miRNA is able to target and repress the translation of as many as hundreds of mRNAs (Enright et al., 2003; Krek et al., 2005; Stevanato & Sinden, 2014).

Numerous studies have investigated the function of EVs particularly in neurodegenerative disorders. However, less is known about their pathological impact on neurodevelopmental disorders (for a recent review see Gomes et al. 2020) (Gomes et al., 2020). The purpose of the present study is to shed light on the role of EVs by presenting a comprehensive review of literature on their role during CNS development. This review suggests new insights into the potential link of exosomes and some of the underlying pathologies in disorders of neurodevelopment.

CNS has the most complex structure in the body consisting of various cell types generated in a spatio-temporal manner and each cell type has a distinct morphology and function. Describing the CNS structure and

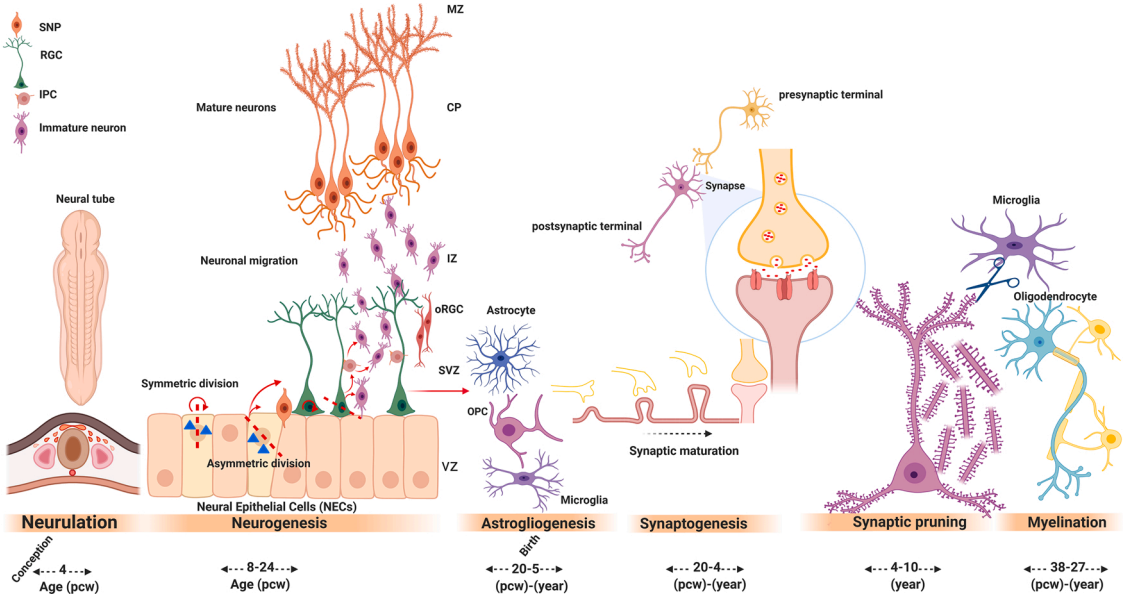

Fig. 1. Summary of major developmental milestones. Neuroepithelial cells (NECs) inside the neural tube populate the ventricular zone (VZ) through symmetric division. Notch signaling, the defining factor in cell fate decision, maintains the balance between proliferation and differentiation. NECs with inactive Notch signaling undergo asymmetric division to produce the first neurons, known as short precursor neurons (SNPs), as well as Radial Glial Cells (RGCs). Besides their proliferation, RGCs in subventricular zone (SVZ) divide asymmetrically to generate intermediate progenitor cells (IPCs) and neurons. The outer radial glial cells (oRGs) reside in the outer subventricular zone (OSVZ) and give rise to the majority of neurons (most notably in human). IPCs divide symmetrically to produce two neurons. Neurons migrate away from VZ and SVZ to reach the cortical plate (CP) and cortical marginal zone (MZ) where they eventually become mature neurons. Newborn neurons start their migration in the intermediate zone (IZ) by using the long basal radial processes of the RGCs as the scaffold (Cooper, 2014; Hirota & Nakajima, 2017; Tan & Shi, 2013). Towards the end of neurogenesis and by receiving signals from neurons, RGCs switch to gliogenesis to produce astrocytes and oligodendrocyte progenitor cell (OPC). During synaptogenesis, axons start to interact with nearby dendrites, a process that undergoes maturation to establish and stabilize synapses. The excess in synapse formation is eliminated during pruning to remove the weak synapses. Mature oligodendrocytes facilitate the fast transport of signals along neurons by wrapping the neuronal axons with myelin sheaths. The approximate timelines provided for each step are from the following references: Neurulation (Muller & O'Rahilly, 1987; Muller & Orahilly, 1988), Neurogenesis (Bystron et al., 2008; Rakic, 1988), Gliogenesis (Choi & Lapham, 1978; Jakovcevski et al., 2009), Synaptogenesis and synaptic pruning (Huttenlocher, 1979; Petanjek et al., 2011), and Myelination (Jakovcevski et al., 2009; D. J. Miller et al., 2012).

function is well beyond the scope of the present review and therefore we only specify and briefly describe the parts highlighted in EVs studies of the developing CNS. We start from neurogenesis followed by gliogenesis and continue to synaptogenesis, synaptic pruning, and eventually myelination. Additionally, since neurogenesis is an ongoing process we will also have a look at the role of EVs in adult neurogenesis. Fig. 1 represents a schematic visualization of the CNS developmental stages.

2. Embryonic neurogenesis

CNS development begins by the neurulation process when the neural plate folds inwardly to eventually form the neural tube. Shortly after being formed, the neural tube undergoes patterning by which three axes are derived: anterior-posterior, medial-lateral, and dorsal-ventral (Hemmati-Brivanlou & Melton, 1997). Neural tube patterning is regulated by morphogens such as Sonic Hedgehog (SHH), Bone morphogenetic proteins (BMPs), Retinoic Acid (RA), Fibroblast Growth Factor (FGF), and Wnt that eventually lead to formation of four different regions in CNS: forebrain, midbrain, hindbrain, and spinal cord (Guerout et al., 2014; Hemmati-Brivanlou & Melton, 1997).

Before the initiation of cortical neurogenesis, Neuroepithelial cells (NECs) located in the neural tube undergo proliferation (at an exponential rate) through symmetric division. Proliferation occurs in the primary proliferative zone, the ventricular zone (VZ), of the neural tube (Fietz & Huttner, 2011; Martinez-Cerdeno & Noctor, 2018; Pinto & Gotz, 2007). Changes in the proliferation mode from symmetric division to asymmetric division is the starting point of neurogenesis (Caviness et al., 2003; Gotz & Huttner, 2005). Asymmetric division produces one NEC while the other daughter cell becomes a progenitor cell, also known as radial glial cell (RGC). NECs are also capable of directly producing short neuronal precursors (SNPs) (Gal et al., 2006; Stancik et al., 2010) which are known to be the first population of neurons generated in CNS. Cell transition from proliferation to differentiation is influenced by Notch signaling that suppresses differentiation and is more in favor of proliferation (Imayoshi et al., 2010; Lutolf et al., 2002). Thus, newly generated NECs with active Notch signaling are programmed to preserve the cell pool by self-renewing divisions while those with inactive Notch signaling take a different path towards differentiation. Symmetric and asymmetric cell divisions are also present in neuronal progenitor cells though at a more restricted manner compared to NECs (Gotz & Huttner, 2005; Holguera & Desplan, 2018). While symmetric proliferative division maintain the neural progenitor cell pool, asymmetric cell division either directly produces neurons or generates intermediate neuronal progenitor cells, also known as amplifying progenitor cells. They migrate radially to the second proliferative zone, known as the subventricular zone (SVZ) (Noctor et al., 2001; Noctor et al., 2004).

Intermediate progenitor cells (IPCs) are subjected to symmetric neurogenic division to produce two pairs of postmitotic neurons (Martinez-Cerdeno et al., 2006; Noctor et al., 2004; Tan & Shi, 2013). Additionally, there is another subtype of progenitor cell, radial glia-like progenitor cells, also known as outer radial glial cells (oRGs). They reside in the outer subventricular zone (OSVZ) in human. They are morphologically different from RGCs and IPCs (Hansen et al., 2010; X. Wang et al., 2011) and able to produce more IPCs through asymmetric division (Hansen et al., 2010; LaMonica et al., 2013).The expansion and complexity of human neocortex is largely due to the OSVZ and most notably due to oRGs. The OSVZ is populated with IPCs and oRGs (Hansen et al., 2010). Recent studies revealed that oRGs are also present in mice (Shitamukai et al., 2011; X. Wang et al., 2011) though the extent to which they contribute to cortical expansion is restricted. In mice, IPC proliferation does not exceed more than one or two cell cycles while in higher mammals such as primates, the IPCs represent higher self-renewing capacity and thus increase the cortical size and complexity (Hansen et al., 2010) (X. Wang et al., 2011) (Martinez-Martinez et al., 2016) (Tiberi et al., 2012).

Neuronal migration to the cortical plate, which ends the neocortex development, is a gradual process. Post-mitotic neurons produced in VZ and SVZ migrate through the intermediate zone to eventually reach the top of the cortical plate, where they further differentiate and turn into their distinct phenotype.

3. Gliogenesis

Generation of neurons and generation of glial cells take place in a sequential order such that neurons are the first to be generated followed by astrocytes and, later on during the postnatal period, oligodendrocyte precursor cells and oligodendrocytes (Adnani et al., 2018; J. Liu & Casaccia, 2010). Transition to gliogenesis occurs towards the end of neurogenesis when RGCs receive signals from newly formed neurons, which trigger their gliogenic fate to take action. The intrinsic and extrinsic factors are the key players in switching from neurogenesis to gliogenesis (Adnani et al., 2018; F. D. Miller & Gauthier, 2007; Zarei-Kheirabadi et al., 2020). For instance, cytokines produced by newly generated neurons in the cortical plate are demonstrated to promote gliogenesis through a negative feedback mechanism (Barnabe-Heider et al., 2005). Newly generated cortical neurons secrete cardiotrophin-1 (CT-1) which acts as an extrinsic gliogenic signal to trigger the conversion from neurogenesis to gliogenesis. Thus, CT-1 instructs multipotent cortical precursor cells to produce astrocytes.

All the aforementioned studies clearly indicate that RGCs are the common precursor cell pool that create heterogeneity in the CNS by generating different cohorts of cell types at different time points and places. This pluripotent property of RGCs therefore makes them a good candidate in cell-based therapy for diseases with neurological phenotypes.

EVs during neurogenesis and gliogenesis

A large number of studies have focused on the potential therapeutic effect of the neural stem cell secretome in neurological disorders. Neural stem/progenitor cells secrete EVs that are instrumental in intercellular communication. In spite of their small size, their content covers a wide variety of biomolecules such as small fragments of DNA, proteins, mRNAs, miRNAs as well as metabolites and lipids. The content of EVs is determined by the parental cell and although its quantity is considered low, its efficacy to induce changes in the recipient cell is inevitable. The study by Stevanato et al. demonstrated that the exosomal miRNA released by human neural stem cells (hNSCs) reflects the miRNA content of the producing cells. Furthermore, the exosomal miRNA can be transferred functionally to recipient cells (Stevanato et al., 2016). They first performed next generation sequencing on cellular and exosomal content of hNSCs that revealed a differentially enriched subset of miRNAs in exosomes including Hsa-miR-1246, hsa-miR-4488, hsa-miR-4508, hsa-miR-4492 and hsa-miR-4516. Stoichiometry findings by real time PCR on highly enriched Hsa-miR-1246 indicated that there were at least 10 copies of this miRNA per exosomes. Moreover, functional analysis by 3' untranslated region dual luciferase reporter assay indicated the reduction in luciferase activity in Hela cells, used as target cells, indicating that the amount of transferred miRNA is sufficient to elicit changes in recipient cells.

EVs efficacy is also determined by their dosage. In a study by Stornati et al, EVs extracted from embryonic mouse neural progenitor cells (NPCs) from spinal cord were shown to differentiate recipient NPCs to astrocytes (Stronati et al., 2019). To assess the dosage efficacy, NPCs were exposed to two different concentration of EVs. Results indicated that cells treated with higher concentration of EVs had a higher percentage of Glial Fibrillary Acidic Protein (GFAP)-positive astrocytes.

EVs can directly participate in neurogenesis. Stornati et al. demonstrated that NPCs derived from embryonic mouse spinal cord can produce and release exosomes both at the proliferation and differentiation phases (Stronati et al., 2019). Cultured in expansion medium, NPCs released exosomes which were confirmed by the presence of exosomal markers TSG-101 and tetraspanin markers CD63 and CD81. Furthermore, when stimulated by differentiation mediums containing either

Fetal Bovine Serum (FBS) or BMP, NPCs showed a highly enriched astrocytic phenotype with less preference for neuronal phenotype. Similarly, the extracted EVs, from both differentiated astrocytes and neuronal cells, also stimulate the proliferating NPCs more towards the astrocytic lineage. This study indeed indicates the important role of EVs in transition of NPCs from a neurogenic to a gliogenic lineage.

Neurogenesis is an ongoing process that continues into adulthood though at considerably lower rates resulting in the creation and maintenance of only a small size pool of NPCs. The paucity of NPCs in adulthood is a major obstacle for therapeutic strategies employing NPCs or NPC-derived EVs. Re-programming of somatic cells into NPCs has provided a new opportunity. Using transcription factors Brn2, Sox2, and Foxg1 Ma et al. reprogrammed mouse fibroblast and astrocytes into induced NPCs (iNPCs) (Ma et al., 2019b). Interestingly, they demonstrated that iNPCs were able to release EVs at higher levels when compared to wild type (WT)-NPCs as determined by Nanoparticle Tracking Analysis and Western Blotting of EV markers Flotillin-1, Flotillin-2 and HSP70. In addition, iNPC-EVs promoted proliferation of WT-NPCs significantly stronger as compared to EVs fromWT-NPC. Proteomics analysis revealed that, in fact, the expression levels of growth factor related domains such as growth factor receptor cysteine rich domain, EGF-like domain, and EGF-like calcium-binding domain are higher both in iNPC and in their EVs. Further analysis by perturbation of function assay suggested that the aforementioned growth factors increase the proliferation rate in NPCs through their downstream MERK/ERK pathways. In a follow up study, it was demonstrated that NPC-originated exosomes (EXO) can induce the differentiation of cortical NPCs into neuronal cells in differentiation medium whereas iNPC-derived exosomes (iEXO) appeared to possess a much lower potency to differentiate cortical NPCs into neuronal cells (Ma et al., 2019a). Neither exosomes population induced significant differentiation towards glial differentiation. Microarray analysis revealed that EXOs carry miRNA-21a at a much higher level than iEXOs. Using a miRNA-21a mimic and a miRNA-21a inhibitor it was demonstrated that the mimic suppressed the proportion of $GFAP^{+}$ glial cells and increased the proportion of $tuj1^{+}$ neuronal cells, whereas the inhibitor caused the opposite effect. Collectively, these results highlight the role of miRNA-21a in determining the NPC cell fate more towards neurogenesis rather than gliogenesis. This is in contrast with the results obtained by Stornati et al. (Stronati et al., 2019). Possible explanations are: Firstly, Stornati et al. collected NPCs from mouse spinal cord while the NPCs in the Ma et al.study were derived from cortex. Both collected NPCs at day 13.5 of mouse embryonic development. Secondly, Stornati et al. investigated the differentiation of NPCs in FBS and BMP4 differentiation medium.

Cell-to-cell communication is a vital part of CNS structure, function, and homeostasis both during development and afterwards in adulthood. Besides electrical and chemical signals, exosome-based cell-cell interaction is also important not only between neurons but also in neuro-glial interactions. A wide range of examples is provided throughout this review. For instance, Morton and colleagues identified a bidirectional interaction between NCSs and microglia, which is mediated by EVs (Morton et al., 2018). They first revealed that murine neonatal subventricular NSCs release EVs. This was confirmed by detecting the EVs markers CD63 and CD9 as well as the exosome cargo protein ALIX. The destiny of released EVs was also determined by tracking CD9-GFP positive particles. The reduction of these particles over the time overlapped with influx of microglia suggesting an active clearance of EVs by microglia. They reported that microglia-iba1 positive cells are co-localized with CD9-GFP positive particles released from electroporated SVZ. For further assessment, NSC-derived EVs of SVZ were labeled with the lipophilic dye DiI and transplanted to P0 murine pups. The labeled EVs were detected in microglia-iba1 positive cells as well as cells that were double positive for CD68 and CD11b (neonatal subventricular zone markers). These results confirmed that SVZ NSC-derived EVs target microglia. Further analysis revealed that these EVs were enriched with miRNAs including miR-9, Let-7, and miR-26, which belong to miRNA families with a role in regulating microglia morphology and physiology (Kumar et al., 2015; Lehmann et al., 2012; Yao et al., 2014; L. Zhang et al., 2015). Transfecting exosomes with synthetic Let-7 miRNA enabeled them to change microglia morphology and induced cytokine release that suppressed proliferation of SVZ NSCs. It was proposed that NSCs and microglia establish an intricate communication network based on EV release that regulates SVZ NSC proliferation. NPC-derived EVs are also instrumental in conveying functional immune responses. The study by Cossetti et al. revealed that the content of NPC-derived EVs mirrors the activation status of the parental cell (Cossetti et al., 2014). Treatment of murine SVZ NPCs with proinflammatory cytokines caused selective sorting of components of the interferon-gamma (IFN-γ) pathway into the EVs. Amongst these components was the interferon gamma receptor 1 (Ifngr1), which was expressed at the EV's surface. These EVs triggered IFN-γ-dependent stat1 signaling in NIH 3T3 cells. Advanced-imaging techniques demonstrated a rapid adhesion and incorporation of these EVs in the target cells. As opposed to their fast incorporation, their degradation was shown to be rather slow due to the lack of lysosomal-associated membrane protein 1 (LAMP1) suggesting a sustained and robust impact of these EVs on the recipient cell. Together these experiments illustrate the role of NPC-derived EVs in immune responses and that this role is modulated by the microenvironment of the EV-producing NPC.

In addition to being conveyors of immune responses, EVs can also provide protection against inflammation. It was previously reported that NPCs in concert with endothelial progenitor cells are able to reduce hypoxia-induced ROS overproduction in brain endothelial cells (ECs) (Wang et al., 2016). The study by Liu and colleagues investigated the underlying mechanism through which NPCs provide protection against oxidative stress in brain ECs and highlighted the role of NPC-derived exosomes (NPC-EXs) (H. Liu et al., 2017). They demonstrated that miR-210 in NPC-EXs mediates the antioxidant effect on ECs by using miR-210 mimic, miR-210 inhibitor and scramble control miRNA. NPC-EXs carrying miR-210 reduced Nox2 levels and apoptosis of ECs exposed to the oxidative stress inducer angiotensin II (Ang-II). In addition, NPC-EXs carrying miRNA-210 diminished Ang-II induced upregulation of ephrin A3 and prevented Ang-II induced loss of ECs ability of tube formation through normalization of the phosphorylated-VEGFR2/VEGFR2 ratio.

It is worth mentioning here that the EV-mediated crosstalk between neural and brain endothelial cells covers a wider array of processes. For instance, neuron-derived exosomes target endothelial cells to regulate the brain vascular integrity (Xu et al., 2017). These exosomes harbor a neuron-enriched miR-132 that upregulates vascular endothelial cadherin protein, also known as Cadherin 5 (Cdh5), by directly targeting eukaryotic elongation factor 2 kinase (eef2k) in endothelial cells. Dysfunction of miR-132 reduces Cdh5 expression (and its partner β-catenin) and is associated with severe intracranial hemorrhage and dysregulation of brain vascular integrity in zebra fish larvae.

EVs seem to have a dynamic interaction with their microenvironment. Not only is their composition determined by the microenvironment (Cossetti et al., 2014), they can also condition the microenvironment. The study by Iraci et al. demonstrated that NPC-derived EVs carry metabolic enzymes and act as independent metabolic units capable of influencing the composition of their microenvironment (Iraci et al., 2017). Metabolic and functional analyses showed that NPC-derived EVs carry functional Asparaginase-like protein 1 (Asrgl1) that converts Aspargine (Asn) into Aspartate (Asp), which is released into the microenvironment.

4. Neurogenesis to be continued (Adult Neurogenesis)

The exciting discovery in 1960's, which revealed that neurogenesis persists to adulthood, revolutionized the dogma around embryonic-born neurons as the solo inhabitants of CNS. Neurogenesis in adulthood takes

place at a limited rate and restricted to only two main regions in the brain also known as neurogenic niches. The subgranular zone of the dentate gyrus in hippocampus (Eriksson et al., 1998) and the subventricular zone in the lateral ventricular wall of the cerebral cortex (Johansson et al., 1999). The NPCs in dentate gyrus represent similar features to radial glial cells and are therefore referred as RG-like cells (Martinez-Cerdeno & Noctor, 2018), their function is shown to be important in cognitive activity of hippocampus such as pattern separation (Aimone et al., 2011), spatial learning and memory (Dupret et al., 2008). The newly produced neurons in the adult SVZ migrate to olfactory bulb and are differentiated to interneurons (Lim & Alvarez-Buylla, 2016). Self-renewal and multipotency of neural stem /progenitor cells of embryonic origin, and to some extent adulthood NSC, have made them potential candidates for therapeutic purposes.

In regenerative medicine, neural stem cell transplantation has found its place as a relatively promising treatment. There has been a vast growth in clinical indications for such therapeutic approach including Parkinson disease, Huntington disease, Alzheimer disease, Amyotrophic lateral sclerosis, spinal cord injury and stroke (Takagi, 2016; Tang et al., 2017). However, there are still many challenges to be addressed. The ethical issues towards NSC transplantation are nowadays rather paved by reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). Nevertheless, one should keep in mind the adverse outcome events associated with using such cell lines including the risk of tumorigenesis and immune response (Li et al., 2008; Nam et al., 2015). In addition, the administered stem cells need to migrate to the target site and differentiate into the desired neuronal cell type. They should also be able to integrate with the neural circuit and form proper synaptic connectivity with the host cells in order to exert their therapeutic effect (Marsh & Blurton-Jones, 2017). Moreover, vascular obstruction is another challenge on the way of achieving desired efficacy from NSC grafting (Luarte et al., 2016b; Xin et al., 2014). Recently, EVs have become more and more appreciated as therapeutic alternative to the cell-based therapy. Increasing lines of evidence support their therapeutic functionality in the brain *in vivo* (Spellicy & Stice, 2020; Zhang et al., 2019b) In addition, due to their size, blood-brain barrier (BBB) is actually not a barrier on their way to target brain cells (Saeedi et al., 2019).

EVs in adult neurogenesis

In addition to NPCs, adult neurogenic niches also host a variety of other cell types such as astrocytes, microglia, neuroblasts, endothelial cells, pericytes as well as mature and immature neurons (Batiz et al., 2016; Luarte et al., 2017). Proper communication between these cellular components is vital both for neurogenesis and for the integration of adult newborn neurons with synaptic circuitry in the hippocampus (Carlen et al., 2002; J. T. Goncalves et al., 2016). A combination of extrinsic factors including morphogens, growth factors, and neurotransmitters together with intrinsic signals such as transcription factors and epigenetic regulators guide neurogenesis through its multiple stages. Although the role of EVs in adult neurogenesis has yet to be firmly established, there are several lines of evidence supporting their involvement. Their potential participation in adult neurogenesis has been reviewed by multiple studies (Batiz et al., 2016; Luarte et al., 2016a; Luarte et al., 2017). For instance, miR-34a, which has a profound role in adult neurogenesis and differentiation of developing neurons, has also been detected in exosomes released in the medium of neuronal cultures (Mollinari et al., 2015). In addition, as mentioned by Bátiz et al, some of the protein modulators of adult neurogenesis are also found in exosomes though from different cell types (Batiz et al., 2016). Among them are Transforming Growth Factor-beta 1 (TGFB1) (C. S. Hong et al., 2014; Raimondo et al., 2015; Sole et al., 2015; Szajnik et al., 2013; Torreggiani et al., 2014), Ephrin-B2 (Mathivanan et al., 2010), Vascular Endothelial Growth Factor (VEGF) (Ekstrom et al., 2014; Thompson et al., 2013; Torreggiani et al., 2014) as well as proteins that are involved in cell fate decision in neurogenic niches such as Pigment Endothelium-Derived Factor (PEDF) (Ramirez-Castillejo et al., 2006) (Hajrasouliha et al., 2013), Insulin-like Growth Factor Binding Protein 6 (IGFBP6) (Barkho et al., 2006), Epidermal Growth Factor Receptor (EGFR) (Graner et al., 2009), and Fibroblast Growth Factor 2 (FGF2) (Hajrasouliha et al., 2013). The same is also reported for some of the proteins of signaling pathways such as Wnt, Notch, and SHH signaling pathways (Batiz et al., 2016; Wendler et al., 2013).

Further studies are needed to investigate their direct role in adult neurogenesis for two important reasons. Firstly, EVs can negatively affect adult neurogenesis. The injection of blood exosomes collected from major depressive disorder patients into mice is shown to cause depressive-like behavior (Wei et al., 2020). Data from miRNA sequencing revealed higher levels of hsa-miR-139-5p in blood exosomes which is a negative regulator of neurogenesis and leads to dysfunction of adult hippocampal neurogenesis (Wei et al., 2020). Secondly, EVs can have therapeutic potential and can improve neurogenesis. Systematic administration of exosomes loaded with miR-124 can promote neurogenesis after ischemia injury (Yang et al., 2017).

It has been suggested that re-activating some of the developmental signalling pathways in the adult might be beneficial to ameliorate the brain injuries (Goncalves et al., 2018). For instance, retinoic acid (RA), as a guidance molecule, is crucial for axon/neurite outgrowth during development (Dmetrichuk et al., 2006). The RA signaling pathway has been shown to regulate remyelination and axonal/neurite outgrowth after spinal cord injury (Goncalves et al., 2019;Goncalves et al., 2018). Interestingly, exosomes were shown as the important intercellular transporter of RA that enable crosstalk between oligodendrocyte precursor cells ($NG2^+$ cells) and neurons to mediate remyelination and axonal/neurite outgrowth. The importance of EVs in mediating the neuron-glia crosstalk was further highlighted by Fruhbeis et al. The authors demonstrated that the small extracellular vesicles released by oligodendrocytes are vital for axonal maintenance and support axonal fast transport. This study suggested that since oligodendroglial exosomes are critical for neuronal integrity, they could be a causative link between glial dysfunction and axonal degeneration (Fruhbeis et al., 2020).

5. Synaptogenesis

Once neuronal migration is accomplished, neurons undergo morphological changes, also known as neuromorphogenesis, to appreciate synaptic connectivity (Cornell & Toyo-Oka, 2017). These changes include the growth of axonal and dendritic cones, which eventually give rise to formation of pre-synaptic terminal and post-synaptic sites. Axons elongate to reach the appropriate post-synaptic targets to form synapses and facilitate neurotransmission. The choice of postsynaptic site is different in glutamatergic and GABAergic neurons. Glutamatergic synapses are formed on dendritic spines while GABAergic synapses take place on somas and proximal dendrites (Sanes et al., 2019). Synaptogenesis is a long-term developmental process that initiates in the developing brain at around the week 28 of gestation and continues to the postnatal period (Huttenlocher, 1990). Synaptic density increases exponentially during infancy and reaches its peak, which is 50% higher than in adult, at around 1-2 years. This trend begins to decline between ages 2 - 16 years (Huttenlocher, 1979) by synaptic pruning.

EVs during synaptogenesis

As with the other stages of CNS development, synaptic differentiation and maturation processes are regulated by a distinct set of proteins and miRNAs. Interestingly, there is evidence that synaptic components are incorporated into EVs, supporting their role at synapses. Results obtained by electron microscopy revealed that somato-dendritic compartments of differentiated cortical and hippocampal neurons can release exosomes *in vitro* (Lachenal et al., 2011). Furthermore, the exosome release was shown to be modulated by glutamatergic synaptic activity and calcium influx. It was first demonstrated that mature rat cortical and hippocampal neurons secrete exosomes. Using two different concentration of KCl, exosome release was shown to be mediated by

KCl-induced depolarization since antibodies against exosomal markers Flotillin-1 and ALIX showed stronger immunoreactivity in the cell culture medium treated with the high KCl concentration. Therefore, exosome release from mature neurons is closely associated with depolarization. In addition, incubation with the calcium ionophore ionomycin, which causes a rise in cytosolic calcium, increased exosome release. Furthermore, antagonists of GABA receptor significantly increased release of exosomes, due to enhancement of glutamatergic spontaneous activity. This was further confirmed with antagonists of NMDA and APMA receptors. These antagonists both inhibited the increase in exosome release confirming that glutamatergic synaptic activity modulates the exosome release from mature neurons. In addition, exosomes were found to contain GluR2/3 subunits of AMPA receptors, which led the authors to suggest that exosomal release of AMPA receptors, which is followed by glutamatergic synaptic activity, is a mechanism to eliminate synaptic receptors as a response to alterations of synaptic plasticity.

The presence of AMPA receptor subunit GluR2/3 was previously reported in exosomes derived from primary cultures of rat cortical neurons. Fauré et al. demonstrated that rat primary cortical neurons are able to secrete exosomes (Faure et al., 2006). Characterization of their content by immunoblotting revealed that they carry GPI anchored prion protein and neuronal cell adhesion molecule L1 as well as AMPA receptor subunit GluR2/3. Authors suggested that exosomes might act as a disposal mechanism in synapses -where lysosomes are not present- to discard AMPA receptors in events such as synaptic depression. However, these exosomes were shown to lack NMDA receptor subunit NR1 indicating that such disposal strategy is not relevant to the other glutamate receptors. It was also demonstrated that exosomal release by primary cortical neurons can be regulated by depolarization and that GluR2 secretion upon depolarization is associated with exosomes. In a review by Smalheiser, multiple scenarios were discussed about the role of exosomes derived from postsynaptic membranes in transferring synaptic proteins e.g. calmodulin-dependent protein kinase II (CAM kinase II) alpha and mRNAs and miRNAs to presynaptic terminals, an action that highlights their involvement in synaptic plasticity (Smalheiser, 2007). In particular, it was proposed that exosomal loading and intracellular transport of synaptic signaling molecules occur at the postsynaptic lipid rafts.

In addition to transfer of synaptic related proteins, EVs are confirmed to carry miRNAs with functionalities at synapses. The study by Morel et al. indicated a role for exosomes in modulating synaptic activity through astrocyte-neuronal communication (Morel et al., 2013). In their study, miR-124a was highlighted as the key component carried by exosomes from neurons to astrocytes where it targets Glutamate Synthase NADH (GLT1). GLT1 is a rodent analog of human excitatory amino acid transporter 2 (EAAT2), an astroglial synaptic protein with a key role in glutamate uptake and clearance at synapses (Kim et al., 2011). It was demonstrated that GLT1 physiological function depends on the signals received by neurons. Protein and mRNA levels of GLT1/EAAT2 in astrocytes were significantly increased when co-cultured with neurons (Gegelashvili et al., 1997; Schlag et al., 1998). Moreover, due to having long 3′untranslated region (UTR), authors proposed that GLT/EAAT2 could be a target for miRNA-mediated regulations. It was first demonstrated that primary cultures from mouse cerebral cortex neurons and astrocytes secrete exosomes. Interestingly, miR-124a was highly enriched in neurons and their harvested exosomes while cultured astrocytes presented only a minimum amount of miR-124a. Neuronal exosomes were then shown to be internalized in astrocytes as detected by time-lapse imaging of the fluorescently labeled exosome membranes. Furthermore, these exosomes were able to increase GLT1 protein levels in cultured astrocytes. Direct transfection of astrocytes with miR-124a increased GLT1 protein expression without any change on mRNA level indicating that miR-124a regulatory effect is at the translational level. *In vivo* experiments in which a specific antisense miR-124a was injected into the striatum part of the mouse brain resulted in reduction of GLT1 and glutamate uptake. Moreover, it was indicated that miR-124a regulatory effect on GLT1 is independent of ligand ephrin3, a putative suppressor of GLT1, confirming that miR-124a association with GLT1 expression is indirect. Moreover, exogenous delivery of miR-124a into the SOD1 mouse model of amyotrophic lateral sclerosis efficiently reduced pathological loss of GLT1 in this model. Collectively, these results underscore the importance of EVs in regulating synaptic function by targeting astrocytes that act alongside of neurons to ensure normal synaptic function.

A recent study by Sharma et al. highlighted the potential role of EVs in improving neurogenesis, synaptogenesis, and circuit assembly in Rett syndrome (Sharma et al., 2019). Rett syndrome is a neurodevelopmental disorder and is caused by mutations in the methyl-CpG-binding protein 2 (MECP2) gene (Amir et al., 1999). MECP2 gene expression is correlated with neuronal maturation and synaptogenesis (Fukuda et al., 2005; Shahbazian et al., 2002). Proteomics analysis conducted by Sharma et al. demonstrated that exosomes from hiPSC-derived neural cultures contribute to neural circuit development owing to the signaling proteins they carry. Authors showed that these proteins are absent from exosomes collected from MECP2 loss-of-function (MECP2LOF) cultures. Furthermore, treatment with control exosomes were effective, both *in vitro* and *in vivo*, in improving neurogenesis. Exosome treatment also improved synaptogenesis and circuit connectivity in MECP2LOF cultures. Based on the results, authors suggested that MECP2 mutation results in the alteration of protein cargos and signaling bioactivity of exosomes.

EVs are also involved in synaptic growth and function. For instance, exosomes are shown to mediate communication between pre-and postsynaptic cells by transferring a retrograde signaling component, Synaptotagmin 4 (Syt4), which is essential during development and for maintenance of synaptic plasticity and growth (Korkut et al., 2013). It was demonstrated that presynaptic neurons at neuromuscular junctions in Drosophila release Syt4 via exosomes. Syt4 is received by postsynaptic muscles and regulate the activity-dependent synaptic growth and potentiation of spontaneous release. In fact, exosomes coordinate the presynaptic function with postsynaptic output by means of Syt4 transfer. The EVs involvement in synaptic plasticity goes beyond transferring retrograde signaling. They also transfer the neuronal Arc mRNA. Arc protein activity is vital for long-term memory and consolidation of information as well as synapse eliminaton (Pastuzyn et al., 2018) and it has been implicated in several neurodevelopmental disorders such as Angelman syndrome (Greer et al., 2010; Pastuzyn & Shepherd, 2017), Fragile X syndrome (Park et al., 2008), and Schizophrenia (Fromer et al., 2014; Manago et al., 2016; Purcell et al., 2014). It has been demonstraed that Arc EVs can transfer highly abundant Arc mRNAs to dendrites e.g. in response to neuronal activity (Pastuzyn et al., 2018). Similarly, the Arc homolog in Drosophila, dArc1, uses EVs for its own mRNA transfer at neuromuscular junctions (Ashley et al., 2018).

6. Synaptic pruning

The excess in synaptic contacts created during early infancy will be eliminated in a process called synaptic pruning. Sculpting the synapses is crucial for proper neural circuit formation and plasticity. It is estimated that nearly half of the synapses and neurons will be removed by pruning and apoptosis (Jiang & Nardelli, 2016; Stiles & Jernigan, 2010). Microglia are the core players in this refinement process (Kettenmann et al., 2013; Schafer et al., 2012). Having close association with presynaptic and synaptic elements, microglia are thought to target weak synapses for phagocytosis (S. Hong et al., 2016; Schafer et al., 2012; Tremblay et al., 2010). Dysfunction of reciprocal interaction between microglia and neurons are observed in neurodevelopmental and neuropsychiatric disorders (Zhan et al., 2014).

EVs role in synaptic pruning

EVs are shown to act as endogenous factors able to eliminate synapses. Lee and collogues revealed an interplay between Wnt and Proline-

Rich 7 (PRR7) via exosomes that results in regulating the number of excitatory synapses (Lee et al., 2018). Wnt signaling is considered as one of the vital synaptogenic factors during both synapse formation and maintenance (Dickins & Salinas, 2013). PPR7 was first identified by proteomic analysis in postsynaptic density fraction of rat forebrain. Although its function was not clear at the time, the interaction with NMDA receptor and Postsynaptic Density Protein 95 (PSD-95) suggests a role for PRR7 in regulating neural activities (Murata et al., 2005). The reciprocal interaction between PPR7 and Wnt reported by Lee et al. unraveled a role for PRR7 at synapses. Both PPR7 and Wnt were shown to be released in exosomes by rat hippocampal neurons. The opposite functions of Wnt and PRR7 maintain the balance between synaptogenesis and synapse removal and interestingly this action is facilitated by exosomes as signal carriers. Authors provided a set of molecular mechanisms through which exosomal PRR7 eliminate synapses. These include inhibiting exosomal secretion of Wnt, protein degradation of PSD-95, and activation of GSK3β as the downstream component of Wnt signaling. First, it was shown that PRR7, Wnt5a, and Wnt7a are highly enriched in exosomes derived from mouse hippocampal neurons. Furthermore, using NMDA receptor antagonist, exosomal release of PRR7 was shown to be reduced while AMPA receptor antagonist did not achieve the same result indicating that neurons release PRR7 in a NMDAR-dependent manner. One of the mechanisms for synaptic elimination is Ubiquitin–Proteasome System (UPS), a strategy that is also applied by PRR7 to reduce synaptic scaffolding proteins including PSD-95, Membrane Associated Guanylate Kinases (MAGUKs), and SAP90/PSD-95-associated proteins (SAPAPs). In addition, PRR7 overexpression increased the total number of poly-ubiquinated proteins confirmed by antibodies against K48-specific poly-ubiquitination. Interestingly, PRR7-contaning exosomes were shown to target excitatory synapses for removal. This was evident when incubation of naïve neurons with these exosomes for 24 h resulted in reduction of excitatory synapse numbers, determined by PSD-95 co-localization with vesicular Glutamate Transporter 1 (vGLUT1), with no alterations in the number of inhibitory synapses.

Interestingly, there has been recently a role identified for exosomes as a regulator of synaptic pruning through their phagocytic ability. Culturing rat pheochromocytoma PC12 cells in a serum-free medium containing Nerve Growth Factor (NGF), Bahrini and colleagues were able to induce the formation of neurite outgrowth and synaptic-like structures (Bahrini et al., 2015). Authors also sought to assess the role of microglia in the clearance of degenerating neurites. It was observed that neurites become degenerated within two days in the absence of NGF, however in DMEM containing 10% FBS the degeneration rate was lower with most of the neurites still reserved. Intriguingly, when PC12 cells were co-cultured with MG6, a microglial cell line derived from mouse, in DMEM/F10 lacking NGF, neurite elimination rate remarkably increased indicating the promotion of pruning by microglia. Considering the previous findings that depolarized neurons secrete exosomes (Faure et al., 2006) and that neuronal derived exosomes target microglia (Cossetti et al., 2012), Bahrini et al. also investigated the association between PC12 cells-derived exosomes and microglial function. PC12 cells were pre-incubated with MG6 cells for 16 h. Comparison of synaptic pruning in pre-incubated MG16 with control MG6 revealed stronger ability for pre-incubated MG6 cells in neurite pruning and it was therefore proposed that exosomes enhance microglia pruning activity. Microarray analysis identified 183 differentially expressed genes in pre-incubated MG6 cells with “Phagosome’’ and “Complement and coagulation cascades’’ being among the enriched terms. Further quantative PCR analysis indicated the up-regulation of complement factor B (Cfb) and complement component 3 (C3) genes. Since C3 mRNA level remained unchanged, authors suggested that exosome regulatory effect on C3 is at the transcriptional level as opposed to directly transferring the mRNA. Thus, exosomes derived from PC12 cells can be engulfed by microglia where they enhance phagocytosis by upregulating the expression of complement factors. However, the factors in exosomes that induce such changes in microglia have yet to be identified.

7. Myelination

Fast transport of action potentials is only possible when axons are enwrapped by myelin sheaths. Myelination is a long-lasting event starting early during postnatal period and extending into adulthood (Semple et al., 2013). Oligodendrocytes and Schwann cells organize myelination in central and peripheral nervous systems respectively. Oligodendrocyte Progenitor Cells (OPC) are produced by NECs in ventricular zones (Bergles & Richardson, 2015; Jiang & Nardelli, 2016). After migration throughout the CNS, OPC will be distributed in gray and white matters where they undergo differentiation to become pre-oligodendrocytes and eventually mature myelinating oligodendrocytes (Bergles & Richardson, 2015; Jiang & Nardelli, 2016). Once differentiated, they extend their plasma membrane towards axons and wrap them with multilayered myelin sheaths. There is also an increase in the gene expression of myelin related genes such as Myelin Basic Protein (MBP), myelin Proteolipid Protein (PLP), Myelin-Associated Glycoprotein (MAG), and Myelin Oligodendrocyte Glycoprotein (MOG) (Bercury & Macklin, 2015; van Tilborg et al., 2018) in OPC prior to differentiation. It is obvious that neuron-oligodendrocyte reciprocal interaction and the exchanged biomolecules between them are the key factors for the myelination process.

EVs role in Myelination

Accumulating evidence points to the involvement of EVs in orchestrating myelination in CNS. We only briefly discuss the evidence in this section and refer the reader to a recent excellent review on this topic by S. Domingues et al. (Domingues et al., 2020).

Exosomes secreted by oligodendrocytes are carriers of proteins that have been recognized to play a role in myelination. The study by Kramer-Albers et al. revealed that exosomes secreted by oligodendrocytes contain several myelin proteins including PLP, 2’3’ -Cyclic-Nucleotide-Phosphodiesterase (CNP), MBP, and MOG (Kramer-Albers et al., 2007). Moreover, it was demonstrated that exosome secretion and heterogeneity of secreted exosomes are regulated by intracellular Ca^{2+} levels suggesting that exosome release by oligodendrocytes is coupled to neuronal activity. Besides myelin proteins, the authors also identified numerous other proteins including chaperones and enzymes with proposed functions in the relief of cell stress. Thus, they suggested that oligodendrocyte-derived exosomes also provide trophic support to the axons. Further characterization of oligodendroglial exosomes revealed the presence of the classic myelin lipids galactocerebroside and sulfatide indicating that oligodendroglial exosomes are uniquely equipped to support myelination of axons.

As mentioned, there is a bidirectional interaction between oligodendrocytes and neurons and EVs are one of the mediators. We noticed a feedback principle mentioned by most of the papers. Exosomes are secreted from oligodendrocytes under the influence received by neurons to arrange a set of alterations in neurons. For instance, in their study Fruhbeis et al. demonstrated that neuronal electrical activity (depolarization) triggers glutamate release, which induces Ca^{2+} influx into oligodendrocytes through AMPA and NMDA receptors and subsequently stimulates exosome release (Fruhbeis et al., 2013b). These exosomes are then internalized by neurons through endocytic pathway. Utilizing microfluidic chambers it was shown that exosomes were taken up by neurons at axonal and somatodendritic sites (Fruhbeis et al., 2013a). To unravel the bioactivity of exosomes, a Boyden chamber co-culture of neurons and oligodendrocytes was prepared. Neurons were subjected to oxidative stress and starvation via hydrogen peroxide and absence of B27 supplement respectively. These changes increased metabolic activity in the presence of oligodendrocyte-derived exosomes suggesting that exosomes confer protection to neurons. The supportive role of oligodendroglial exosomes on neurons is not limited to neuroprotection.

Fröhlich et al. reported a broad spectrum of roles for oligodendrocyte-derived exosomes, which include activation of signaling pathways such as MEK/Erk and PI3K/Akt, neuronal gene expression, and enhancement of action potential, as well as resistance to oxidative stress and promotion of neuronal survival (Frohlich et al., 2014).

In addition to serving as initiating factor in myelination, neurons can also negatively regulate myelination. Bakhti et al. reported that oligodendrocytes secrete exosome-like vesicles that has autoinhibitory effect on cell differentiation by reducing the cell surface expansion and subsequently inhibiting myelination (Bakhti et al., 2011). Regarding the regulatory role of Rho-associated kinase (RhoA-ROCK) pathway in oligodendrocyte branching and cell surface expansion (Kippert et al., 2009; Kippert et al., 2007; Liang et al., 2004), authors tested the effect of Rock inhibitor and Myosin II inhibitor on cell surface size. It was observed that such inhibitors prevented the negative regulatory effect of exosome-like vesicles on cell expansion. Thus, exosome-like vesicles inhibit cell surface size through the activation of Rho-ROCK-myosin signaling axis. Furthermore, incubation of oligodendrocytes with neuronal conditioned medium robustly reduced exosome release and therefore authors proposed the likelihood of one or more neuronal factors in neuronal conditioned medium that prevent the exosome secretion. Therefore, neuronal signals regulate myelin membrane biogenesis through controlling the exosome release.

Oligodendrocytes have been shown to receive instructive signals -incorporated in exosomes- also from other cell types such as astrocytes and dendritic cells. For instance, it was observed that direct contact culture of OPC with astrocytes significantly enhanced exosome secretion and OPC proliferation (Zhang et al., 2020) when compared to OPC co-cultured with AST supernatant group. Transcriptome sequencing revealed a set of differentially expressed genes between the two groups among which was the upregulation of Integrin subunit Beta 4 (ITGB4), a protein which mediates cell adhesion suggesting an important role for ITGB4 in OPC proliferation. This was confirmed by ITGB4 gene knockdown, which resulted in reduction of exosome release and proliferation rate. When exosomes were added to the ITGB4 deficient OPC/astrocyte co-culture proliferation rate was restored. It was therefore concluded that OPC proliferation is regulated by astrocytes through ITGB4 mediated exosomal secretion.

A considerable number of papers have highlighted the therapeutic effects of extracellular vesicles and exosomes in promoting oligodendrocytes proliferation and/or differentiation as well as remyelination and axon regeneration. For example, in prenatal brain injury, which affects white and gray matter and causes severe neurodevelopmental phenotypes, Mesenchymal stromal cell-derived exosomes were effective in rescuing myelination and reducing injuries of gray and white matter (Thomi et al., 2019). In another study, treatment with Mesenchymal stem cell-derived EVs in a rat model of preterm brain injuries efficiently ameliorated inflammation induced hypomyelination, neuronal cell degeneration and long-term white matter microstructural abnormalities (Drommelschmidt et al., 2017). With promising outcomes, environmental enrichment (EE) also seems an effective treatment in improving brain function. EE is described as enhancement of physical, social and intellectual activity (Pusic et al., 2016). Exosomes have been found as one of the effective neuroprotection elements in EE (Pusic & Kraig, 2014). Application of both young and EE-serum exosomes on

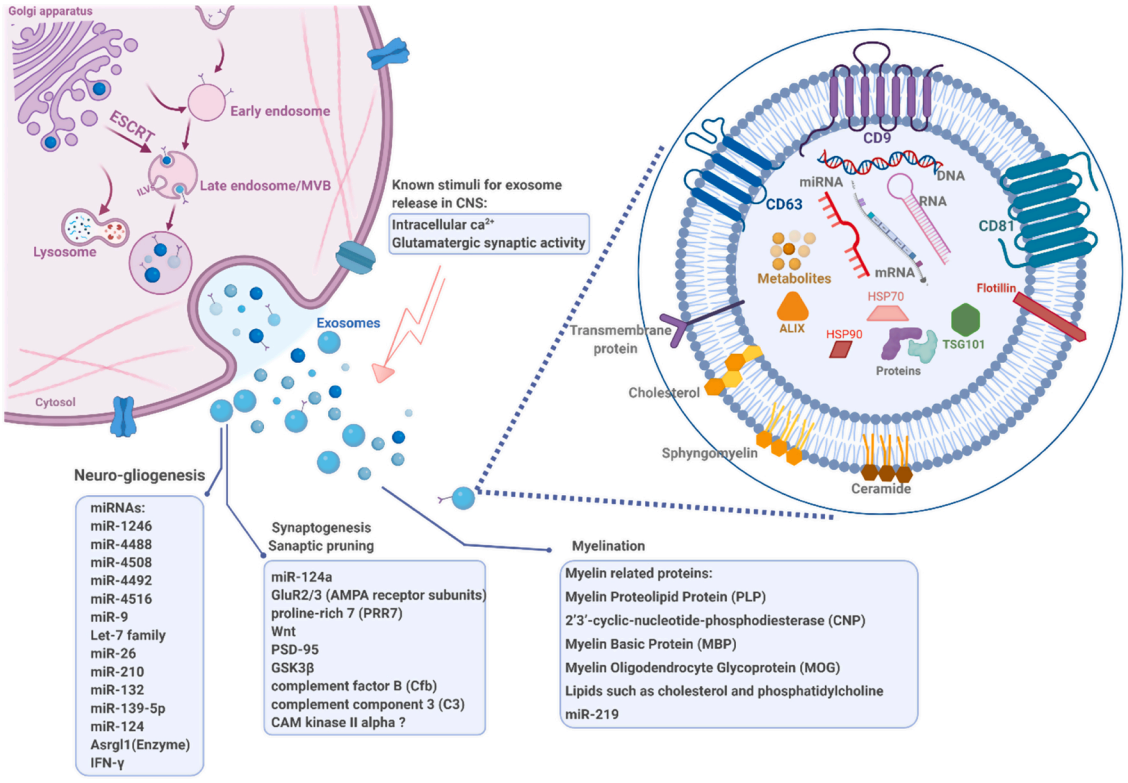

Fig. 2. Schematic of exosome secretion, structure, and involvement during CNS development. The exosome cargos are sorted into early endosomes through either endocytosis, inward folding of plasma membrane, or directly from Golgi network (Palmulli & van Niel, 2018), a process that is mediated by the endosomal sorting complexes required for transport (ESCRT) machinery. Invagination of the late endosomes, also referred as Multivesicular bodies (MVBs), forms intraluminal vesicles (ILVs). These ILVs are eventually released as exosomes into extracellular space by fusion of MVBs with plasma membrane. The precise mechanism of release is not clear however, intracellular calcium and depolarization, mediated by glutamatergic synaptic activity, are indicated as triggers for exosome secretion. Some of the exosome contents are highlighted at each stages of CNS development. As it is shown, miRNAs are the most studied element of exosomes during neuro-gliogenesis. CAM kinase II alpha is suggested as one of the candidate cargos of synaptic exosome, a scenario which needs to be investigated (Smalheiser, 2007). Interestingly, exosomes actively involve in myelination mainly by transporting myelin related proteins between neurons and oligodendrocytes.

hippocampal slice culture, an *in vitro* myelination model, significantly increased OPCs proliferation. They were also effective in increasing the baseline levels of BMP, indicating that myelination is also increased. Analysis of miRNA expression profile identified miR-219 as the effective factor in such exosomes. Interestingly, miR-219 was also able to increase OPCs differentiation to myelinating oligodendrocytes. When exosomes derived from young rats were nasally administered in aging rats, a significant myelination was observed in the motor cortex suggesting the regenerative therapeutic potential of such exosomes. Fig. 2 represents a summary of exosome secretion process and its contribution at each CNS developmental stage.

8. Challenges and Future directions

Despite a plethora of studies that have enlightened our understanding of EVs' biogenesis, structure and function, some fundamental and critical questions remain to be answered. For instance, there is still insufficient knowledge about the distinct roles of each subtype and the precise mechanisms that regulate their biogenetic pathways. Why are some EVs produced through the ESCRT-dependent pathway while others are generated by the ESCRT- independent pathway? And how does the biogenetic pathway of the parental cell affects the impact of the EV on a distant recipient cell? Such questions need in depth answers in order to understand the full paradigm of EVs in CNS development in physiology and pathology. Such understanding is also elementary to successful exploitation of EVs in therapeutic strategies to treat CNS disorders. There is an increasing interest in engineering EVs for therapeutic purposes; the success of such approach highly relies on basic understanding of the relationships between biogenesis, structure and function of natural EVs.

Another challenge is posed by the fact that our current understanding about EVs mainly stems from *in vitro* studies. It needs to be determined whether this knowledge is transferrable to the *in vivo* situation. Interestingly, it has recently been demonstrated that EVs released by 3D-culture systems differ from EVs released by 2D-culture systems, underscoring the impact of the microenvironment on the production of EVs (Rocha et al., 2019; Thippabhotla et al., 2019; Villasante et al., 2016).

In vitro studies also lack a dynamic BBB posing a challenge on the exploitation of *in vitro* EV knowledge in designing strategies to treat CNS disorders. Although EVs are capable of crossing the BBB, the rate at which they pass such dynamic barrier *in vivo* might affect their treatment efficacy as determined *in vitro*. For instance, the study by Banks et al. showed that exosomes from different cell lines were able to pass the BBB, albeit at different rates and engaging different molecular transport mechanisms (Banks et al., 2020).

Last but not least, designing successful EV-based strategies to treat CNS disorders requires consideration of a few complicating points. The brain is an intricate organization of neural and glial cells that displays regional differences in structure and function and most likely in EV repertoir and dynamics. Also, during CNS development *in situ* EV-properties probably vary in correspondence with developmental milestones. Therefore, future investigations should also focus on the impact of the brain regional differences and the CNS developmental stages on EV structure and function and *vice versa*. Since the brain is a hard to access tissue such studies will certainly benefit from recent and future developments in the generation of human-derived brain organoids.

9. Conclusion

The current review surveys the enormous amount of research conducted on the role of EVs in neural cell communication and highlights the fact that exosomes dynamically accompany neural cells throughout CNS development from initial stages such as neurogenesis and synaptogenesis to the final steps when cells are fully functional.

EVs are appreciated as vehicles carrying messages to which recipient cells respond. The messages are composed by the parental cell and vary with its genotype, phenotype and microenvironment. Reciprocity involving neural and non-neural cells makes this membrane-encapsulated communication system apt to orchestrate dynamics of the developing and adult brain. We are just beginning to understand the complexity of EV-mediated communication in CNS. On the basis of current knowledge, we already see opportunities to utilize EVs for diagnosis and therapy of CNS disorders. For example, the onset of autism spectrum disorders and Rett syndrome begins approximately below the age of two when synaptogenesis and neuro-gliogenesis are occurring. EVs offer a promising therapeutic avenue to instruct these geneses to develop normally. More research is needed to fully understand this intricate communication system of the brain and to become able to exploit clinically its diagnostic and therapeutic potentials.

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Declaration of Competing Interest

The authors report no declarations of interest.

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The schematic representations were created using BioRender.com.

Appendix A. The Peer Review Overview and Supplementary data

The Peer Review Overview and Supplementary data associated with this article can be found in the online version, at doi:https://doi.org/10.1016/j.pneurobio.2021.102124.

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