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Subventricular Zone Neural Stem Cells Release Extracellular Vesicles During Early Postnatal Neurogenesis

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SUBVENTRICULAR ZONE NEURAL STEM CELLS RELEASE EXTRACELLULAR VESICLES DURING EARLY POSTNATAL NEUROGENESIS

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ABSTRACT

Extracellular vesicles (EVs) are nanometer sized particles released from all cells. EVs are found in all biological fluids, including cerebrospinal fluid (CSF) and blood. EVs modulate intercellular communication through the transfer of nucleic acids and proteins from donor to recipient cells. During early postnatal neurogenesis, subventricular zone (SVZ) neural stem cells (NSCs) asymmetrically divide to give rise to neuroblasts that migrate along the rostral migratory stream (RMS) and populate the olfactory bulb (OB). Other, non-neuronal cells populate the SVZ, such as microglia and endothelial cells. Microglia have been shown to regulate SVZ NSCs, but it remains unclear if this communication is bidirectional. The purpose of this study was to determine if SVZ NSCs release EVs and what physiological impact this has on postnatal SVZ development. First, we generated a protocol in which SVZ NSCs were placed in culture and EVs were isolated from NSC conditioned media. To study EVs *in vivo*, EVs were labeled with DiI and transplanted into the SVZ of neonatal mice. Immunocytochemistry, immunohistochemistry, and electron microscopy were used to confirm the isolation and transplantation of NSC EVs. Using this methodology, SVZ NSC EVs were transplanted into the lateral ventricles of neonatal mice. We found a majority of DiI particles coalesced with Iba1-positive microglia in the SVZ. Furthermore, Iba1-positive microglia underwent a morphological shift from a stellate to rounded phenotype. RNA sequencing and analysis of EV treated microglia revealed that immune system processes and inflammatory responses were the most highly

enriched and represented terms. Small RNA sequencing of NSC EVs uncovered families of miRNA, such as Let-7, that have been shown to regulate microglia physiology and morphology. The upregulation of inflammatory response transcripts included interleukin 1α (IL-1α), IL-1β, and IL-6. In agreement with RNA sequencing data, Luminex assays revealed cytokines, such as IL-1α, IL-1β, and IL-6, were significantly upregulated in treated microglia. EV-depleted microglia media was transplanted into the lateral ventricles of neonatal mice, and media collected from EV-treated microglia reduced SVZ NSC proliferation. To further investigate if SVZ NSCs release EVs *in vivo*, we generated a transgenic model system in which EV marker protein tetraspanin CD9 was fused to Turbo-GFP (CD9-GFP), which is derived from the copepod *Pontellina plumata.* CD9-GFP was inserted downstream of a STOP codon flanked by loxP sites. CD9-GFP was found in Nestin-positive cells in the SVZ of transgenic mice electroporated with Cre-recombinase. Taken together, our data supports the release of EVs from SVZ NSCs both *in vivo* and *in vitro.* Furthermore, EVs released from SVZ NSCs regulate microglia during early postnatal development.

DEDICATION

To Dixie, Kitty and Ben.

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CHAPTER ONE

INTRODUCTION

Extracellular Vesicles

Extracellular Vesicles (EVs) are primarily membranous-derived particles ranging in size from 30 nm – 500 nm (Cocucci and Meldolesi 2015; Théry, Zitvogel, and Amigorena 2002; Maas, Breakefield, and Weaver 2017). EVs are found in most biological fluids, including cerebrospinal fluid (CSF), blood, urine and saliva, and carry an array of biologically active cargo such as proteins, lipids and RNA (Valadi et al. 2007; Ramachandran and Palanisamy 2012; Raposo and Stoorvogel 2013; Théry, Zitvogel, and Amigorena 2002; Wei et al. 2017). Three types of EVs have been described: exosomes, microvesicles and exomeres (H. Zhang et al. 2018; Théry, Zitvogel, and Amigorena 2002; Cocucci and Meldolesi 2015). Exosomes are generated from the inward budding of an early endosome to generate intraluminal vesicles (ILVs) that populate multivesicular bodies (MVBs; **Figure 1A-C**) (Théry, Zitvogel, and Amigorena 2002). MVB biogenesis relies on the ESCRT (endosomal sorting complex required for transport) pathway. Comprised of five complexes, the ESCRT pathway intricately delegates specific steps in MVB biogenesis to each of the complexes (Henne, Buchkovich, and Emr 2011). ESCRT-0 is required at the site of initiation to generate the early endosome (Henne, Buchkovich, and Emr 2011). Invagination of the cell membrane to generate the early endosome requires the recruitment of ESCRT complexes I and II by ESCRT-0 (Bache et al. 2003; Lu et al. 2003; Katzmann et al. 2003). ESCRT-

II is required to initiate ESCRT-III complex (Booth et al. 2006; Babst et al. 2002; Langelier et al. 2006). ESCRT-III tethers the outer edges of the early endosome which has been suggested to allow for Vps4-Vta1 complex (V complex) to assemble and complete the scission of the endosome (Henne, Buchkovich, and Emr 2011). Interestingly, protein cargo sorting into ILVs can occur in an ESCRT-dependent or -independent manner (van Niel et al. 2011). MVBs then fuse with the cell membrane and release the vesicles, now called exosomes, into the extracellular space (**Figure 1D**). Although it is unclear precisely how MVBs are sequestered to, dock and fuse with the cell membrane, many studies point to the involvement of RAB GTPases. The first example of RAB GTPase involvement in exosome secretion came from studies utilizing reticulocyte cell lines which demonstrated that Rab11 was required for exosome release (Savina, Vidal, and Colombo 2002). Rab27a and Rab27b have also been shown to reduce exosome release from cells by inhibiting the targeting and docking of MVBs to the plasma membrane (Ostrowski et al. 2010; A. Bobrie et al. 2012; Peinado et al. 2012). Another study discovered that Rab-GTPase activating proteins TBC1D10A-C in oligodendrocytes target Rab35 function which is required for exosome release (Hsu et al. 2010). Reduction of Rab35 function resulted in the accumulation of ILVs in MVBs within the cytoplasm, thus suggesting Rab35's function in docking at the plasma membrane (Hsu et al. 2010). Conversely, microvesicles are generated following scission of the outward budding of the cell membrane (**Figure 1H**) (Cocucci and Meldolesi 2015). Exosomes are characteristically smaller in size (<

150 nm), whereas microvesicles are larger in size (200-500 nm) (Maas, Breakefield, and Weaver 2017). Finally, non-membranous EVs, called exomeres, are protein-like vesicles comprised of metabolic, translational and coagulation regulating proteins (H. Zhang et al. 2018). Elucidating the biogenesis of these non-membranous vesicles will be critical to understand their physiological roles (H. Zhang et al. 2018).

Extracellular Vesicle Cargo

EVs carry an array of biological material such as miRNA, mRNA and protein (Valadi et al. 2007; Ramachandran and Palanisamy 2012; Raposo and Stoorvogel 2013; Théry, Zitvogel, and Amigorena 2002; Wei et al. 2017). Historically, EVs were thought to act as a cellular disposal system in which damaged or unnecessary RNA and protein were shuttled into and released from the cell (Harding, Heuser, and Stahl 1983). However, recent discussion has shifted to favor a more intentional role of EVs and their cargo. To uncover the role EVs play in cellular communication, EV content has been investigated. Studies have ranged from small scale hypothesis driven experimentation to next generation RNA sequencing and microarrays to proteomics and lipidomic studies.

Since the discovery that EVs can transfer functional miRNA and RNA from donor to recipient cells, studies have focused on the regulatory roles of EVs in both development and disease. However, many questions remain regarding EV miRNA quantity, biological significance and the functional transfer of miRNA between cells (**Figure 1E-F**). Predominately, circulating miRNAs are bound to protein

complexes, such as Ago2, with only a small subset of miRNAs found within exosomes (Melo et al. 2014; Arroyo et al. 2011). Quantity of EV content per EV was discovered to follow a low occupancy model (Chevillet et al. 2014). It is estimated that for every 100 circulating exosomes, only one will contain miRNAs, but for non-circulating exosomes, this ratio is higher (1:10) (Chevillet et al. 2014). miRNA sorting is dependent on miRNA consensus sequences or binding motifs, miRNA shuttling proteins and endogenous target mRNA levels (Melo et al. 2014; Squadrito et al. 2014). Heterogenous nuclear ribonucleoprotein A2B1 (hnrnpA2B1) sorts miRNA into exosomes through specific motifs found on EV miRNAs (Villarroya-Beltri et al. 2013). Sorting of miRNAs can be altered by mutagenesis of the binding motif or through the down-regulation or loss of hnrnpa2b1 (Villarroya-Beltri et al. 2013). In breast cancer (BC) cells, miRNA processing components were identified within exosomes released from BC cells (Melo et al. 2014). Exosomes were shown to process pre-miRNA utilizing Dicer, AGO2 and the RISC complex in a cell-independent manner (Melo et al. 2014). Additionally, miRNA sorting relies on the level of endogenous miRNAs. If the target mRNA transcript is elevated within the donor cell, then the miRNA which recognizes that target mRNA will less frequently be sorted into exosomes (Squadrito et al. 2014). Therefore, the converse is true, meaning if target transcripts are low and miRNAs are not actively binding to their targets, they are shuttled into ILVs and released from the cell encapsulated in exosomes. miRNAs are more readily found in exosomes compared to microvesicles. For instance, in

adult CSF, miRNAs found in exosomes and microvesicles varied greatly with exosomes containing the majority of circulating miRNAs (Akers et al. 2015). Given that exosomes are generated within the cytoplasm of the cell and thus have greater exposure to endogenous miRNAs than microvesicles, it is not surprising that exosomes, and not microvesicles, carry the majority of EV associated miRNAs. miRNAs have the capacity to regulate translation and can work in combination, thus researchers have focused their studies on EVs role in development and disease. During development, miRNAs, such as miR-124 and miR-9, have been identified within EVs in the central nervous system and from mesenchymal stem cells, respectively (Cao, Pfaff, and Gage 2007; Visvanathan et al. 2007; Feliciano et al. 2014). Since embryonic neuronal differentiation requires the regulatory actions of both these miRNAs and since fibroblast can be re-programmed into neurons following co-expression of both miR-9 and miR-124, it is possible that combinations of EV encapsulated miRNAs can regulate cell fate (Yoo et al. 2009, 2011). Through the utilization of EVs, distally located cells could coordinate development without direct cell-to-cell transfer of molecular signals. EV miRNAs have also been shown to contribute to the onset and progression of disease. For example, in patients with metastatic breast cancer, exosomes containing miR-181c were found to fuse with the cells constructing the blood brain barrier (BBB), and through miRNA dysregulation, resulted in the break-down and subsequent metastasis of breast cancer cells in the brain (Tominaga et al. 2015). Additionally, EVs released from cancer cells *in vivo* have the potential to be used as a diagnostic

and biomarker tool, and bioengineered EVs can be generated as a more potent and specific therapeutic (EL Andaloussi et al. 2013). In addition to miRNAs, EVs contain other non-coding RNAs, mRNA and even DNA, although generally DNA is not found in EVs (Maas, Breakefield, and Weaver 2017).

EVs also transport a wide variety of proteins. Specifically, research has identified a group of proteins referred to as EV marker proteins that are highly abundant in exosomes, microvesicles or both. Tetraspanins, such as CD9, CD63 and CD81, are composed of four transmembrane domains (Andreu and Yáñez-Mó 2014). These proteins cluster together to form microdomains at the cell surface and thus are found enriched in both exosomes and microvesicles (Théry et al. 2006; Raposo and Stoorvogel 2013; Andreu and Yáñez-Mó 2014). Additionally, EVs are associated with proteins specific to lipid rafts including flotillin and glycosylphosphatidylinositol-anchored proteins (Müller et al. 2009; López-Cobo, Campos-Silva, and Valés-Gómez 2016). Exosomes are also enriched in sphingomyelin and cholesterol (Kosaka et al. 2013; Bianco et al. 2009). Other than protein identifiers, EVs carry protein cargo specific to their cell of origin. For instance, neural stem/progenitor cells (NSCs) from the subventricular zone (SVZ) of adult mice release metabolically active EVs containing asparaginase (Iraci et al. 2017). Another studied identified the release of interferon gamma (IFN-gamma) on the surface of NSC EVs activated Stat1 signaling in target cells (Cossetti et al. 2014). EVs also contribute to the pathogenesis of certain diseases, including but not limited to Alzheimer's disease and cancer (Rajendran et al. 2006). However,

how protein cargo is sorted into EVs has yet to be fully defined. Post-translational modifications of EV protein cargo may act as specific molecular signatures, such as ubiquitinoylation or SUMOylation, which might allow for protein sorting into EVs (Villarroya-Beltri et al. 2013).

Extracellular Vesicles and Disease

EVs have a role in the pathogenesis of neurodegenerative diseases and cancer. Most cells release EVs at a pre-determined rate; however, cells under stress, such as hypoxia, DNA damage, cellular senesce or exposure to exogenous pathogens, increase their rate of release. EVs potentially play a pathogenic role in some neurodegenerative diseases. For instance, Alzheimer's disease arises from the aggregation of amyloid-β plaques in neurons and the secretion of abnormally processed Tau protein. Initially, exosomes isolated from HeLa cells expressing a mutant Amyloid Precursor Protein (APP) were found to be enriched in amyloid-β proteins (Rajendran et al. 2006). From the CSF of Alzheimer's patients, isolated EVs were found to be enriched for tau protein (Saman et al. 2012). In models of Parkinson's disease, α-synuclein was discovered in exosomes (Emmanouilidou et al. 2010). Exosome conditioned media was sufficient to induce neuronal death, but immunodepletion of α-synuclein reversed this finding (Emmanouilidou et al. 2010). Since α-synuclein was found in exosomes, conditioned media depleted of exosomes would be required in addition to and independent of immunodepletion to prove indeed exosomes were the causal factor in the neuronal cell death. EVs

have also been implicated in the pathogenesis of prion-associated neurodegeneration (Fevrier et al. 2004; L. Vella et al. 2007).

Cancer cells also release EVs that promote angiogenesis, regulate immune cells and prime tissue for metastasis (Becker et al. 2016; Cantaluppi et al. 2014). A hallmark characteristic of tumors is their ability to generate their own blood supply (Webber, Yeung, and Clayton 2015; Plaks, Kong, and Werb 2015). Earlier studies identified the release of pro-angiogenic factors from cancer cells, but recent data demonstrates that EVs also contribute to the angiogenic potential of tumors (Todorova et al. 2017). EVs containing tetraspanin-8 (tspn-8) released from rat adenocarcinoma cells induced angiogenesis by up-regulating VEGF and VEGF-R2 in endothelial cells (Nazarenko et al. 2010). Another study identified that EVs released from colorectal cancer cells promoted endothelial cell proliferation and migration through ERK1/2 and JNK signaling pathways via Egr-1 activation (Yoon et al. 2014). Pro-angiogenic miRNAs have also been identified in EVs (Feliciano et al. 2014; Skog et al. 2008). Immunomodulatory alterations allow for cancer cells to persist without infraction *in vivo*. Because of their ability to transfer bioactive molecules between cells, EVs released from cancer cells are thought to have immunoregulatory functions (Robbins and Morelli 2014). EVs transport immunosuppressive ligands and ncRNA, such as programmed cell death 1 ligand (PD-L1) and hY4 ncRNA, from cancer to immune cells thus suppressing monocyte function and supporting tumor growth (G. Chen et al. 2018; Haderk et al. 2017; Ricklefs et al. 2018). In addition to angiogenic and immunomodulatory roles,

cancer-associated EVs self-promote tumor growth and prime the pre-metastatic niche to promote metastasis (Tominaga et al. 2015; Y. Zhang and Wang 2015; Liu et al. 2016; Peinado et al. 2012; Angélique Bobrie et al. 2012). For example, miR-122 containing exosomes released from breast cancer cells suppressed glucose metabolism of non-tumor cells in the pre-metastatic niche (Fong et al. 2015). EVs released from cancer cells act as a homing mechanism in cancer metastasis in an organ specific manner (Hoshino et al. 2015). When transplanted into nude mice, exosomes released from tissue-specific cancer cells preferentially target and prime their tissue of origin for metastasis (Hoshino et al. 2015). Interestingly, pre-treatment with exosomes isolated from lung-tropic cancer cells changed the metastatic potential of bone-trophic tumor cells, thus exosomes have the capacity to influence the metastatic pattern of cancer cells (Hoshino et al. 2015). Cancer exosomes also promote tumor growth. Exosomes released from glioblastoma cells were capable of promoting proliferation of tumor cells *in vitro* (Skog et al. 2008). Additionally, these exosomes contained pro-angiogenic proteins capable of eliciting tubule formation of endothelial cells within the cancer niche (Skog et al. 2008). Taken together, these studies indicate a fundamental role for EVs in the pathogenesis of disease.

Biomarkers and Designer Vesicles:

Almost all cell types release EVs which carry a unique molecular profile that can be traced back to its cell of origin. Interestingly, one study identified the enrichment of low-abundance miRNA in exosomes, meaning that miRNA sorting

into exosomes is not random, but rather a calculated effort by the cell (Koppers-Lalic et al. 2014). Profiling EVs released from various types of cancers will potentially allow for the use of EVs as a tool for diagnosis. In the case of cancer, most diagnoses rely primarily on tissue biopsies. Whereas EVs offer a sensitive, non-invasive option which may increase cancer detection at an earlier stage. One study isolated exosomes from the urine of prostate cancer patients and were able to identify tumor-specific mRNA biomarkers in the exosomes (Nilsson et al. 2009). Other studies have utilized exosomes as potential biomarkers of both breast and ovarian cancer through the identification of cancer specific miRNA and protein, respectively (Corcoran et al. 2011; Li et al. 2009).

Since the discovery of pro-neurodegenerative miRNAs and proteins in EVs isolated from the CSF of patients, researchers have identified the potential of EVs as biomarkers of neurogenic disease. In theory, to diagnose a disease such as Alzheimer's disease, EVs would be collected from patient CSF and analyzed for specific pathogenic markers, such as amyloid-β protein (Rajendran et al. 2006; Saman et al. 2012; Tapiola et al. 2009). This would allow doctors to more accurately diagnose patients at an earlier stage and thus offering any early treatment that may be vital to slowing the onset of the disease. However, many obstacles must be overcome before EVs can indeed be used as biomarkers of disease. To properly profile EVs, regardless of cell of origin, a pure sample of EVs must be isolated from the biological sample (Thind and Wilson 2016). Different methods have been described, but currently there is no universal standard for EV

isolation. Additionally, EVs released by cancer cells will inevitably carry cargo similar to non-cancerous cells thus making cancer EV detection seemingly complex (Thind and Wilson 2016). However, utilizing specific combinations of cargo as a unique molecular barcode, cancer EVs likely can be separated from other non-cancerous EVs. This approach has limitations. While some cancers might have similar molecular barcodes between patients, in some cases it is not unreasonable to speculate that EV cargo might be patient specific. Nevertheless, the more data acquired of cancer EVs and their cargo, the more likely these EVs will be of broader use as biomarkers of disease.

EVs have also been investigated as a therapeutic. EVs, with the exception of exomeres, are derived from the cell membrane and therefore have a lipid bilayer structure. Due to this structure, contents encapsulated within EVs are protected from RNases and proteases (Weber et al. 2010; Babu et al. 2011). In fact, EV miRNAs have remain stable at -20ºC for five years (Koga et al. 2011). EVs can be engineered to carry miRNA and mRNA and elicit transcriptional and proteomic regulation in target cells (EL Andaloussi et al. 2013; Bolukbasi et al. 2012; Wahlgren et al. 2012; Alvarez-Erviti et al. 2011; Maguire et al. 2012). Additionally, EVs can be targeted to specific cells via ligand-receptor modification or by direct transplantation into target tissue (Ohno et al. 2013). Finally, in terms of pharmaceutical therapy, EVs offer a more specific and direct method of drug delivery potentially resulting in better over-all effectiveness of the drug and reduction of cost (EL Andaloussi et al. 2013). Many studies have already

investigated the use of EVs as nano-therapy units. For example, *RAD51,* a eukaryotic DNA repair protein, was sufficiently knocked down *in vitro* through the delivery of an EV-loaded siRNA, and another *in vivo* study demonstrated EV targeting of neuronal cells after intravenous transplantation (Shtam et al. 2013; Ohno et al. 2013). While further research, including clinical trials, is needed, EVs prove to be a promising tool for medical technology.

Brief Overview of Subventricular Zone (SVZ) Neural Development & Neurovesicles in Brain Development (Morton and Feliciano 2016)**:**

The subventricular zone (SVZ) is one of two neurogenic niches in the adult mammalian brain (Lim and Alvarez-Buylla 2016). During early embryonic gestation (embryonic day 8.5-10, E8.5-10), neuroepithelial stem cells (NESCs) line the wall of the ventricles, unlike that of the adult SVZ in which ependymal cells make up the ventricular wall (Taverna, Götz, and Huttner 2014). NESCs project a primary cilium and microvilli into the CSF filled ventricle (Dubreuil et al. 2007; Marzesco et al. 2005). The primary cilium has the ability to sense mitogenic and morphogenic cues, such as Sonic Hedgehog (SHH), from the CSF through receptors on the cilium itself (Corbit et al. 2005; Guemez-Gamboa, Coufal, and Gleeson 2014). NESC's primary cilium contains prominin-1 (CD133), a stem cell marker protein (Dubreuil et al. 2007; Marzesco et al. 2005). Vesicles containing CD133 are released from NESC microvilli, cilium and the midbody during NESC division (**Figure 2A**) (Marzesco et al. 2005; Dubreuil et al. 2007). Interestingly, the number of CD133 decreased after E12 in both mouse and chick models of development

(Marzesco et al. 2005; Dubreuil et al. 2007). It is unclear as to the purpose of the release of CD133 positive particles from NESCs, but it could potentially be a method to remove cues from the cells themselves.

At approximately embryonic day 11 (E11) in mice, NESCs give rise to choroid plexus epithelial cells (CPEs) and Radial glia (RG; **Figure 2B**) (Lun, Monuki, and Lehtinen 2015; Taverna, Götz, and Huttner 2014). CPEs are located in the four ventricles in the brain and generate CSF. The ventricular system, comprised of interstitial fluid and CSF, is the nutrient delivery and waste disposal system that bathes the brain throughout life (Lun, Monuki, and Lehtinen 2015; Lehtinen and Walsh 2011; Damkier, Brown, and Praetorius 2013; Nedergaard 2013). EVs have been identified in the CSF (Grapp et al. 2013; Akers et al. 2015; Bachy, Kozyraki, and Wassef 2008; Chiasserini et al. 2014; Marzesco et al. 2005; Dubreuil et al. 2007; Feliciano et al. 2014; Fraser et al. 2013; Akers et al. 2013; Harrington et al. 2009; Huttner et al. 2008; Saman et al. 2012; Street et al. 2012; Tietje et al. 2014; L. J. Vella et al. 2008; Yuyama et al. 2015). Since CPEs generate CSF and CSF contains EVs, it is likely that a portion of CSF-EVs originate from CPE (**Figure 2B**). Primary cultures of CPEs produce EVs that contain EV marker proteins including CD63, hnRNPA2/B1 and folate receptor (Grapp et al. 2013; Tietje et al. 2014). Other studies have identified morphogens and growth factors released by CPEs that regulate neurogenesis. One study in particular demonstrated that Insulin-like growth factor 2 (IGF2) is released by the choroid plexus and induces NSC proliferation(Lehtinen et al. 2011). *In vitro* studies of CSF-

EVs show that CSF-EVs can modulate NSC behavior thus CPE EVs may regulate brain development (Feliciano et al. 2014). CSF EVs also undergo an age-dependent decline, suggesting a more neurogenic role of CSF EVs than a modulator of homeostasis (Tietje et al. 2014). Indeed, loss-of-function mutations in *CHMP1A* was shown to cause microcephaly in patients through the reduction of exosome biogenesis and exosome mediated sonic hedgehog (SHH) release during neurogenesis (Coulter et al. 2018).

RG reside at the latero-ventricular interface of the developing cortex (Taverna, Götz, and Huttner 2014). RG have a distinct morphology with an apical process that projects towards the lateral ventricle and through a primary cilium interact with the CSF, and a basal projecting process that anchors at the pial surface (Higginbotham et al. 2013; Noctor et al. 2001). Beginning at embryonic day 12 (E12) to E18-19 RGs first symmetrically divide to generate a progenitor pool, and then asymmetrically divide to give rise to neuroblasts (NBs). NBs initially exhibit a multipolar morphology, but transitions into a bipolar phenotype (Kriegstein and Noctor 2004). Using the basal projecting process as a scaffold, NBs migrate out radially to generate the excitatory glutamatergic pyramidal neurons of the laminar structured mammalian cortex in an inside-out fashion (Kriegstein and Noctor 2004; Taverna, Götz, and Huttner 2014). Interestingly, during spinal cord neurogenesis in the chick embryo, when NBs delaminate from the ventricular surface, they abscise a portion of their apical luminal projecting primary cilium (**Figure 2D**) (Das and Storey 2014). This particle is then left behind in the ventricle,

thus begging the question as to whether or not 1) this abscission product functions in intercellular signaling and 2) could apical abscission also occur for NBs produced by radial glia in the developing cortex.

Neurogenesis persists in the postnatal SVZ. NSCs in the SVZ arise from RGs (**Figure 2G**; Merkle et al. 2004). During early perinatal development, one population of SVZ NSCs undergo self-renewal symmetric divisions (Obernier et al. 2018). Whereas a separate, larger population of SVZ NSCs give rise to Type C cells that divide three or four times to generate NBs which migrate along the rostral migratory stream to the olfactory bulb (OB) where they differentiate into periglomerular or granule interneurons (Bjornsson et al. 2015; Luskin 1993; Lois, Garc a-Verdugo, and Alvarez-Buylla 1996; Petreanu and Alvarez-Buylla 2002; Imayoshi et al. 2008). SVZ NSCs continue to generate NBs destined for the OB until approximately postnatal day 15 (P15) when the SVZ begins to resemble that of an adult SVZ (Tramontin et al. 2003). SVZ NSCs also produce astrocytes, oligodendrocytes and ependyma cells (**Figure 2F**). Astrocytes and oligodendrocytes are considered the support cells of the brain, and ependyma cells line the ventricular interface and generate a cellular barrier between the CSF and the SVZ (Bjornsson et al. 2015; Mirzadeh et al. 2008). Both astrocytes and oligodendrocytes generate EVs for which the developmental impact has only begun to emerge (**Figure 2F**) (Bianco et al. 2009; Gosselin, Meylan, and Decosterd 2013; Wang et al. 2011; Proia et al. 2008; Frühbeis et al. 2013). SVZ NSCs have a bipolar morphology, similar to RG. SVZ NSCs project an apical

process through a pinwheel structure of ependyma cells to contact the ventricle (Mirzadeh et al. 2008). This apical process also contains a primary cilium that interact with the CSF (Mirzadeh et al. 2008). Additionally, SVZ NSCs project a basal process onto a nearby blood vessel where they interact with pericytes on endothelial cells to regulate blood flow (Lacar et al. 2012). Unlike RG, SVZ NSCs have been shown to release EVs *in vitro* (**Figure 2G**). For instance, SVZ NSC EVs were shown to be taken up by NIH3T3 cells, and through EV-associated interferon gamma (IFN-γ) and interferon gamma receptor-1 (Ifngr1) complexes, activated Stat1 signaling in target cells (Cossetti et al. 2014). Another study demonstrated that EVs released from SVZ NSCs *in vitro* were selectively targeted to taken by microglia *in vivo* resulting in a transcriptional and cytokine profile alteration in the resident microglia (Morton et al. 2018). When transplanted into the lateral ventricles of P0 mice, microglia conditioned media, after treatment and depletion of EVs, resulted in reduction of SVZ NSC proliferation (Morton et al. 2018). While it is clear that EVs are prevalent during SVZ neurogenesis, it is still unclear how EVs contribute to the establishment of complex circuitry and structure in brain development.

Conclusions:

From development to disease, EVs play a substantial role. Understanding EV biology will allow for advances in not only our understanding of development, but also medical biomarkers and therapeutics. To understand the complexity of EVs, future studies will be required to parse apart EV sources, targets and

functions. EV studies continue to push the limit of scientific technology. As researchers investigate these nanometer sized particles, better and more precise technology must be established to more accurately study EVs. From garbage disposals to molecular messengers, EVs have altered the current understandings of intercellular communication.

Dissertation Goals and Objectives

The goal of this dissertation is to determine if EVs are released from early postnatal SVZ NSCs and their physiological function. Previous studies have found an abundance of EVs within embryonic and adult CSF, but the sources, targets and functions are unclear. SVZ NSCs line the latero-ventricular wall during neonatal neurogenesis and interact with CSF. Therefore, SVZ NSCs were a potential source of CSF EVs. I hypothesized that SVZ NSCs release EVs carrying specific molecular cargo and are taken up and regulate target cells. This dissertation provided three objectives:

Objective 1: Generate an experimental paradigm to study the effects of primary SVZ NSC EVs *in vivo*. We constructd a protocol specific to the culturing of primary SVZ NSCS, isolation of EVs and subsequent transplantation of labeled EVs *in vivo*.

Objective 2: Analyze and assess the uptake of SVZ NSC EVs in target cells. Using the protocol established in the first objective, we assessed the uptake of

SVZ NSC EVs *in vivo* in target cells largely through next-generation RNA sequencing, western blotting, immunohistochemistry, Luminex assays and transplantation experiments.

Objective 3: Generate an *in vivo* model system to label and study EVs. Since EV experimentation is limited by available scientific tools, we generated a transgenic mouse model system to fluorescently label and track tetraspanin CD9-containing vesicles in a temporally and spatially regulated manner. This model system was used to validate the generation of SVZ NSC EVs *in vivo*.

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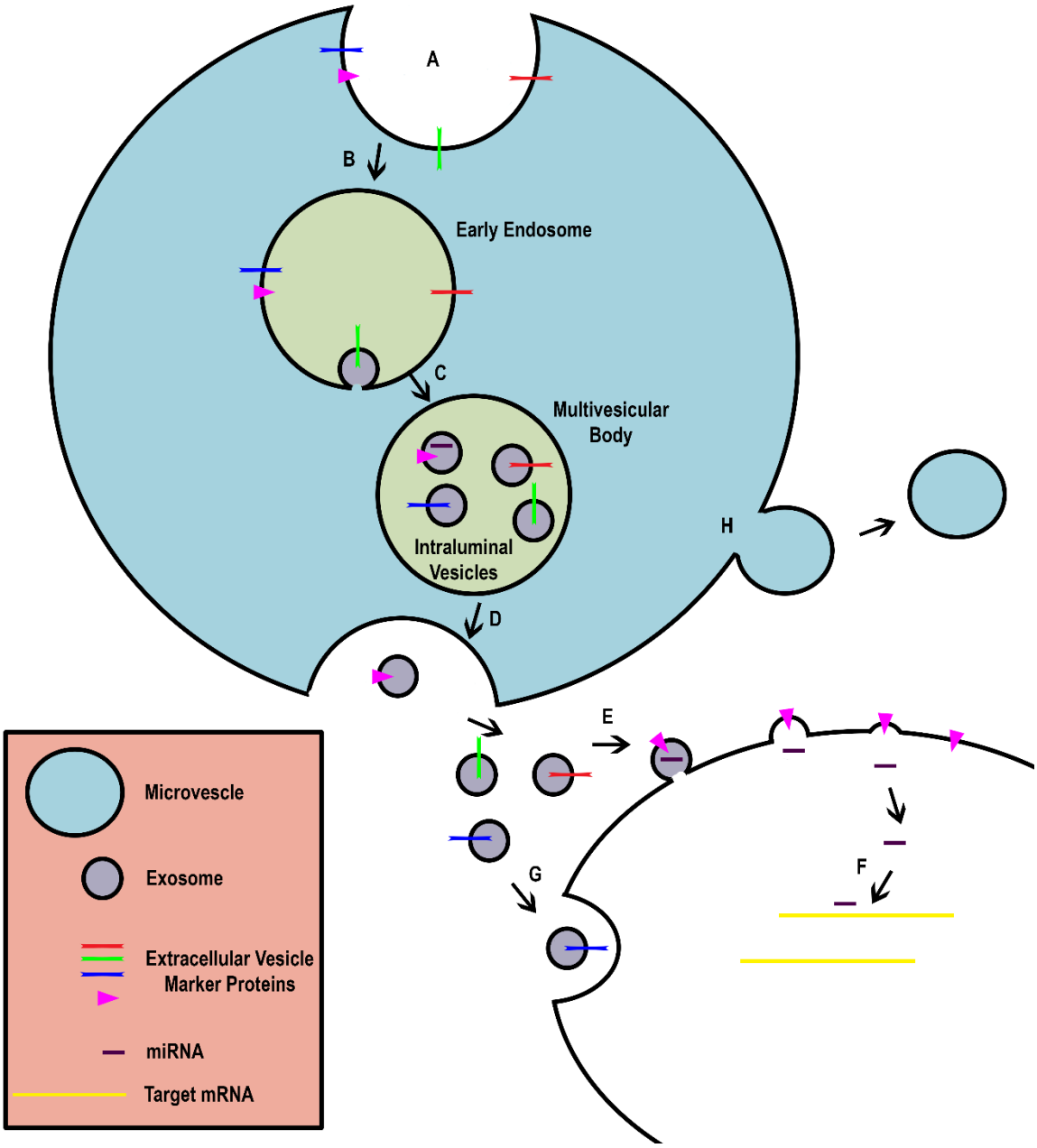
A
B
Early Endosome
C
Multivesicular Body
Intraluminal Vesicles
D
H
E
G
F
Microvescle
Exosome
Extracellular Vesicle Marker Proteins
miRNA
Target mRNA

Figure 1.

Schematic of Extracellular Vesicle Release and Exosome Uptake. A-B) Cell membrane invaginates to generate an early endosome. **C)** The early endosome undergoes further inward budding to generate a multivesicular body (MVB) encapsulating intraluminal vesicles. EV marker proteins (red, green, blue and pink) and miRNA (purple) are found integrated into the lipid bilayer and inside of the vesicles, respectively. **D)** MVB then fuses with the cell membrane and exosome are released into the extracellular space. Exosomes are taken up by target cells in multiple ways. **E)** Exosomes can fuse with the cell membrane releasing its molecular contents into the target cell. For instance, exosomal miRNA can be released into the recipient cell and target specific mRNA **(F)**. Conversely, exosomes can be taken up through endocytic pathways **(G).** Finally, microvesicles are generated through the outward budding the cell membrane **(H)**.

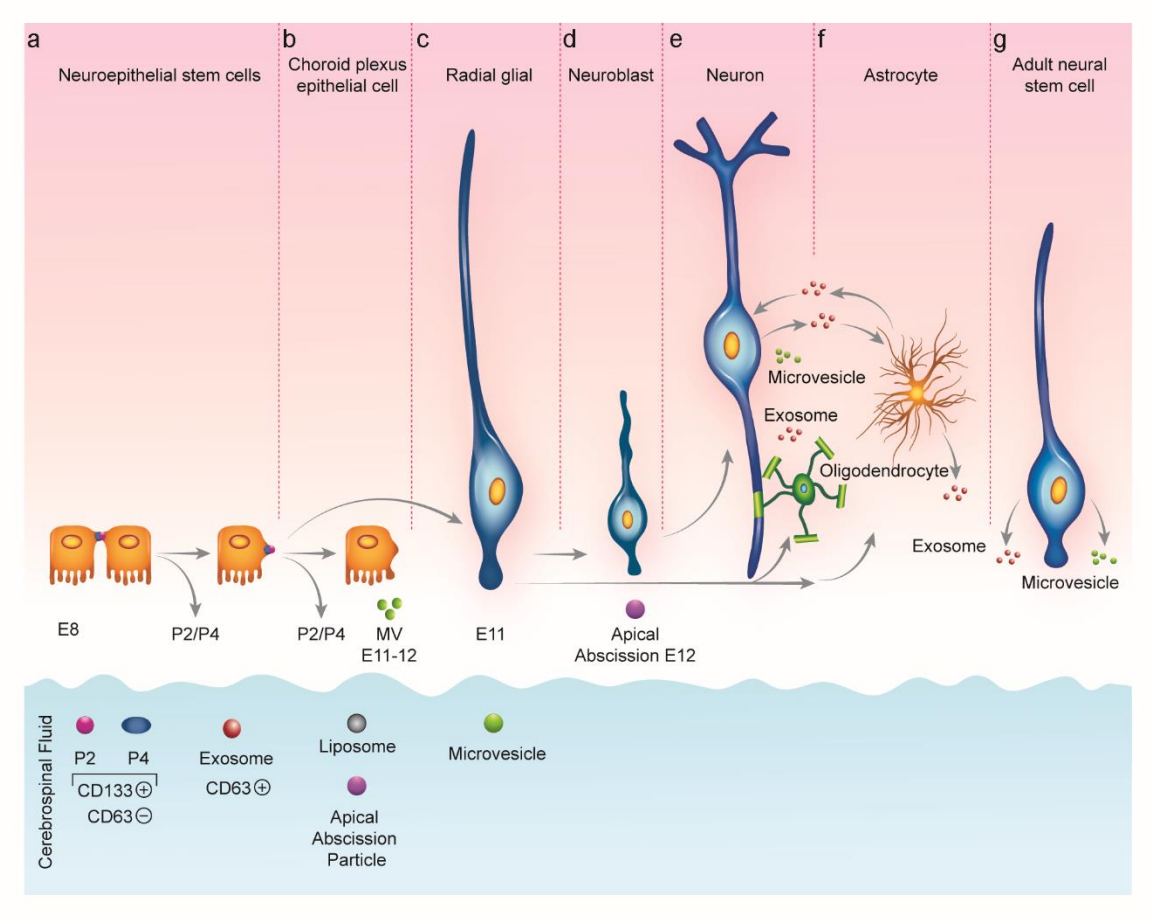

Figure 2.

Schematic of Extracellular Vesicles and Cortical Development. A) During formation of the neural tube at embryonic day eight (E8), invagination of the epithelium results in neuroepithelial stem cells (NESCs) that orient their apical surface toward the lumen of the neural tube. NESCs contain CD133 on the ventricular projecting primary cilia, mid-body, and microvilli. NESCs release CD133 positive large ~600 nm, (P2 fraction; *pink*) and 50–80 nm (P4 fraction; *blue*) vesicles into the lumen of the neural tube. **B)** NESCs produce choroid plexus epithelial cells (CPEs) beginning at E11−12. Cultured CPEs

release extracellular vesicles including microvesicles (MV, green). **C** At E11−12, radial glia are generated from NESCs, and span the developing cortex, beginning at the ventricular surface and extending a radial process to the pial surface. It is unknown whether EVs are released from radial glia. **D)** Radial glia undergo asymmetric division and give rise to immature glutamatergic neurons, called neuroblasts, that migrate into and populate the cortical plate. Through a similar process, neuroblasts that are produced in the developing chick embryo delaminate from the ventricular surface and release a particle into the ventricle during apical abscission (Apical Abscission Particle; *purple*). **E)** Once neuroblasts mature into neurons, excitatory stimuli such as glutamate and depolarization can induce the release of microvesicles (*green*) and exosomes (*red*) that may target astrocytes while others target neurons. Oligodendrocytes that myelinate the axons of neurons also release EVs in response to glutamate. **F)** NESCs eventually give rise to astrocytes which release EVs that target neurons. As with oligodendrocyte EVs, those from astrocytes seem to be neurotrophic. **G)** NSCs that persist in the subventricular zone (SVZ) are contiguous with the lateral ventricles. SVZ NSCs generate both exosomes (*red*) and microvesicles (*green*; Morton and Feliciano 2016).

CHAPTER TWO

Isolation of Extracellular Vesicles from Subventricular Zone Neural Stem Cells.

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ABSTRACT

The neonatal subventricular zone (SVZ) is a neurogenic niche that contains neural stem cells (NSCs). Extracellular vesicles (EVs) are released from many cell types. EVs encapsulate a wide array of biological material including nucleic acids. EVs are proposed to be targeted to recipient cells. Recent studies have demonstrated that SVZ NSCs release EVs. A classic developmental approach to uncovering bio-activity of molecules is to perform *in vivo* transplantations. Here we demonstrate how to culture neonatal SVZ NSCs and to isolate and subsequently transplant EVs into the neonatal brain. Using this approach, we demonstrate that NSC EVs label microglia.

INTRODUCTION

Extracellular vesicles (EVs) are membranous derived particles that range in size from 50 – 500nm, and are categorized by their mechanism of biogenesis (Cocucci and Meldolesi 2015). First, exosomes are derived from multivesicular bodies (MVBs) which fuse with the cellular membrane to release the vesicles from the cell (Théry, Zitvogel, and Amigorena 2002). Conversely, microvesicles are produced through the outward budding of the cell membrane (Théry, Zitvogel, and Amigorena 2002). Finally, a new species of non-membranous EVs has recently been identified called exomeres (Zhang et al. 2018). EVs are thought to modulate intercellular communication through the transfer of biological material, such as mRNA, miRNA, and protein from donor to recipient cells (Ramachandran and Palanisamy 2012; Valadi et al. 2007). For example, EVs have been shown to activate viral-sensing receptors, such as Toll-like receptors (TLRs) and RIG-1 in recipient cells (Eckard et al. 2014). Additionally, other reports have discovered the transfer of biologically active proteins, such as asparaginase (Iraci et al. 2017).

Various methodologies have been used for isolating EVs. Polymer-based isolation utilizes polyethylene glycol to isolate a high yield of EVs (Momen-Heravi et al. 2015), but is limited in purity of EVs due to lipoprotein contaminants (Vickers et al. 2011; Lobb et al. 2015). Additionally, other studies have employed ultracentrifugation to isolate a “dirty” EV pellet, but inconsistencies with reproducibility have been reported (Livshits et al. 2015; Bobrie et al. 2012). Some methods call for a clean-up step to further purify exosomes following

ultracentrifugation. EVs pellets are resuspended in high molarity (M) sucrose, and then sucrose of decreasing M are layered on top. It is recommended that the finished gradient containing EVs is centrifuged at a very high speed for at least 16 hrs to ensure proper EV sedimentation (Taylor and Shah 2015). Sucrose density gradient fractionation relies on the density of EVs for isolation. Exosomes are collected from fractions at a sucrose concentration of 1.1 – 1.9 g/mL, but other studies have discovered that, depending on the density, EVs overlap and are not separated completely (Taylor and Shah 2015; Théry et al. 2006). Sucrose density gradients have their own limitations including structural compromise, EV fusion, and vesicle rupture (Linares et al. 2015). Currently, there is no universal standard for EV isolation.

The subventricular zone (SVZ) is one of two neurogenic niches in the perinatal brain (Lim and Alvarez-Buylla 2016). Located contiguous with the lateral ventricles, one population of SVZ neural stem cells (NSCs) symmetrically divide to as mode of self-renewal (Obernier et al. 2018). Whereas a separate, larger population of NSCs give rise to Type C cells that divide three or four times to generate neuroblasts which migrate along the rostral migratory stream to the olfactory bulb where they differentiate into periglomerular or granule interneurons (Bjornsson et al. 2015; Luskin 1993; Lois, Garc a-Verdugo, and Alvarez-Buylla 1996; Petreanu and Alvarez-Buylla 2002; Imayoshi et al. 2008). Reduction in the number of SVZ NSCs occurs in correlation in both aging and in continued NSC proliferation (Bouab et al. 2011; Daynac et al. 2016). Additionally, the number of

SVZ NSCs decrease by ~60% between early postnatal life (0-7 days) and adults at 26 months (Maslov et al. 2004). Interestingly, the neurogenic zone in the SVZ decrease significantly between 0 and 15 days postnatally (P0 – P15), and at P15, the SVZ begins to resemble that of an adult SVZ (Tramontin et al. 2003). SVZ NSCs produce astrocytes and oligodendrocytes, the support cells of the nervous system (Bjornsson et al. 2015), and ependyma cells that act as a barrier between the SVZ and the fluid-filled lateral ventricles (Mirzadeh et al. 2008). The early perinatal SVZ (P0-P1) is comprised mainly of NSCs. Resident central nervous system (CNS) immune cells infiltrate the developing nervous system from the yolk sac at embryonic day 8.5-9.5 (Ginhoux et al. 2010). These cells, called microglia, congregate in proliferative zones in the perinatal brain, that later in postnatal development migrate out to and populate the cortex (Cunningham, Martínez-Cerdeño, and Noctor 2013; Swinnen et al. 2013). Microglia release factors that regulate NSC proliferation during embryonic development and into perinatal neurogenesis (Battista et al. 2006; Zhu et al. 2008; Antony et al. 2011; Snyder et al. 1997). Recent studies have identified SVZ-NSCs as sources of EVs that regulate neighboring cells, including microglia (Morton et al. 2018; Cossetti et al. 2014; Asai et al. 2015).

When culturing primary SVZ NSCs, various methodologies have been described in which their utility is dependent on the goal of the experiment. Primary SVZ NSCs have been cultured through neurosphere assays (Reynolds and Weiss 1996), adherent monolayer systems (Ray, Raymon, and Gage 1995; Palmer, Ray,

and Gage 1995), and matrixgels. Neurosphere assays allow for the examination of differences in cell proliferation and cell potential (Walker and Kempermann 2014). Neurospheres themselves are heterogenous clusters of NSCs, progenitor cells, and differentiated cells (Suslov et al. 2002; Parmar et al. 2003) that, together, generate a stem cell niche with more differentiated cells residing in the center (Azari et al. 2010; Bez et al. 2003). Unlike neurosphere culturing, adherent monolayer systems more closely recapitulate *in vivo* proliferation and have a more homogenous population of NSCs (Walker and Kempermann 2014; Babu et al. 2011). The homogeneity of adherent monolayer systems allow for a better interpretation of NSCs rather than NSC niches of neurospheres. Finally, matrixgels are used to construct a 3D structure that mimics the extracellular matrix in which NSCs reside *in vivo* (Aligholi et al. 2016). Different from non-adherent cell culture systems, such as neurospheres, cells cultured in matrixgels have a distinct advantage in that they are cultured in a degradable biomaterial which provides structural integrity (Thonhoff et al. 2008). Matrixgel culturing systems are an important avenue of culturing methodologies, specifically in terms of neuro-regeneration *in vivo* (Thonhoff et al. 2008; Moradi et al. 2012).

Here we demonstrate in detail the culturing of the neonatal SVZ, which is enriched in NSCs (Walker and Kempermann 2014) the isolation of NSCs EV with the production of a “dirty” fraction and a subsequent clean-up step, followed by the labeling and transplantation of EVs into the developing perinatal lateral ventricles.

MATERIALS

1. Primary Cell Culture and Microdissection

a. Mouse Laminin

b. Neurobasal A Complete Culture Media: Neurobasal A Medium, 2% B27, 1X Glutamax, 50 units/mL Penicillin/Streptomycin, 20 ng/mL purified mouse receptor-grade epidermal growth factor (EGF) and 20 ng/mL recombinant bovine fibroblast growth factor (FGF-2).

c. Disassociation Buffer: 0.05% Trypsin-EDTA, Neurobasal A Medium

d. Cascade Biologicals Trypsin Inhibitor

e. Sterile Microdissection Kit

f. Scalpel

2. Sucrose Density Gradient Exosome Isolations

a. Thickwall Polycarbonate Tube or Ultracentrifuge Tubes

b. Sucrose for Density Gradient: 2.5M Sucrose, 1X d-Phosphate Buffered Saline Solution (dPBS)

c. Beckman Coulter Optima MAX-XP centrifuge with TLA 100.3 rotor.

3. Exosome Labeling and Transplantation

a. Vibrant DiI Cell-Labeling Solution.

b. 10 cm fire polished borosilicate glass capillary tubes or Hamilton Neuros Syringe.

c. Table-top centrifuge.

PROTOCOL

1. Preparation of Culture Medium

1.1. 24 h prior to culturing SVZ NSCs, prepare Laminin-coated wells for adherent monolayer NSC cultures. To prepare culture wells, add 8.5 ug/mL in DiH_2O per well and incubate at 37°C overnight. Wash wells three times using DiH_2O and use wells immediately.

1.2. Prepare fresh culture media on the day of dissections by mixing Neurobasal A Medium with 2% B27, 1X Glutamax, 50 units/mL Penicillin/Streptomycin, 20 ng/mL purified mouse receptor-grade epidermal growth factor (EGF) and 20 ng/mL recombinant bovine fibroblast growth factor (FGF-2). Warm culture media to 37°C. (*see* **Note 1**)

1.3. Prepare glass Pasteur pipettes with varying hole sizes by rotating tip of glass pipette over an open flame until desired hole size is obtained. Ideally a “small,” “medium,” and “large” hole are best suited for culturing. (*see* **Note 2**)

1.4. Prepare 0.05% Trypsin-EDTA in Neurobasal A for the Disassociation Buffer, and 1X Trypsin Inhibitor (Cascade Biologicals). Pre-warm to 37°C.

1.5. Sterilize dissection tools (forceps x2, microdissection scissors, dissection scissors, dissecting microscope, scalpel). (*see* **Note 3**)

2. Harvesting perinatal brains and SVZ microdissections

2.1. Anesthetize P0 – P1 pup by placing it on ice for 5 minutes or by following the facility's proper guidelines.

2.2. Decapitate pup using scissors and carefully remove the skull by cutting between the two hemispheres and using forceps to peel back the skin and skull thus exposing the brain. (*see* **Note 4**)

2.3. Carefully remove the brain by using the curved or soft edges of the forceps and place it on ice-cold 1X Phosphate Buffered Saline (PBS) solution under the dissecting scope with the ventral side up.

2.4. Using the scalpel, remove any remaining olfactory bulbs. Then make a single coronal section 1/3 of the distance from the most rostral portion of the cortex, severing the cortex into two pieces. Discard the most caudal piece.

2.5. To microdissect the SVZ place the rostral portion of the cortex with the cut side facing up. Carefully tease apart the SVZ from the cortex at the lateral ventricle walls and place dissected SVZ into Neurobasal A incubated on ice. For further reference, please see Walker and Kemperman, 2014 [13].

3. SVZ Tissue Dissociation

3.1. Place dissected SVZ into 750 µL Disassociation Buffer (0.05% Trypsin-EDTA in Neurobasal A), gently invert the sample two or three times and incubate at 37°C for 7 min.

3.2. Add 1:1 ratio of 1 X Cascade Biologicals Trypsin Inhibitor to Disassociation Buffer and centrifuge for 5 min at 300 x g in Eppendorf Centrifuge 5415 D.

3.3. Aspirate supernatant and re-suspend cell pellet in 1000 µL pre-warmed Neurobasal A Complete Media

3.4. Using fire polished glass pipettes, triturate cells. Starting with the pipette with the “large” hole and moving to the pipette with the “small” hole. (*see* **Note 5**)

3.5. Spin down cells for 5 min at 300 x g, and re-suspend in 200 µL and plate cells at 1 x 10^6 cells/mL.

3.6. Place cells in 37 ℃ incubator with 5% CO_2. Do not disturb cells for at least 24 hours. After 24 h, replace half of the media. (*see* **Note 6**)

4. Exosome Isolation from SVZ NSC Culture Media using Sucrose Density Gradients

4.1. Prepare 2.5 M sucrose in 1 X dPBS immediately prior to exosome isolation. Dilute 2.5 M sucrose to 2.0 M, 1.5 M, 1.0 M, 0.5 M and 0.25 M. (*see* **Note 7**)

4.2. 48 hr after SVZ NSC culture initiation, collect culture media and begin exosome isolations. Centrifuge media for 10 min at 300 x g and then 10 min at 2000 x g in an IEC Centra GP8 centrifuge. (*see* **Note 8**)

4.3. Dispense media into ultracentrifuge tubes. Ultracentrifuge tubes should be equal in weight to prevent unequal weight distribution in the rotor. (*see* **Note 9**)

4.4. Centrifuge exosome containing media for 90 min at 100,000 x g at 4 °C in Beckman Coulter Optima MAX-XP centrifuge with TLA 100.3 rotor. This will result in a pellet hereon referred to as the P100 pellet. (*see* **Note 10**)

4.5. Resuspend the P100 fraction in 2.5 M sucrose and layer the other sucrose solution in descending order (2.0 M, 1.5 M, 1.0 M etc.). Take extra caution not to disturb any layer otherwise the gradient will not form properly. Weigh the completed sucrose gradients and ensure equal weight before moving on to the next centrifuge step. (*see* **Note 11**)

4.6. Place gradients in ultracentrifuge rotor and place the rotor in the ultracentrifuge. Spin gradient for 18 h at 4°C at 100,000 x g.

4.7. Discard top layer and collect ten (1-10), equal fractions and place each fraction in a separate ultracentrifuge tube. Dilute 1:10 in 1 X dPBS and spin each fraction for 1 h at 100,000 x g at 4°C. (*see* **Note 12**)

4.8. Discard supernatant and resuspend each fraction in 30 µL 1 X dPBS. Collect exosome fractions 5 – 8. Exosomes can be stored at -20°C or immediately labeled and used for transplantation. (*see* **Note 13**)

5. DiI Labeling of Exosomes and Transplantation

5.1. Centrifuge exosomes at 14,000 x g for 30 min or at 100,000 x g for 1 hr.

5.2. From a 1 mM stock solution of DiI labeling solution, resuspend pellets in 1 µM DiI labeling solution in 1 X dPBS and incubate fractions for 10 min at room temperature and vortex periodically during incubation.

5.3. Centrifuge fractions at 14,000 x g for 30 min at Room Temperature.

5.4. Resuspend fractions in 1 X PBS and repeat steps 5.2. – 5.3. 3x times. (*see* **Note 14**)

5.5. Resuspend final pellet of DiI labeled exosomes in 50 µL 1 X dPBS and either store exosomes at -20 °C or move immediately to transplantation.

6. Transplantation of exosomes into lateral ventricle of P0 pups.

6.1. Prior to transplantations, prepare pulled glass pipettes by placing 10 cm fire polished borosilicate glass capillary tubes (O.D.: 1.5mm, I.D.: 1.1mm) into a Sutter Instrument Company Model P-97 pipette puller. Place pipettes in a sterile container for transplantations. Alternatively, a Hamilton Neuros Syringe can also be used for injections.

6.2. Using an aspirator connected to the capillary tube or a Hamilton Neuros Syringe constructed with a Neuros Adapter, load 1-2 µL of DiI labeled exosomes.

6.3. Anesthetize P0 pup on ice for 5 min or until pup slows down. Hold pup in between thumb, index and middle finger (nose should be anchored using thumb). Pull skin back to identify injection site.

6.4. Identify midline of the brain. Place capillary needle or Hamilton Neuros Syringe needle near the rostral portion of the brain at the midline. Move the needle approximately 1 mm laterally in either direction and 0.5 mm caudally and insert needle into the lateral ventricle approximately 2 mm deep. Inject 1-2 µL DiI labeled exosomes into the lateral ventricles.

6.5. Place pups on heating pad briefly and put back with mother. Sacrifice pups 24 or 48 h after transplantation, collect and fix brain and begin immunohistochemistry.

Notes:

1. Neurobasal A Complete Media can be stored at 4°C for two weeks. It is recommended that fresh Complete Media be made for this protocol.
2. It is recommended that three glass 58asteur pipettes with varying hole sizes should be prepared each with decreasing bore size. Before using on SVZ tissue, be sure that liquid can pass through the

newly sized hole.

3. All tools should be sterilized prior to tissue dissection. Primary cultured cells are extremely susceptible to contamination. Any steps involving the dissected tissue should be completed under a cell culture hood using 70% Ethanol to sterilize when appropriate.
4. When removing the brain, be sure to not cut too deep between the hemispheres or use the sharp edges of the dissection forceps to remove the skull. Either could result in puncturing the brain and damaging the tissue.
5. Media should have a cloudy appearance indicating proper tissue dissociation. Do not triturate cells excessively. 7-10 times total is more than sufficient.
6. Save media. Do not discard.
7. For each exosome isolation procedure, be sure to prepare fresh 2.5M Sucrose solution in 1X dPBS.
8. Transfer media into a new tubes in between centrifugation steps. Be sure to not disturb the pellet.
9. When weighing the ultracentrifuge tubes, the tubes should be equal in weight to the 100^{th} decimal place to prevent systemic mechanical breakdown.
10. Prior to ultracentrifugation, ensure tubes plus the sample are equal in weight to the 100^{th} decimal place. Additionally, P100 fractions can

be used for postnatal transplantations. P100 fraction contains both exosomes and microvesicles.

11. In some cases a syringe is most useful to construct the sucrose gradient layers. When constructing the sucrose density gradient, do not add the next layer of sucrose directly onto the previous layer in a destructive manner. Instead, pipet each layer of sucrose down the side of the tube, gently, to ensure that the prior layer is not disrupted. Again, weigh each tube containing the sucrose gradients and equilibrate using 1X dPBS.
12. When collecting fractions, collect from the top down. Be careful not to insert the pipet tip too far into the gradient, thus disrupting lower layers.
13. Exosome pellets can appear as a gray or translucent pellet. Take note of tube orientation in the rotor before resuspending pellet.
14. To ensure no DiI labeling particles are left in the exosome pellet, repeat the washes in 1x dPBS as many times as needed.

DISCUSSION

Here we have demonstrated the transplantation of SVZ NSC EVs from primary cultures to *in vivo* perinatal lateral ventricles. Transplantation experiments have been widely used in the field of developmental biology, and continue to be implemented today; however, transplantation experiments have their limitations.

In this study, EVs from primary cultures of SVZ NSCs were isolated, labeled, and transplanted into the lateral ventricles of perinatal mice. One limitation to this technique is the use of primary SVZ NSCs culture as the source of EVs and not SVZ NSCs *in vivo*. To circumvent this caveat, an *in vivo* EV labeling system would need to be generated to capture and isolate EVs directly from *in vivo* sources. Additionally, cultured SVZ NSCs are highly susceptible to contamination. Contamination would result in alterations to the EV profile of primary SVZ NSCs, therefore special precaution should be taken to ensure sterilization of all tools, solutions, and tubes. Furthermore, this methodology of SVZ NSC isolation yielded a high percentage of cells that stained positive for the NSC marker Nestin, however without selectively isolating NSCs, via differential centrifugation or flow cytometry, it is likely that the primary cultures were a mixed population of SVZ cells. Although none of the cells stained positive for the neuroblast marker Doublecortin (DCX), NSCs in transition from a stem cell to an astrocyte, for example, are likely to stain positive for both the NSC marker Nestin and the Astrocyte marker GFAP (Tramontin et al. 2003; Bouab et al. 2011; Petreanu and Alvarez-Buylla 2002; Mirzadeh et al. 2008). Further purification of NSCs should be explored to ensure a pure population of NSC EVs.

While most studies report a high abundance of EV release from cells, the amount of miRNA and mRNA contained within these EVs remains under scrutiny (Morton and Feliciano 2016; Morton et al. 2018; Cossetti et al. 2014; Chevillet et al. 2014). One study reported that for every 100 EVs, only one contained miRNA

(Chevillet et al. 2014). If this is the case, *in vivo* EV function may only require a very low abundance of miRNA to be present, thus transplantation experiments may not recapitulate *in vivo* conditions. In order to address this issue, the rate of release from SVZ NSCs would need to be taken into consideration. If transplantation experiments elevate EV numbers by 10^8 per mL, then any biological response may only be superfluous (Morton et al. 2018). EVs might stimulate a cellular response in neighboring cells for which this occurrence is 1 in 100, so introducing an abundance of EVs might result in a cellular response above endogenous levels. Additionally, sucrose density gradients can result in vesicle disruption or fusion (Linares et al. 2015). The phenomenon would likely alter the profile of EVs collected and biological data (miRNA, RNA, and protein) might be lost or disrupted. To avoid this issue, P100 fractions of EVs can be used for transplantations, although other studies have identified molecular contaminates within this fraction (Livshits et al. 2015; Bobrie et al. 2012). Going forward, alternate methods of EV isolation should be taken into consideration, such as size exclusion, FACS or immunocapture.

The field of EVs has rapidly expanded in the past 10 years. Thus nomenclature, isolation techniques, and characterization of these vesicles are still undergoing constant reconstruction. EVs are thought to modulate intercellular communication through the transfer of miRNA, RNA, and protein, and furthermore, each EV contains its own unique molecular bar code. This begs the question, what information can be transferred between cells or organisms? Could EVs carry

molecular information pertaining to consciousness or memories? As the field continues to expand, more precise techniques will need to be employed to answer these questions.

FIGURES:

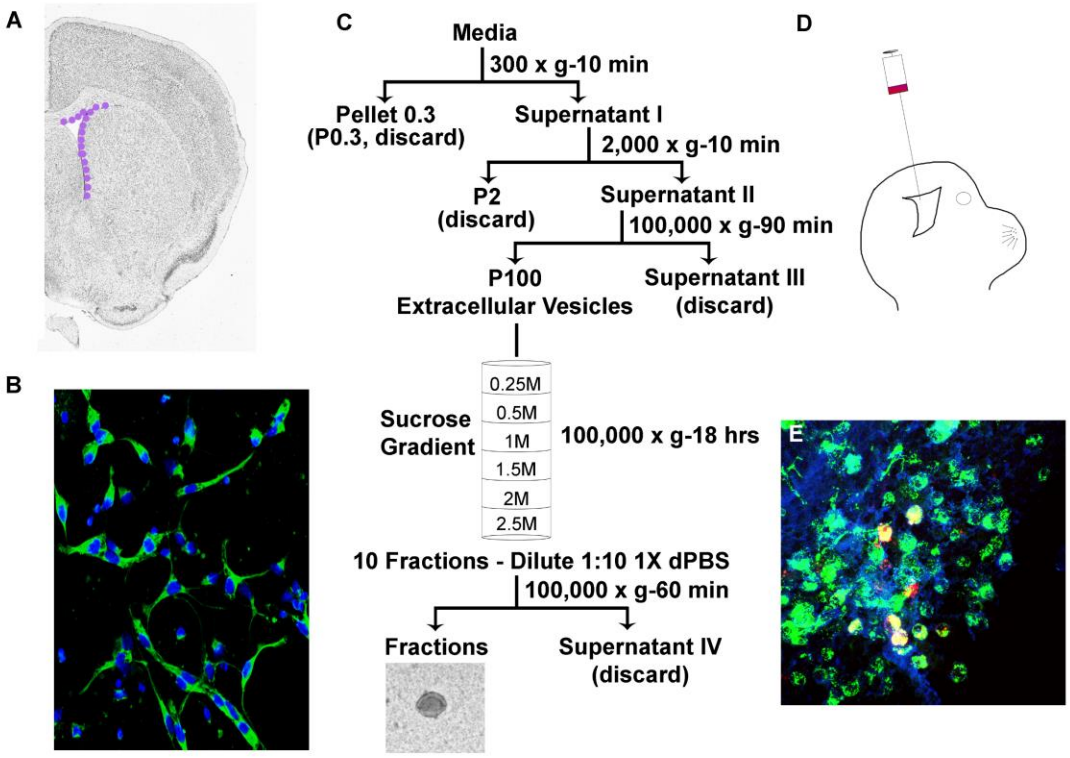

Figure 1. Isolation and Transplantation of EVs from SVZ NSCs. A) Schematic brain section highlighting regions dissected (purple). B) Following isolation, these cells were cultured as monolayers. Cells stained positive for the NSC marker Nestin (green). DNA was counterstained using TOPRO-3 (blue). C) The conditioned media was subjected to a serial centrifugation protocol that produces a “dirty fraction” that contains EVs (P100). These EVs were then further purified using a sucrose gradient. D) Isolated EVs were then labeled with DiI (red) and injected into the lateral ventricles of a P0 pup. E) Brains were harvested from P0-P7 mice and EVs (red) targeting cells were detected.

Labeled cells were Iba1 positive (blue) and CD11b positive (green).

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DISCLOSURES

The authors have nothing to disclose.

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CHAPTER THREE

Neonatal Subventricular Zone Neural Stem Cells Release Extracellular Vesicles that Act as a Microglia Morphogen

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ABSTRACT

Subventricular Zone (SVZ) Neural Stem Cells (NCSs) persist in the perinatal neurogenic niche and give rise to neurons early and late into adulthood. Microglia are the immune cells of the central nervous system that help form the intricate neural circuitry of the mammalian brain. Extracellular vesicles (EVs) are cellular-derived, nanometer-sized vesicles that encapsulate miRNA and proteins. It is thought that EVs transfer molecular information from donor to recipient cells which may play a role in normal development or could potentially contribute to the pathogenesis of disease. In this study, we tested the communicative potential of SVZ NSC EVs and microglia in the perinatal brain. Using a fluorescent fusion EV protein, CD9-GFP, to study EV release, it was found that SVZ NSCs generated EVs. The fusion protein was expressed in Nestin-positive NSCs in the SVZ and could be detected outside of labeled cells. Scavenging microglia selectively took up tagged NSC EVs. Small RNA sequencing identified miRNAs within NSC EVs that regulate microglia physiology and morphology. NSC EVs induced a transition of microglia to a non-stellate, rounded morphology. This morphological shift was accompanied by an altered microglial transcriptional state and cytokine profile which contributed to a negative feedback loop that controlled NSC proliferation. These data suggest that SVZ NSCs generate EVs that are targeted to and alter microglia within the perinatal brain. These finding offer insight into normal and pathophysiological functions of EVs during brain development.

INTRODUCTION

Neural stem cells (NSCs) reside in discrete regions, called neurogenic zones, of the adult mammalian brain. In rodents, these NSCs produce neurons throughout life that populate the olfactory bulb and are imperative for olfactory sensation (Lledo and Valley 2016). The subventricular zone (SVZ) is one of two neurogenic zones in the adult mammalian brain (Lim and Alvarez-Buylla 2016). Intercellular communication is required for producing the proper number and types of cells in the SVZ (Choe, Pleasure, and Mira 2016). Alterations in SVZ environmental conditions or genetic signaling in SVZ NSCs likely generate certain tumors and malformations (Dietrich, Imitola, and Kesari 2008; Vescovi, Galli, and Reynolds 2006; Zhou et al. 2011). Thus, understanding mechanisms that regulate their development and maintenance is of upmost importance.

Microglia are the primary immune cells of the nervous system. Interestingly, a population of microglia reside in the neonatal SVZ, and in adulthood, migrate out and become evenly distributed through the brain (Shigemoto-Mogami et al. 2014). Microglia are haematopoietically derived myeloid cells that originate from yolk sac macrophages (Ginhoux et al. 2010; Prinz, Erny, and Hagemeyer 2017). Using the developing vasculature, microglia migrate into the brain during mid-gestation and go on to produce a majority of the resident microglia during the first postnatal weeks (F Alliot et al. 1991; Francoise Alliot, Godin, and Pessac 1999). Microglia have been shown to influence neural progenitor cells (NPCs), and other studies have shown that NPCs likely influence microglia (Pluchino and Cossetti 2013; Sato

2015; Lehtinen and Walsh 2011; Su et al. 2014; Shigemoto-Mogami et al. 2014). One study in particular demonstrated the accumulation of activated microglia in the neonatal SVZ and the combinatorial effects of microglia released cytokines on NSC proliferation (Shigemoto-Mogami et al. 2014). This report alone provides evidence for a neuro-developmental role of microglia.

Extracellular vesicles (EVs) are found in most biological fluids, including blood, urine, and cerebrospinal fluid (CSF) (Morton and Feliciano 2016; Feliciano et al. 2014). EVs are primarily membranous vesicles that range in size from 30-500nm and have the capacity to carry an array of cargo, including miRNA, mRNA, and protein (Raposo and Stoorvogel 2013; Maas, Breakefield, and Weaver 2017). Three types of EVs have been documented: exomeres, exosomes, and microvesicles. Exomeres are non-membranous particles comprised of metabolic, translational, and coagulation regulating proteins (H. Zhang et al. 2018). Unlike other documented EVs, exomere biogenesis is not well understood (H. Zhang et al. 2018) Exosomes are produced when a multivesicular body fuses with the cell membrane (Théry, Zitvogel, and Amigorena 2002). Exosomes are distinct from other types of EVs in that they are less than 150nm in size and exhibit a cup-like morphology when viewed under electron microscopy (EM) (Théry, Zitvogel, and Amigorena 2002). Finally, microvesicles, the largest type of EV (200-500nm), are generated by the outward budding and scission of the cell membrane (Cocucci and Meldolesi 2015; Maas, Breakefield, and Weaver 2017). Recent studies have demonstrated that *in vitro* SVZ-derived NSCs/precursor cells release EVs.

(Cossetti et al. 2014). When used to treat NIH 3T3 cells, EVs isolated from cultures of primary SVZ NPCs activated Stat1 signaling in target cells (Cossetti et al. 2014). However, *in vivo* targets and functions of NSC EVs remain undetermined.

In this study, we investigated whether SVZ NSCs produce EVs, and what the *in vivo* targets were. Primary cultures of neonatal SVZ NSCs were found to release EVs. Transplantation experiments revealed that NSC EVs were selectively taken up by microglia in the neonatal SVZ. EV uptake was observed in CD11b/Iba1-positive microglia with a distinct rounded cellular morphology. RNA sequencing and cytokine profiling of microglia treated with NSC exosomes showed a changed in the transcriptional network and cytokine profile. Alterations in the microglia cytokine profile resulted in a negative feedback loop determined by EdU labeling in SVZ NSCs *in vivo*.

RESULTS

Neonatal SVZ NSCs Release EVs

Primary cultures of postnatal day zero (P0) SVZ NSCs cultured as a monolayer were subjected to immunocytochemistry and western blotting for the NSC marker protein Nestin. Most cells (~92%) were Nestin-positive (**Figure 1A**). Conversely, SVZ cultures were negative for the migrating neuroblast marker doublecortin (DCX; data not shown). Nestin could also be detected by western blot of primary SVZ NSC lysates. (**Figure S1A**) Neonatal SVZ NSC conditioned media was subjected to low-speed centrifugation to remove dead cells and cellular debris. To assess number and particle size present within the NSC conditioned media,

nanoparticle tracking analysis (NTA) was utilized. Four peaks were identified at 89, 129, 199, and 263nm with an average size of 164.9±7.2nm. NSC conditioned media was also subjected to an ultracentrifugation protocol to further isolate EVs. Ultracentrifugation yielded an NSC P100 fraction and NTA revealed four peaks at 109, 154, 209. and 312 with an average size of 190.7±5.2nm (**Figure 1B**). Electron microscopy confirmed the size of P100 EVs, and some EVs presented a cup-like morphology, characteristic of exosomes (**Figure 1D**).

Tetraspanin CD9 is frequently used as a protein marker of EVs (Raposo and Stoorvogel 2013; Andreu and Yáñez-Mó 2014). CD9 was detected by P2 in dorsal forebrain extracts and upregulated by P7 and increased into adulthood (**Figure 1F**). Additionally, CD9 and another EV marker protein, ALIX, were detected in primary neonatal SVZ NSC EVs by western blot (**Figure 1F and 1G**). CD9 expression was also confirmed in primary SVZ NSC cultures by immunocytochemistry similar to previous reports (**Figure 1H and 1I**) (Llorens-Bobadilla et al. 2015). *In vivo* immunohistochemistry of P0 and P4 brains revealed CD9 expression along the ventricular wall in Nestin-positive NSCs beginning at P0 and increasing by P4 (**Figure 1J-1L**). Furthermore, CD9 enrichment in the neonatal SVZ was consistent with *in situ* hybridization data collected by the Allen Brain Institute (**Figure 1M**)(Henry and Hohmann 2012). Taken together, these data demonstrate that SVZ NSCs release EVs *in vitro*.

SVZ NSCs Release CD9 In Vivo

To study EV release *in vivo*, Neuro2a (N2A) cells were first transfected with a fluorescent EV marker fusion plasmid, CD9-GFP, and tomato (fluorescent protein) (**Figure S2A**). CD9-GFP was detected in the cytoplasm of N2A cells and exosome fractions from sucrose gradients along with ALIX, CD63 and a His-tag present within CD9-GFP (**Figure S1B**). 47% (n = 9, 46.59 ± 4.943) of CD9-GFP-positive particles were found outside of transfected cells (**Figure S2B**), consistent with the hypothesis that CD9 is released from cells. Exosomes isolated from transfected N2A cell conditioned media were incubated with naïve N2A cells. CD9-GFP could be detected surrounding by not within N2A nuclei suggesting cytoplasmic uptake (**Figure S2C**). To confirm uptake, CD9-GFP-positive exosomes were incubated with N2A cells transfected with Tomato fluorescent protein. Indeed CD9-GFP could be detected in Tomato-positive cells (**Figure S2D**). Live imaging of N2A cultures transfected with CD9-GFP and Tomato fluorescent protein demonstrated considerable movement of intracellular CD9-GFP (**Figure S2E**). Finally, release of CD9-GFP was confirmed by live imaging (**Figure S2F**).

To study SVZ NSC EVs *in vivo*, CD9-GFP and Tomato fluorescent protein encoding plasmids were electroporated into the SVZ of P0 mice (**Figure 2A**). Tomato fluorescent protein could be detected throughout the soma of electroporated cells, whereas CD9-GFP expression was more localized to the apical and basal processes of SVZ NSCs (**Figures 2B – 2I**). CD9-GFP-positive cells were more readily found along the ventro-lateral ventricular wall (**Figure 2B**

– 2D). CD9-GFP was expressed in cells with an SVZ NSC morphology, namely an apical process that projects to the lateral ventricle and a basal process which projected onto a nearby blood vessel (**Figure 2E – 2H**). Cells with an NSC morphology stained positive for Nestin (**Figure 2L and 2M**), and for glial fibrillary acid protein (GFAP) by P7 (**Figure 2J and 2K**). Additionally, CD9-GFP could be detected outside of electroporated cells.

At 48 hr post electroporation, we could detect approximately 52 Tomato-positive cells (**Figure 2P**). From P2-P7, the number of tomato-positive cells decreased by 80% and remained unchanged at P21-P28. This is not surprising given that SVZ NSCs generate transit-amplifying cells that produce neuroblasts which migrate along the rostral migratory stream to the olfactory bulb. Therefore, fluorescence from NSCs expressing plasmid DNA is diluted overtime. NSCs were co-electroporated with Tomato and either of two CD9-GFP plasmids containing cytomegalovirus (CMV) or CAG promoters. Under the CMV protomer, CD9-GFP expression was limited to 7.52% of Tomato-positive cells, whereas CAG-CD9-GFP labeled 24% of Tomato-positive cells in the SVZ. Given that CAG-CD9-GFP was generated by cloning CD9-GFP into the Tomato plasmid, it is not surprising that CAG-CD9-GFP and Tomato co-expression was more abundant. Thus CAG-CD9-GFP was used for quantification. The percentage of cells co-expressing Tomato and CAG-CD9-GFP was compared to the total number of Tomato-positive cells decreased from 24% to 7.32% at P7 (**Figure 2Q**). For every electroporated cell 48 hr post-electroporation, at least one extracellular particle could be detected (54.77

CD9-GFP particles/image) (**Figure 2R**). The number of CD9-GFP particles found outside of co-electroporated cells decreased to 18.20 particles 7 days post-electroporation and remained similar (19.06) in P21-P28 mice (**Figure 2R**). Taken together, these results illustrate that SVZ NSCs release CD9-GFP, which is soon after cleared from the extracellular space.

SVZ NSCs Selectively Target Microglia

The reduction of extracellular CD9-GFP pointed towards a model in which NSCs EVs were targeted for removal. Whether or not this process was passive (i.e. CSF waste removal) or active was unclear. During early perinatal SVZ neurogenesis, there is an influx of microglia. This increase of microglia correlated with the observed loss of extracellular CD9-GFP. Therefore, it is likely that microglia are responsible for the loss of extracellular CD9-GFP-positive particles. To test this hypothesis, immunohistochemistry was used to stain brain sections electroporated with CD9-GFP and Tomato with the microglia marker ionized calcium-binding adaptor molecule 1 (Iba1). CD9-GFP-positive particles were found in proximity of or colocalized with Iba1-positive microglia (**Figures 3A and 3B**). Although *in vivo* labeling of EVs provided an initial insight into possible targets of NSC EVs, the lack of abundance of EVs forced us to investigate other methods to study EVs *in vivo*. As an initial experiment, exosomes from N2A cells transfected with CD9-GFP were isolated and labeled with a fluorescent lipophilic dye, DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) and transplanted into the lateral ventricles of P0 mice (**Figure 3C**). One to seven days post-

transplantation, brains were isolated, sectioned, and imaged. A population of cells near the lateral ventricles were labeled with DiI and GFP (**Figures 3D and 3E**). Some cells labeled with DiI had a stellate morphology and stained positive for the microglia marker Iba1 (**Figure 3F**). ~93% (92.84 ± 32.44, n = 8) of DiI exosomes co-localized with microglia at P2 and 80% at P7 (79.77 ± 5.448, n = 14) (**Figure 3G**). Because of their cancerous origin, N2A cells likely do not mimic NSC-derived EVs. Therefore, SVZ NSCs were isolated from P0 mice and placed in culture. P100 fractions were collected from the SVZ NSC conditioned media and labeled with DiI and transplanted into the lateral ventricles of P0 mice. DiI labeled NSC EVs predominately targets Iba1-positive microglia (**Figure 3H**). Labeled microglia were often contiguous with the lateral ventricles and found within the SVZ. DiI labeled cells also stained positive for two other microglia markers, CD68 and Cd11B, in addition to Iba1 (**Figures 3I – 3K**). Quantification revealed that a majority of NSC P100 EV-labeled cells were Iba1 positive (**Figure 3L**). EVs carry nucleic acids, such as mRNA and miRNA. We hypothesized that miRNAs were partially responsible for selective targeting to microglia. Therefore, prior to treatment, NSC P100 EVs were subjected to UV-treatment, but no significant change in Iba1 co-localization was detected (control 24 hr = 76.52 ± 4.608, UV-treated 24 hr = 69.64 ± 9.661, control 48 hr = 77.57 ± 6.263. UV-treated 48 hr = 61 ± 8.860) (**Figure 3L**). Exosomes were purified from SVZ NSC EVs, labeled with DiI, and transplanted into P0 mice. More than half of all DiI labeled cells were Iba1-positive microglia (P1 – 57.07 ± 8.134, P2 = 51.46 ± 7.996, P7 = 51.28 ± 12.13) (**Figure 3M**). Although

highly abundant in the developing SVZ, microglia account for less than 10% of total cells in the SVZ (Shigemoto-Mogami et al. 2014). Thus, NSC exosome uptake is not random, but is selectively targeted to microglia.

SVZ NSC EVs Act as a Microglia Morphogen

NSC EVs selectively target microglia, but to what effect this has on microglia is unclear. To understand the functions of NSC EVs, small RNA sequencing was performed. RNA sequencing identified numerous miRNA in NSC P100 EVs, with the most abundant miRNAs having reads 10-fold greater than the average miRNA (**Figure 4A**). Specifically, miR-9, Let-7, miR-26, and miR-181 families were highly enriched in NSC EVs. Interestingly, members of these miRNA families regulate microglia physiology and morphology(Kumar et al. 2015; Lehmann et al. 2012; Yao et al. 2014; L. Zhang et al. 2015). Based on previous reports, we hypothesized that NSC EVs induce a change in CD11b/Iba1-positive microglia morphology from a stellate to a rounded non-stellate phenotype (**Figure 4B**). NSC P100 EVs increased CD11b expression when compared with the contralateral hemisphere of transplanted brains (**Figure 4E**). 24 hr post-transplantation, Iba1/CD11b-positive microglia were found contiguous with and on the apical side of the lateral ventricle, and in addition, in clusters within the choroid plexus. DiI/CD11b- positive microglia found within the ventricle and in the choroid plexus were rounded, having an average ellipticity of 0.64, whereas unlabeled CD11b-positive microglia were more stellate with a lower ellipticity of 0.36 (**Figure 4F**). Due to this observation, microglia morphology was assessed following NSC P100 EV treatment. Microglia

are complex cells that project processes out into the extracellular environment in order to sense and survey their surroundings (Nimmerjahn, Kirchhoff, and Helmchen 2005). Therefore, we assessed the number of processes in Iba1-positive microglia both with and without DiI labeling. NSC EV-labeled microglia were less complex than non-labeled cells within the same SVZ. This phenotype persisted up to seven days (**Figure 4G**). UV pretreatment of SVZ NSC exosomes reversed the reduced complexity phenotype (**Figure 4H**). Because UV treatment was able to partially rescue the non-stellate morphology of microglia, we concluded that the content of NSC exosomes, and likely EVs, is important for the morphological changes of microglia. The Let-7 family was the most abundant miRNA found in NSC EVs, therefore we hypothesized that Let-7 might regulate microglia morphology. Exosomes isolated from N2A cells transfected with a Let-7 sponge were collected. Exosomes were then transfected with either a mock or synthetic Let-7 and subsequently transplanted into P0 lateral ventricles. Interestingly, synthetic Let-7 miRNA transfected exosomes, but not the mock, induced a morphological shift in microglia, similar to the shift caused by NSC EVs (**Figures 4I-4L**). Taken together, these results confirm that SVZ NSCs release EVs that are targeted to, taken up by, and modify microglia.

NSC EVs Activate a Microglia Transcriptional Network, Resulting in an NSC Feedback Loop

Primary cultures of microglia were subjected to next-generation RNA sequencing following NSC EV treatment. In cultures treated with NSC EVs, 1,713 transcripts

were upregulated by greater than 2-fold, and 1,175 were significantly downregulated by greater than 2-fold (**Figure 5A**). Gene ontology analysis revealed that immune system processes and inflammatory responses were the most highly enriched and significantly represented terms (**Figure 5B**). A heat map of altered transcripts revealed that the most highly upregulated transcripts included cytokines such as interleukin-1α (IL-1α), IL-1β, and IL-6 (**Figure 5C**). This observation was consistent with network analysis which included a cytokine node at the core of this network (**Figure 5D**). Luminex assay was performed on microglia conditioned media either treated or untreated with NSC EVs. The cytokine profile from treated vs. untreated microglia was consistent with the transcriptional data in that upregulated transcripts, such as IL-1α, IL-1β, and IL-6, were also increased in the cytokine profile of treated microglia (**Figure 5E**). Regulation of cytokines is likely dependent on NSC exosome content. Since Let-7 was the most abundant miRNA transcript detected and since Let-7 activates the endosomal TLR-7 receptor(Lehmann et al. 2012), we investigated whether Let-7 family members could modulate transcriptional changes that induce cytokine responses in microglia. Plasmids expressing Let-7 sponges were transfected into N2A cells. N2A media was collect and EVs were isolated using an ultracentrifugation protocol (P100 EVs). Synthetic Let-7 miRNAs were transfected into isolated P100 EVs and transfected EVs were used to treat cultures of microglia. Measured cytokine responses revealed that synthetic Let-7-containing P100 EVs stimulated robust release of cytokines from microglia that mimicked the effect of NSC EVs (**Figure**

5F). Interestingly, when NSC EVs were used to treat RAW 264.7 macrophages, the cytokine response differed from microglia (**Figure S3**). For example, macrophages released IL-1α, IL-6, and granulocyte-colony stimulating factor (G-CSF), whereas G-CSF was not increased for treated microglia, and IL-1β was unchanged in macrophages after NSC EV treatment. Suggesting that depending on the source of EVs, varied responses in target cells are observed. We hypothesized that the release of factors from microglia following exosomes uptake may exert a feedback response to SVZ NSCs. To test this, microglia conditioned media from NSC EV treated and untreated microglia was depleted of EVs and injected into the lateral ventricles of P0 mice. EdU (5-ethynyl-2'-deoxyuridine) labeling was performed 2 hr prior to tissue harvesting (**Figure 5G**). Conditioned media from treated microglia with NSC EVs, but not untreated microglia, caused a reduction in the number of dividing NSCs in the SVZ (**Figure 5H**). Taken together, these results demonstrated a robust and significant effect of SVZ NSC EVs on microglia signaling that results in a negative feedback loop to NSCs during early postnatal development.

Discussion:

In this study, we provide evidence of EV release from SVZ NSCs that are selectively targeted to microglia. NTA, western blotting and EM were used to confirm the release of EVs from primary SVZ NSCs. Fluorescent staining *in vivo* and *in vitro* confirmed the presence of EV marker protein CD9 in Nestin-positive NSCs. CD9 was found to be highly enriched in EV fractions, including EVs isolated

from N2A cells. These findings support other reports of CD9 in stem and stem-like cells, including adult SVZ NSCs (Bolukbasi et al. 2012; Karlsson et al. 2013; Kolle et al. 2009; Leung et al. 2011; Llorens-Bobadilla et al. 2015; Terada et al. 2002).

The CSF of embryonic and postnatal brains contain an abundance of EVs, but the sources, targets. and function are mostly undiscovered (Feliciano et al. 2014; Tietje et al. 2014). One study reported that CSF EVs isolated from rat embryonic CSF had a proliferative effect on mixed NSC cultures (Feliciano et al. 2014). Due to their location at the latero-ventricular interface, we investigated whether SVZ NSCs were a possible source of CSF EVs. Neonatal electroporations were preformed using CD9 fused to a green fluorescent tag (CD9-GFP). Extracellular CD9-GFP could be detected 48 hrs post-electroporation near Nestin-positive cells in the SVZ. Previous studies have identified the release of particles during neurogenesis whose targets and functions are not well understood (Das and Storey 2014; Dubreuil et al. 2007). For instance, neuroepithelial stem cells (NESCs) release a particle from the midbody during NESC progenitor pool expansion (Dubreuil et al. 2007). Thus, SVZ NSC EVs simply add to a growing list of the ever-increasing developmental EVs.

The number of extracellular CD9-GFP particles decreased over time. This reduction correlated with the influx of microglia into the SVZ of the neonatal brain. To unbiasedly identify the targets of SVZ NSC EVs, EVs were labeled with the lipophilic dye DiI, and transplanted into the lateral ventricles of P0 mice. We discovered that a majority of DiI labeled SVZ NSC EVs target Iba1/CD11b/CD68-

positive microglia. However, how EVs are targeted to microglia remains unclear. Other studies have also documented the uptake of oligodendrocyte and glioma exosomes by microglia (Fitzner et al. 2011; van der Vos et al. 2016). Since EVs carry various RNA species, including miRNA, and that other studies have identified the regulatory role of miRNA on microglia morphology and physiology, next-generation small RNA sequencing was used to identify miRNA content within SVZ NSC EVs. Several miRNA families were enriched in SVZ NSC EVs including miR-9, lethal-7 (let-7), and miR-181. Due to previous findings of miRNA regulation of microglia, we hypothesized that NSC EVs may function as a non-canonical microglia morphogen. Indeed, NSC EV uptake in microglia resulted in a morphological shift from a stellate to a rounded morphology, in agreement with previous reports (Kumar et al. 2015; Lehmann et al. 2012; Yao et al. 2014; L. Zhang et al. 2015). Since EVs also carry non-nucleic acids components, we cannot rule out the possibility of other NSC EV cargo contributing to the observed effects on microglia.

RNA sequencing of NSC EV treated microglia revealed a significant change in transcriptional networks. Of the transcripts, a notable change was associated with the immune system gene ontology terms, including immune system process, inflammatory response, defense response to viruses, neutrophil chemotaxis, positive regulation of cytokine secretion, and I-kappa B kinase/nuclear factor κB (NF-κB) signaling. In agreement with transcriptional changes, Luminex analysis of cytokine profiles showed an increased secretion of IL-6, IL-1α, and IL-1β from

treated microglia. Let-7 transfected EVs were sufficient to alter treated microglia cytokine profiles, although how this occurs is unclear. To understand the consequences of the altered cytokine profiles of treated microglia, microglia conditioned media was depleted of EVs and injected into the lateral ventricles of P0 mice. Transplantation of conditioned media from treated microglia resulted in a reduction of the number of proliferating SVZ NSCs, as determined by EdU labeling. Other studies have also reported the regulatory effect of microglia on dentate gyrus and embryonic ventricular zone NSCs (Cunningham, Martínez-Cerdeño, and Noctor 2013; Gebara et al. 2013).

The results of this study are two-fold: 1) EVs act as a method of intercellular communication and 2) SVZ NSC EVs are targeted to and alter microglia physiology and morphology. We propose that during neonatal SVZ neurogenesis, SVZ NSCs release EVs that act as a non-canonical microglia morphogen which affect the phenotype, location, and function of microglia in the developing brain. Many studies have reported the presence of EVs in normal and pathophysiological states during development and disease. While NSC EVs are just one population of EVs that likely contribute to normal brain development, future studies will be required to understand the far-reaching implications of SVZ NSC EVs in development and disease.

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and D.M.F. Exosome isolations were carried out by M.C.M and V.N.N. Labeling of EVs was done by M.C.M. and transplantations were done by M.C.M. and D.M.F. Media transplantations and Click-iT EdU labeling was performed by M.C.M. Western blotting was conducted by J.C.H., V.N.N. and M.C.M. Culturing of primary SVZ NSCs was primarily conducted by M.C.M. with technical assistance from V.N.N. and C.M.S. All analysis was performed by M.C.M. Microglia were cultured by M.C.M. Exo-fection was conducted by J.C.H. All statistics were done by D.M.F. This manuscript was written by M.C.M with assistance from D.M.F.

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DISCLOSURES

The authors have nothing to disclose.

Experimental Procedures

Animals

Experiments were performed according to guidelines set forth by the Clemson University Institutional Animal Care and Use Committee and NIH Guide for the Care and Use of Laboratory Animals. Pregnant CD-1 mice obtained from Charles River Laboratories were housed under pathogen-free conditions with a 12-hr light/dark cycle. For primary cell culture, EV and exosome preparation, and sequencing, pooled samples of both genders were used.

Cell Culture, Transfections, and Immunocytochemistry

N2a mouse neuroblastoma cells (American Type Culture Collection CCL-131) were maintained in tissue culture-treated polystyrene multi-well plates or flasks (Falcon; BD Biosciences Discovery Labware) in a 37°C incubator with 5% CO_2. N2a cells were routinely propagated and DNA transfections were performed with PolyJet (SignaGen Laboratories) as previously described (Feliciano et al. 2013b).

For immunocytochemistry experiments, when Neuro-2a cells reached 80-90% confluence, they were passaged onto circular coverslips in six-well plates 24 hours prior to transfection. Cells were then transfected with PolyJet (SignaGen Laboratories) with DNA vectors according to manufacturer's recommendations. 48 hours post-transfection, cells were subjected to live imaging (see below) or fixed using 4% paraformaldehyde in 1X DMEM by replacing half of the media three consecutive times. In short, half of the DMEM media was removed and replaced with 4% PFA. This was repeated three times over a course of 10 minutes.

Coverslips were then rinsed three times in wash buffer (PBS, 0.1% Tween-20) and incubated with 1 nM TO-PRO-3 Iodide (Topro) in DMSO (Life Technologies). Coverslips were then washed sequentially in wash buffer and 1X PBS and mounted in ProLong Gold antifade reagent (Life Technologies) and imaged at described.

Live Imaging

24-48 hrs after transfection of N2a cells with CD9-GFP and Tomato plasmids, coverslips containing cells were placed in a perfusion chamber. Cells were continuous perfused using pre-warmed culture media (37°C) through a pump (Harvard Apparatus, Catalog # 70- 2027)/vacuum system (Warner Instruments, Model # 64-1940 (DMV). Cells were subjected to live imaging in 4 hr intervals. Movies were collected, and representative snapshots were chosen for further analysis.

Electroporation

Electroporations were performed as described previously (Feliciano et al., 2013). Briefly, DNA combined with fast green was injected into the lateral ventricle. Pups were electroporated with a BTX ECM 830 Square Wave Pulse generator and Tweezertrodes (Harvard Apparatus).

Slice Preparation and Immunohistochemistry

Slices were prepared as described previously (Feliciano et al., 2011). Antibodies used included Nestin (Novus Biologicals, NB100-

1604), CD9 (eBioscience, 14-0091-81), Iba1 (Novus, NB100-2833), CD68 (Bio-Rad, MCA1957), CD11b (Bio-Rad, MCA711G), or GFAP (Cell Signaling Technology, 12389S). Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 633 (Life Technologies) were incubated overnight at 4°C.

Imaging

Images were taken on a Leica TCS SPE spectral confocal microscope using 20×, 40×, or 63× oil immersion lenses. Images were processed using Leica Application Suite X software (Leica Microsystems).

Exosome Isolation

Supernatant was collected and centrifuged at 300 × *g* for 10 min and 2,000 × *g* for 10 min, followed by 100,000 × *g* centrifugation in a Beckman Coulter Optima MAX-XP with a TLA 100.3 rotor for 90 min (P100 fraction). EVs were then subjected to further purification via sucrose density gradients consisting of 8%, 30%, 45%, and 60% layers in PBS and centrifuged at 232,000 × *g* for 30 min to 18 hr at 4°C. 10 fractions were collected, diluted 1:10 in PBS, and centrifuged for 1 hr at 100,000 × *g*. Each fraction was re-suspended in 4× Laemmli buffer and subjected to western blotting or PBS for further analysis. For exosome transfection experiments, P100 fractions were resuspended in 2.5 M sucrose, and density gradients were constructed from 2.5 M to 0.25 M (2.5 M, 2 M, 1.5 M, 1 M, 0.5 M, and 0.25 M). Gradients were centrifuged at 100,000 × *g* for 18 hr at 4°C. 10 Fractions were collected and diluted 1:10 in PBS and centrifuged

for 1 hr at 100,000 × *g* at 4°C. Fractions were resuspended in Dulbecco's PBS and stored at –20°C.

Electron Microscopy

Preformed at Department of Cellular and Molecular Physiology, Yale University School of Medicine.

EVs suspended in 4% paraformaldehyde were embedded in a formvar carbon-coated grid, washed in PBS, fixed in 1% glutaraldehyde, and stained with saturated aqueous uranyl oxalate. EVs were embedded in 0.4% w/v uranyl acetate and 1.8% w/v methylcellulose. Samples were imaged with a Carl Zeiss 910 electron microscope (Carl Zeiss Microscopy, Thornwood, NY).

Neonatal Transplantation and Click-iT EdU Labeling

DiI-labeled P100 fractions were preloaded into capillary tubes and loaded with 2 µL of EV/DiI/PBS mixture, and EVs were injected into the lateral ventricle. Pups were placed onto a heating pad for 5 min until recovered and placed back into cages. Medium collected from cultured microglia was loaded into capillary tubes, and approximately 2 µL of medium was transplanted into the lateral ventricles of P0 pups. The Click-iT EdU imaging kit (Invitrogen, C10338) was used according to the manufacturer's protocol to assess proliferation.

DiI Labeling of P100 Fractions

P100 fractions were isolated as described above. P100 fractions were centrifuged at 14,000 × *g* for 30 min. Pellets were re-suspended in PBS and subjected

to DiI labeling (1:1,000 at room temperature [RT], Life Technologies, V22889) for 10 min while vortexing periodically during incubation. DiI-labeled P100 fractions were centrifuged at 14,000 × *g* for 30 min and re-suspended in 1× PBS. Centrifugation was repeated a total of three times. The final pellet was re-suspended in 50 µL 1× PBS and stored at –20°C.

Western Blot

Samples were lysed in radioimmunoprecipitation assay (RIPA) buffer, 2% SDS, and Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Samples were placed on ice, sonicated with a Q55 sonicator (Qsonica), and centrifuged, and the supernatant was placed into fresh tubes. The following antibodies were used: CD63 (1:1,000, System Biosciences), CD9 (1:1,000, System Biosciences), ALIX (3A9, 1:1,000, Cell Signaling Technology, 21711), β-tubulin III (Aves Labs, TUJ), or His tag (D3I1O, 1:1,000, Cell Signaling Technology, 12698). Horseradish peroxidase (HRP)-conjugated antibodies from Life Technologies were used as a secondary antibody (1:3,333–1:5,000). Polyvinylidene fluoride (PVDF) membranes were incubated with ECL substrate (Pierce) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

Primary Neural Stem Cell Culture and Immunocytochemistry

The protocol was derived from Walker and Kempermann (2014). Cells were cultured in 500 µL Neurobasal A complete medium (1X Glutamax, 50 units/mL penicillin/streptomycin, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL fibroblast growth factor 2 (FGF-2), and 2% B27 Supplement). Cells were

placed on laminin-coated plates or coverslips in 24-well plates in Neurobasal A complete medium. The following day, complete media was refreshed, and the supernatant was stored at –20°C.

24 hr later, culture medium was collected and used for exosome and P100 isolation. Cells were lysed with 1X RIPA, 2% SDS, and PBS and used for western blotting or fixed by adding equal volumes of 37°C fixative solution (4% paraformaldehyde in 300 mM sucrose and Neurobasal A) directly to the wells and incubated at 37°C for 10 min. Coverslips were then washed three times, blocked in antibody buffer (PBS, 2% BSA, and 0.1% Tween 20) with 0.1% Triton X-100 and then washed again three times. Coverslips were incubated with antibodies against anti-CD9 mouse (eBioscience, 1:1,000), Nestin (Novus, 1:1,000), Iba1 (Vector Laboratories, VP-RM04, 1:500), and DCX (Santa Cruz Biotechnology, C-18, 1:500), washed three times, and then incubated in antibody buffer with the appropriate secondary antibodies (1:1,000, Life Technologies). Following four 15-min washes, coverslips were mounted in ProLong Gold Antifade with DAPI (Life Technologies) on Superfrost microscope slides (Thermo Scientific).

Analysis: Distribution

Co-localization and distribution of CD9-GFP in electroporated postnatal neural stem cells were analyzed using Fiji. Z stack images were loaded into Fiji software, Z-projected (Max Intensity), and separated from stacks to images based on fluorescent channels (i.e., green, red, or blue). Brightness/contrast was adjusted for each Z-projected image to reduce background noise. Using the Co-localization

plugin in Fiji, Z-projected images were assessed for co-localization of CD9-GFP and Tomato. The co-localization threshold of each image was set between 85–130 using 5-point increments when adjusting. Images were subjected to particle analysis through the Analyze Particle plugin. Green particle size was set from 1 micron to infinity to detect small CD9-GFP particles, and Tomato particle size was set from 10 microns to infinity to only count the number of cells and not cell debris. The percentage of CD9-GFP and Tomato co-localization was recorded.

DiI and Cell Type Co-localization (Iba1, CD11b, and CD68)

Images were loaded into Fiji (ImageJ) and converted into composite images. Z sections were selected based on DiI and Iba1/CD11b/CD68 co-localization within the selected Z section. Selected sections were Z-projected using max project and separated into individual color channels. Brightness and contrast were adjusted to correct for background noise within each image. Co-localization analysis was performed on Z-projected DiI and Iba1/CD11b/CD68 images. Co-localized images were converted to 8 bits, inverted, and thresholded to 0 and 255 (only co-localized particles were visible). Thresholds of Z-projected DiI and Iba1/CD11b/CD68 images were set to 10/255 and 85–100/255, respectively. All images were then subjected to particle analysis. The percentage of co-localized DiI particles was in relation to the total number of DiI particles.

Morphological Analysis: Ellipticity and Process Number

Cellular ellipticity was measured using Shape Description in Particle Analysis in ImageJ. Images were loaded into ImageJ, Z-projected, and separated into

individual channels (green, red, and blue). Thresholds were set at 100–120 and 255. Cells either in the SVZ or in the ventricular wall were outlined using the freehand tool and assessed through particle analysis. Circularity was reported as a value between 0 and 1, with 1 describing a perfect circle. Microglia complexity analysis was conducted using Leica Application Suite X (LASx) 1.1.0.12420 3D module software. Processes of Iba1+ cells were quantified manually in the 3D module. The average number of processes in DiI+/Iba1+ cells was compared with DiI-/Iba1+ cells.

Nanosight Particle Tracking Analysis

Samples were shipped on wet ice to the Nanomedicine Characterization Core facility in the Center for Nanotechnology in Drug Delivery at the University of North Carolina (UNC) at Chapel Hill. Samples were prepared in a laminar flow hood and thawed at RT. Sample dilutions were based on an initial run with PBS, 10 mM salt. Samples were loaded onto a pre-cleaned and pre-warmed Nanosight NS 500 nanoparticle characterization system (NanoSight, UK) equipped with a 532-nm laser and a 565-nm long pass filter. Mean size and particle concentration values were calculated by the nanoparticle tracking software. The Nanosight NS 500 was calibrated with 100 nm polystyrene latex microsphere standards (Nanosight, UK), and readings were acquired at 23.3°C.

RNA Sequencing

Small RNA sequencing was performed by System's Biosciences (SBI). RNA Sequencing of microglia was performed by GeneWiz.

For EV small RNA sequencing, RNA was isolated by Trizol extraction from EVs from P100 fractions of primary SVZ NSCs. Samples were quantified using the Agilent Bioanalyzer small RNA assay. Libraries were prepared according to manufacturer's protocol (Illumina small RNA preparation kit). Samples were subjected to 1 × 75 bp single-end reads at an approximate depth of 10–15 million reads per sample on an Illumina Hi-Seq.

RNA was isolated from microglia cell cultures using Trizol in accordance with the manufacturer's protocol. Three samples from 1–2 RNA isolations for each condition were used to generate libraries. RNA concentrations and purity were assessed by Nanodrop, Qubit assay, and Agilent Tapestation. RNA library preparation with poly(A) selection was performed using the NEBNext Ultra RNA Library Preparation Kit according to the manufacturer's protocol (New England Biolabs). 2 × 150 bp reads were generated at a depth of 56–84 million reads per sample on an Illumina HiSeq. Samples had a mean quality score of 38.52, with 92.76% of bases having a ≥ Q30 score. Sequence reads were trimmed to remove possible adaptor sequences and nucleotides with poor quality using Trimmomatic v0.36. The reads were then mapped to the *Mus musculus* GRCm38 reference genome available on ENSEMBL using the STAR aligner. The RNA sequencing (RNA-seq) aligner is executed using a splice aligner that detects splice junctions and incorporating them to help align the entire read sequences. Unique exon hit counts were calculated using feature counts from the Subread package. After mapping and unique exon hit count calculations, downstream

differential expression analysis was performed using DESeq2. Gene ontology (GO) analysis was performed on the statistically significant set of genes by implementing the software GeneSCF. The Mouse Genome Informatics GO list was used to cluster the set of genes based on their biological process and determine their statistical significance. Gene interaction networks were determined by Cytoscape 3.60 using Genemania.

Luminex

Cytokine concentrations were quantified with the cytokine multiplex assay from Bio-Rad as described previously (Racicot et al., 2017). Wells of a 96-well filter plate were loaded with 50 µL of prepared standard solution or 50 µL of cell-free supernatant and incubated with the Bio-Plex Pro mouse 23-plex assay from Bio-Rad at ± 800 rpm for 30 min in the dark at RT. Wells were vacuum-washed three times with 100 µL wash buffer. Samples were then incubated with 25 µL of biotinylated detection antibody at ± 800 rpm for 30 min at RT in the dark. After three washes, 50 µL of streptavidin-phycoerythrin was added to each well and incubated for 10 min at ± 800 rpm at RT in the dark. After a final wash, the beads were resuspended in 125 µL of sheath buffer for measurement with the Luminex 200 (Luminex, Austin, TX)

Microglia Culture

24 hr prior to plating microglia, 6 well plates were coated with 10 µg/mL poly-L-lysineaccording to the supplier's instructions. The protocol was derived from Bohlen et al. (2017). Primary microglia cells isolated from CD1 mice were

reconstituted in a 37°C water bath immediately prior to plating. For immunohistochemistry following exosome treatment experiments, 3,500 cells were plated in 500 µL microglia complete medium (2 ng/mL TGF-β2, 100 ng/mL IL-34, 1.5 mg/mL cholesterol, 100 units/mL penicillin-streptomycin [pen-strep], 1× Glutamax, and microglia medium [Sciencell, 1901]) and allowed to adhere for 10 min at RT in 24-well plates.

Exo-fection

After sucrose density gradient isolation of Neuro-2a exosomes or extracellular vesicles, the respective vesicles were transfected (System Biosciences, EXFT10A-1). In brief, isolated vesicles were mixed with 10 µL Exo-Fect solution, synthetic Let-7 miRNAs (Sigma; HMI0007, HMI0009, and HMI0017, which also correspond to mouse Let-7), and Dulbecco's phosphate-buffered saline (dPBS). Tubes were incubated at 37°C for 10 min then immediately placed on ice. Samples were centrifuged at 14,000 x g for 15 min at 4°C. Samples were either used to treated cultured primary microglia or labeled with DiI and injected into the lateral ventricles of P0 pups as described previously.

Statistics

Statistics were performed with Prism software (version 6; GraphPad). Significance was calculated using unpaired t tests and one-way ANOVA with Tukey's multiple comparisons test. Statistical significance was defined as $p < 0.05$. All data are presented as mean ± SEM. The Wald test, p values, and absolute Log_2Fold changes were generated for gene expression analysis. Genes with adjusted p <

0.05 and absolute Log_2Fold change > 2 were called as differentially expressed genes for each comparison.

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FIGURES AND LEGENDS:

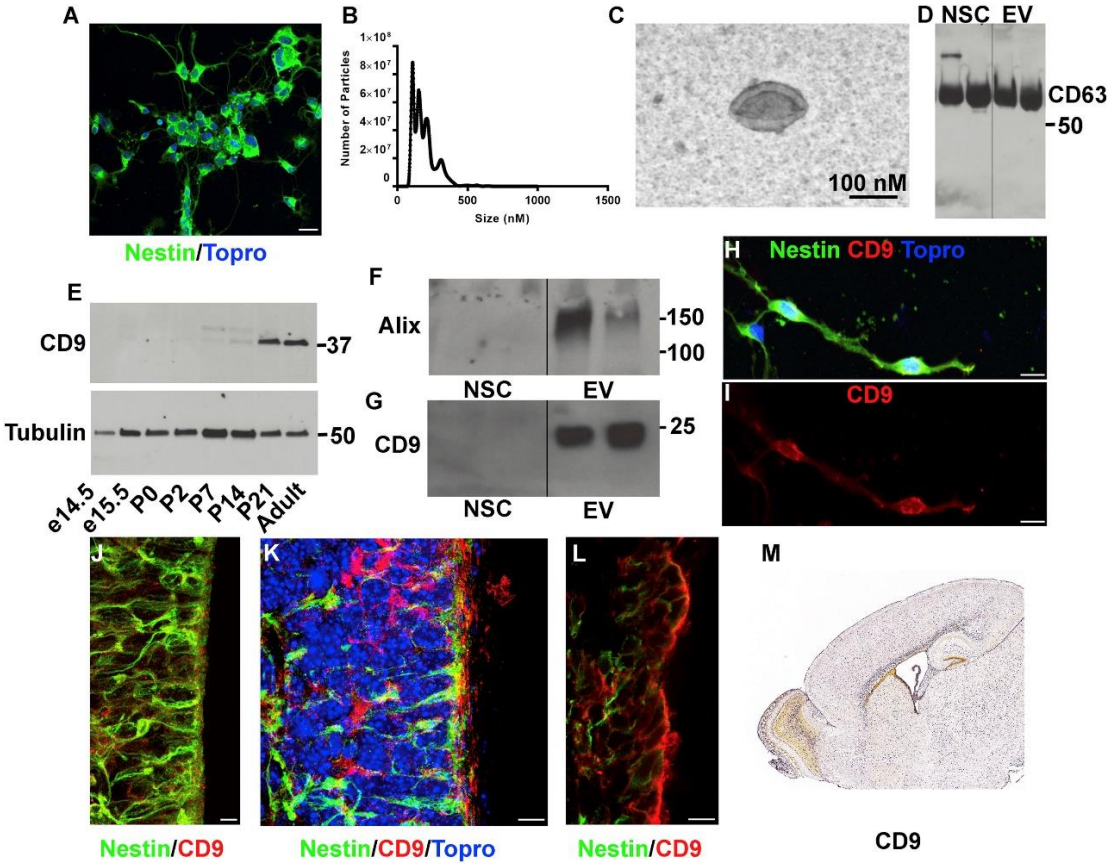

Figure 1. Neonatal SVZ NSCs Release EVs

(A) Immunocytochemistry for Nestin (green) and TO-PRO-3 (blue) in primary cultures of P0 SVZ NSCs 48 hrs after culture initiation. Scale bar, 25 μm.(B) Nanosight particle profile of EVs derived from the P100 fraction of SVZ NSCs. (C) Electron micrograph of an NSC-derived exosome. Scale bar, 100 nm. (D) Western blot of CD63 in NSC lysate or NSC EV P100 fractions. (E) Western blot of CD9 and β3-tubulin from dorsal forebrain extracts. (F) Western blot for ALIX in SVZ NSC EV P100 fractions as in (D). (G) Western blot for CD9 from NSC EV P100 fractions as in (D). (H and I) Immunohistochemistry of Nestin (green), TO-PRO-3 (blue), CD9 (red) (H), and CD9 (I) in P0 SVZ NSC cultures. Scale bars, 12.5 μm. (J) Nestin (green) and CD9 (red) expression within the SVZ at P0. Scale bar, 5 μm. (K and L) Nestin (green), CD9 (red), and TO-PRO-3 (blue) within the SVZ at P4. Scale bars, 25 μm. (M) *In situ* hybridization of CD9 in a P14 sagittal brain section from the Allen Brain Institute Developing Mouse Brain Atlas. Image credit: Allen Institute.

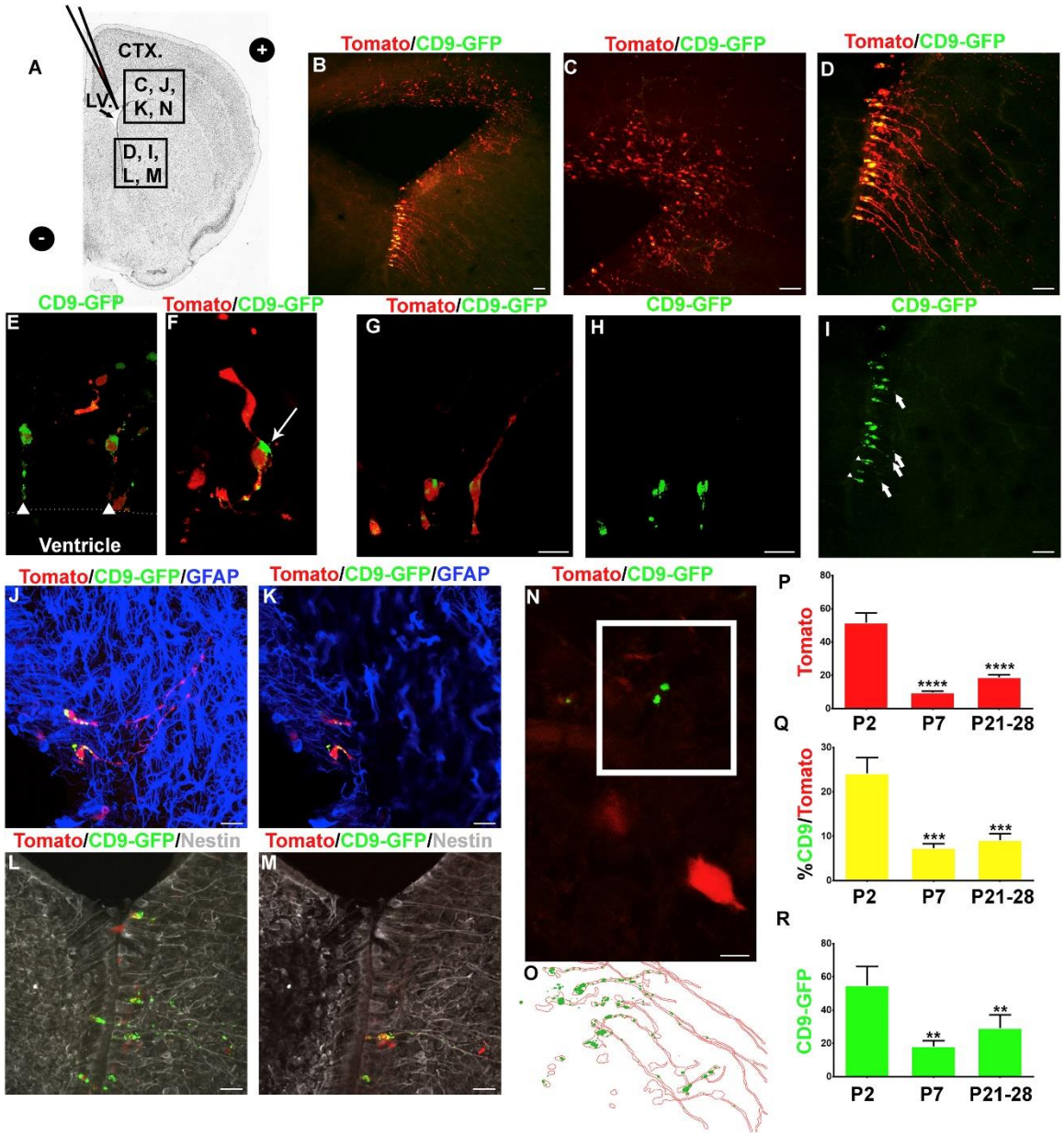

Figure 2. SVZ NSCs Release CD9 *In Vivo*

(A) Schematic diagram of a coronal section, indicating the direction of electrodes used for neonatal electroporation; black rectangles indicate the corresponding regions imaged. Image credit: Allen Institute. (B) 5x image of the SVZ of a P2 mouse following P0 electroporation with Tomato (red) and CD9-GFP (green).

Scale bar, 100 μm. (C) 20x image of the dorsolateral SVZ from (B). Scale bar, 50 μm. (D) 20x image of the ventro-lateral SVZ from (B). Scale bar, 50 μm. (E–H) 63x images of SVZ NSCs electroporated with Tomato (red) and CD9-GFP (green) demonstrating differences in CD9-GFP localization in apical and basal processes (E) and soma(F). Note the distribution of Tomato and CD9-GFP (G, merge) and CD9-GFP alone (H). Scale bars, 50 μm. (I) CD9-GFP distribution in relation to the Tomato-positive SVZ NSC soma located at the ventricle. Arrows point to CD9-GFP in the basal fiber, and arrowheads point to apical fibers with CD9-GFP present. Scale bar, 50 μm. (J) SVZ from a Tomato-electroporated (red) and CD9-GFP-electroporated (green) mouse stained for GFAP (blue). Scale bar, 25 μm. (K) Individual Z section from (J). Scale bar, 25 μm. (L) 20x image of a CD9-GFP-electroporated (green) and Tomato-electroporated (red) brain stained for Nestin (white). Scale bar, 25 μm. (M) Individual Z section from (L). Scale bar, 25 μm. (N) 63x zoom-2 image within the SVZ of Tomato-electroporated (red) and CD9-GFP-electroporated (green) mice showing extracellular CD9-GFP-positive particles. Scale bar, 5 μm. (O) A tracing of an individual slice showing a Tomato-positive cell body (red) and CD9-GFP (green). (P) Quantification of the number of Tomato-positive SVZ cells from P2–P28. (Q) Quantification of CD9-GFP and Tomato-positive cells in the SVZ from P2–P28. (R) Quantification of the number of extracellular CD9-GFP-positive particles in the SVZ from P2–P28. See also Figure S1. Data are represented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

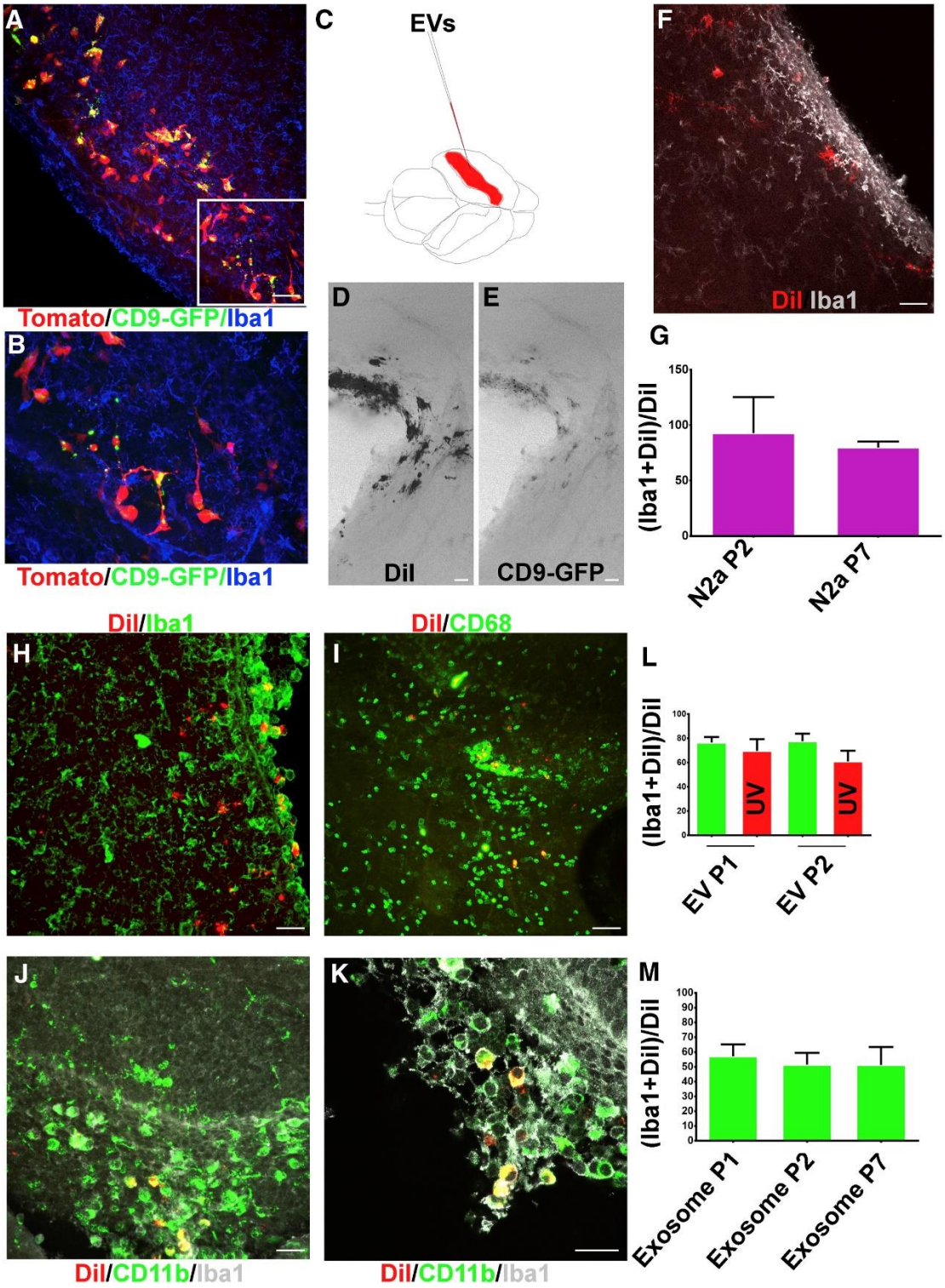
A
Tomato/CD9-GFP/Iba1
B
Tomato/CD9-GFP/Iba1
C
EVs
D
DiI
E
CD9-GFP
F
DiI Iba1
G
(Iba1+DiI)/DiI
150
100
50
0
N2a P2
N2a P7
DiI/Iba1
H
DiI/CD68
I
L
(Iba1+DiI)/DiI
100
80
60
40
20
0
UV
UV
EV P1
EV P2
J
DiI/CD11b/Iba1
K
DiI/CD11b/Iba1
M
(Iba1+DiI)/DiI
100
90
80
70
60
50
40
30
20
10
0
Exosome P1
Exosome P2
Exosome P7

Figure 3. NSC EVs Selectively Target Microglia

(A) 20x image showing Iba1-positive microglia (blue) in proximity to Tomato-electroporated (red) cells and CD9-GFP-positive particles (green). Scale bar, 50 μm. (B) 20x image of (A). (C) Schematic of EV transplantations. (D and E) DiI (D) and CD9-GFP (E, green) fluorescence within the SVZ of P7 brains following CD9-GFP N2A EV transplantation. Scale bar, 50 μm. (F) Brain transplanted with DiI-labeled EVs (red) and stained for the microglia marker Iba1(white). Scale bar, 50 μm. (G) Quantification of co-localization of Iba1 and DiI following N2A transplantations and analysis at P2 and P7. (H) 20x image of a lateral SVZ following primary NSC DiI-labeled EV (red) transplantation stained for Iba1 (green). Scale bar, 50 μm. (I) 20x image of a dorso-lateral SVZ following primary NSC DiI-labeled EV (red) transplantation stained for CD68 (green). Scale bar, 50 μm. (J and K) 20× (J) and 63x (K) image of SVZ following primary NSC DiI-labeled EV (red) transplantation stained for CD11b (green) and Iba1 (white). Scale bars, 25 μm. (L) Quantification of Iba1 co-localized EVs without (green) or with (red) UV treatment from NSC EV transplantations. (M) Quantification of the number of Iba1-positive, DiI-positive cells in the SVZ from P1–P7 from NSC exosome transplantations. Data are represented as mean ± SEM.

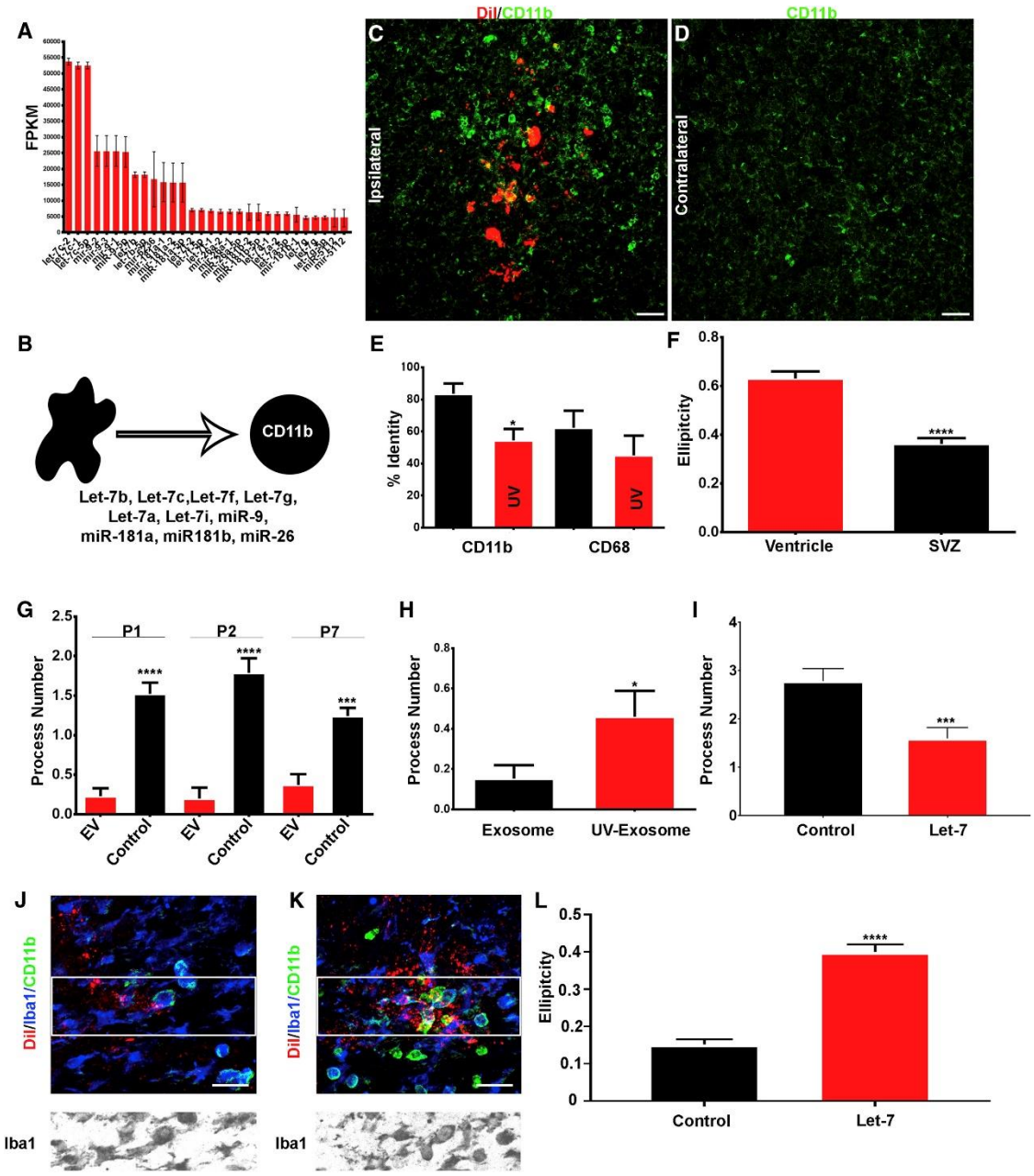

Figure 4. NSC EVs Act as a Microglial Morphogen

(A) Identity and quantity of NSC EV miRNAs. (B) Schematic of predicted NSC EV miRNA functions. (C and D) Image demonstrating CD11b-labeled (green) cells in DiI-labeled EV-transplanted (red) ipsilateral (C) or contralateral (D) hemispheres.

Scale bar, 25 μm. (E) Quantification of the percentage of CD11b- or CD68-positive microglia. (F) Measurement of regional microglia ellipticity. (G) Average number of cellular processes in Iba1-positive microglia following EV transplantation. (H) Quantification of the number of cellular processes in Iba1-positive microglia labeled by control or UV pre-treated NSC exosomes. (I) Quantification of the number of cellular processes following treatment with control N2A exosomes or N2A exosomes packaged with synthetic Let-7. (J and K) Representative images of control (J) or Let-7 (K) EV transplantations from (I). Scale bars, 50 μm. (L) Quantification of microglia ellipticity following control or Let-7 exosome uptake. Data are represented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

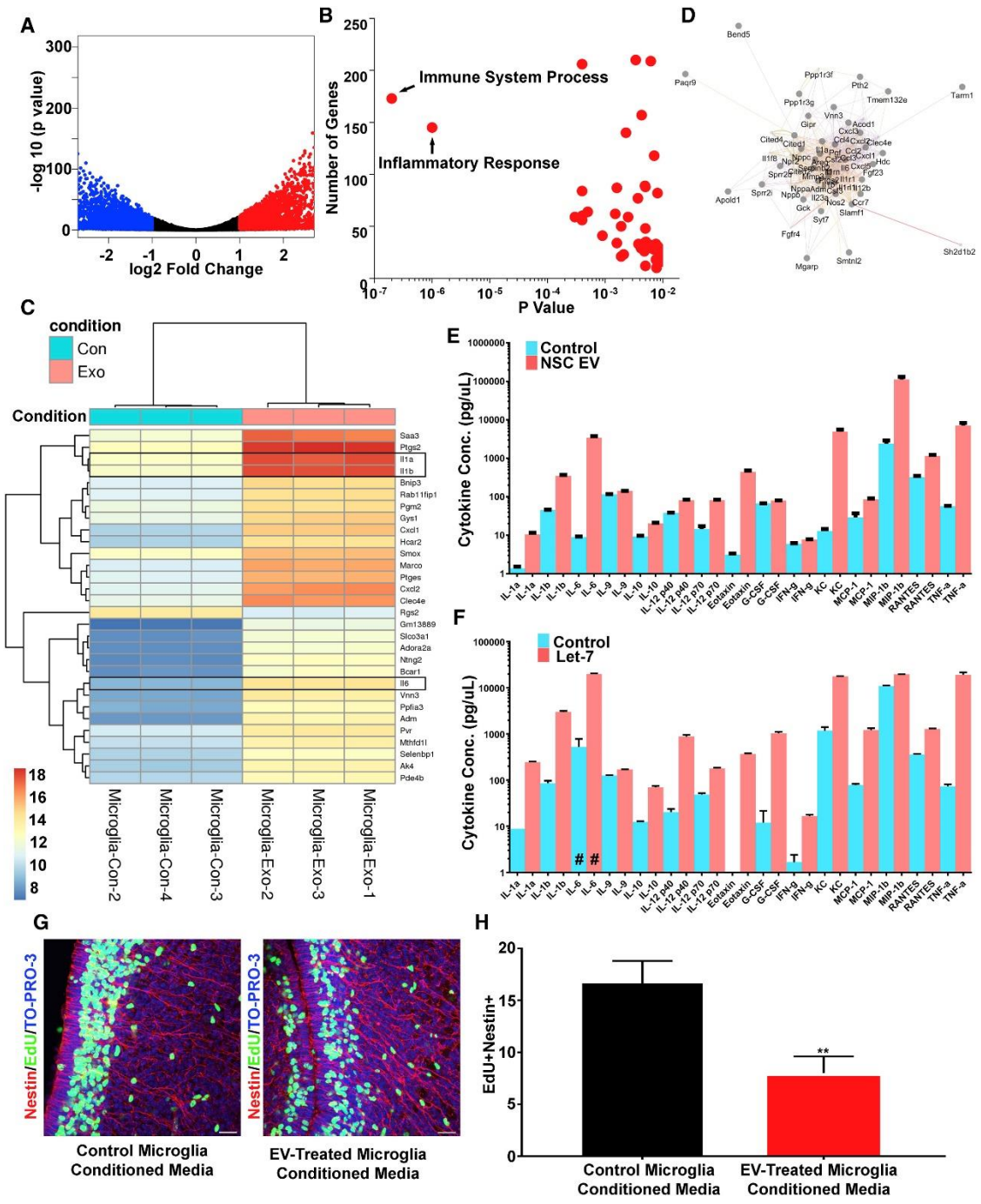
A
-log 10 (p value)
log2 Fold Change
B
Number of Genes
P Value
Immune System Process
Inflammatory Response
C
condition
Con
Exo
Condition
Microglia-Con-2
Microglia-Con-4
Microglia-Con-3
Microglia-Exo-2
Microglia-Exo-3
Microglia-Exo-1
D
E
Control
NSC EV
Cytokine Conc. (pg/uL)
F
Control
Let-7
Cytokine Conc. (pg/uL)
G
Nestin/EdU/TO-PRO-3
Control Microglia Conditioned Media
EV-Treated Microglia Conditioned Media
H
EdU+Nestin+
Control Microglia Conditioned Media
EV-Treated Microglia Conditioned Media

Figure 5. NSC EVs Activate a Microglia Transcriptional Network, Resulting in an NSC Feedback Loop

(A) Volcano plot of p values and fold changes of microglia mRNAs after NSC EV treatment. (B) Gene ontology terms represented by p value and number of genes changed in the microglia gene network. (C) Bi-clustering heatmap of the top 30 significantly altered mRNAs from control or NSC EV-treated microglia. (D) Gene network analysis of the top differentially expressed microglia mRNAs demonstrates clustering of cytokines and cytokine receptors. (E) Cytokine levels in medium from control and NSC EV-treated microglia. (F) Cytokine levels from microglia-conditioned medium from N2A control and synthetic Let-7 transfected exosomes. #, = fluorescence. (G) Representative images of brains injected with control or NSC EV microglia-conditioned medium, Edu-labeled (green), subjected to immunohistochemistry for Nestin (red), and counterstained with TO-PRO-3 (blue). Scale bar, 25 μm. (H) Quantification of the percentage of EdU-positive, Nestin-positive neural stem cells. See also Figure S2. Data are represented as mean ± SEM. $^{**}p < 0.01$.

Figure S1:

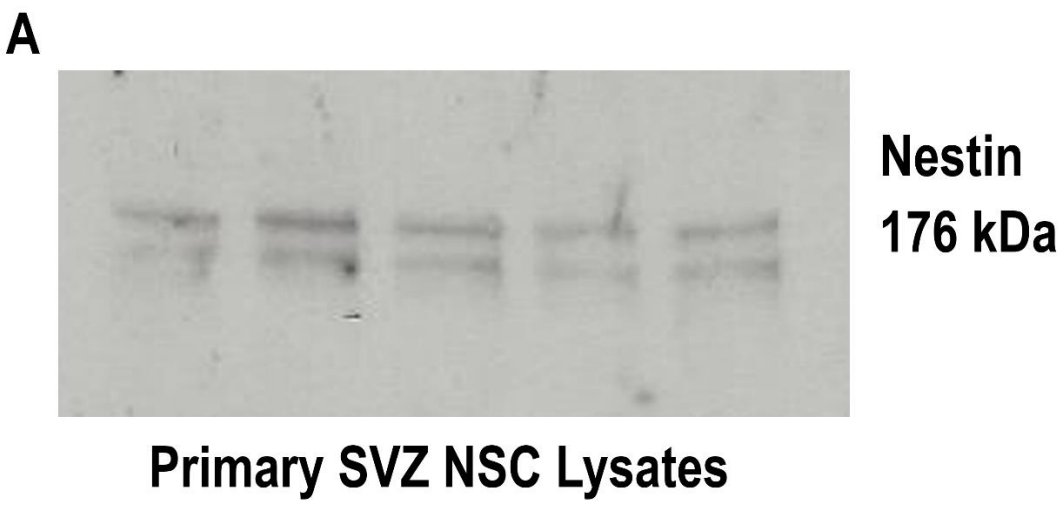

Figure S1. Western blot of primary SVZ NSC lysates. A) Western blot of primary SVZ NSC lysates were probed for the NSC marker Nestin. Nestin = 176 kDa.

Figure S2:

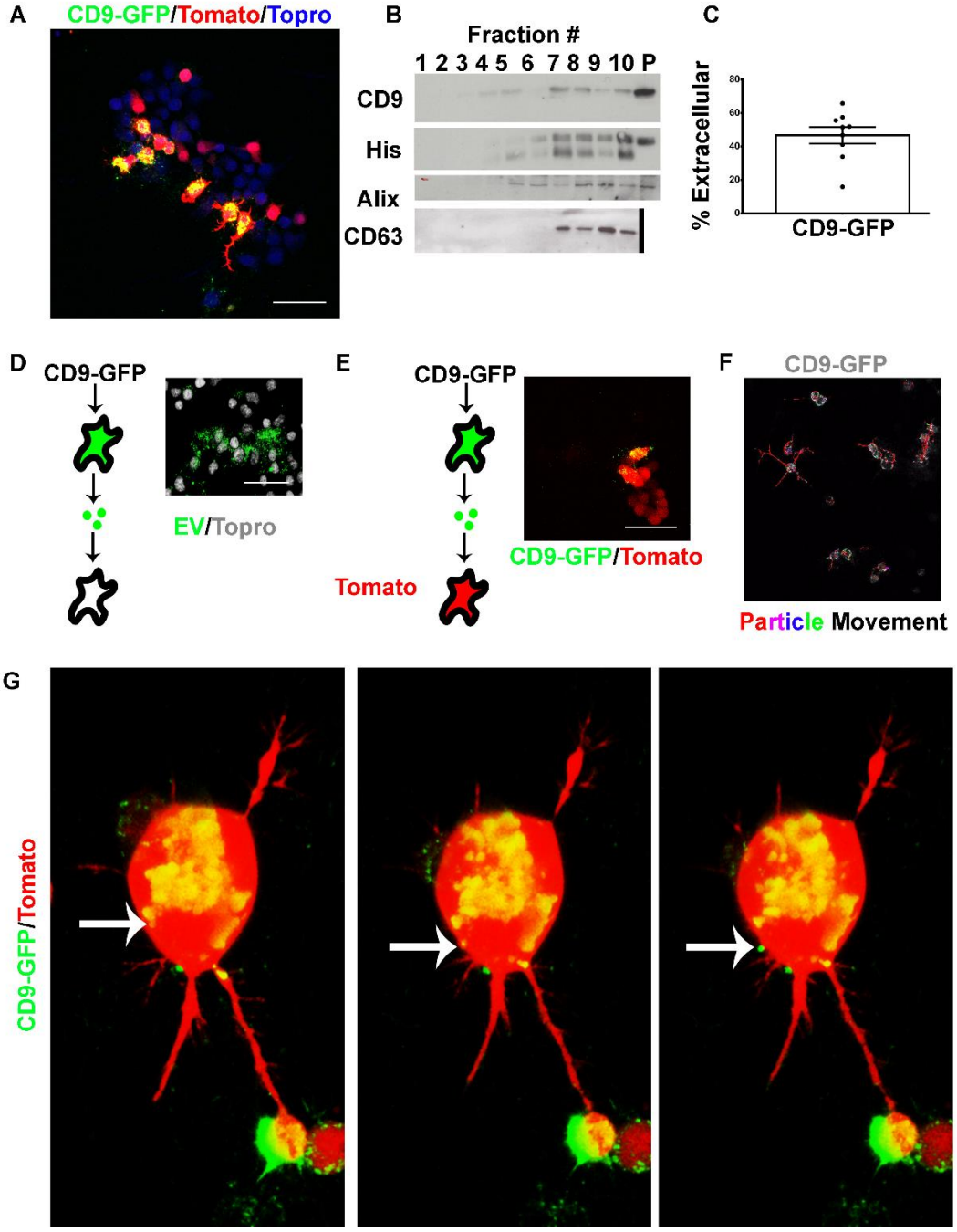
A
CD9-GFP/Tomato/Topro
B
Fraction #
1 2 3 4 5 6 7 8 9 10 P
CD9
His
Alix
CD63
C
% Extracellular
CD9-GFP
D
CD9-GFP
EV/Topro
E
CD9-GFP
Tomato
CD9-GFP/Tomato
F
CD9-GFP
Particle Movement
G
CD9-GFP/Tomato

Figure S2. CD9 is Released with N2A EVs, Related to Figure 2.

A) Image of N2A cells transfected with CD9-GFP (green) and Tomato (red) and DNA counter-stained with TO-PRO-3 (blue). B) N2A EVs were subjected to sucrose gradient fractionation and subsequently western blotted for CD9, Alix, CD63, and His-tag. P=P100 fraction. C) Quantification of the percentage of extracellular CD9-GFP. D) Exosomes from CD9-GFP (green) transfected N2A cells were isolated and incubated with naïve N2A cells and counterstained for TO-PRO-3 (grey). E) Same experiment as D with the exception that CD9-GFP exosomes (green) were added to tomato (red) transfected N2A and imaged. F) Live imaging of CD9-GFP (grey) and tomato transfected N2A cells with CD9-GFP particle trajectories labeled (multi-color). G) Live imaging of tomato (red) and CD9-GFP (green) transfected N2A cells. Arrow points to CD9-GFP being released. Data are represented as mean ± SEM.

Supplemental Figure 3:

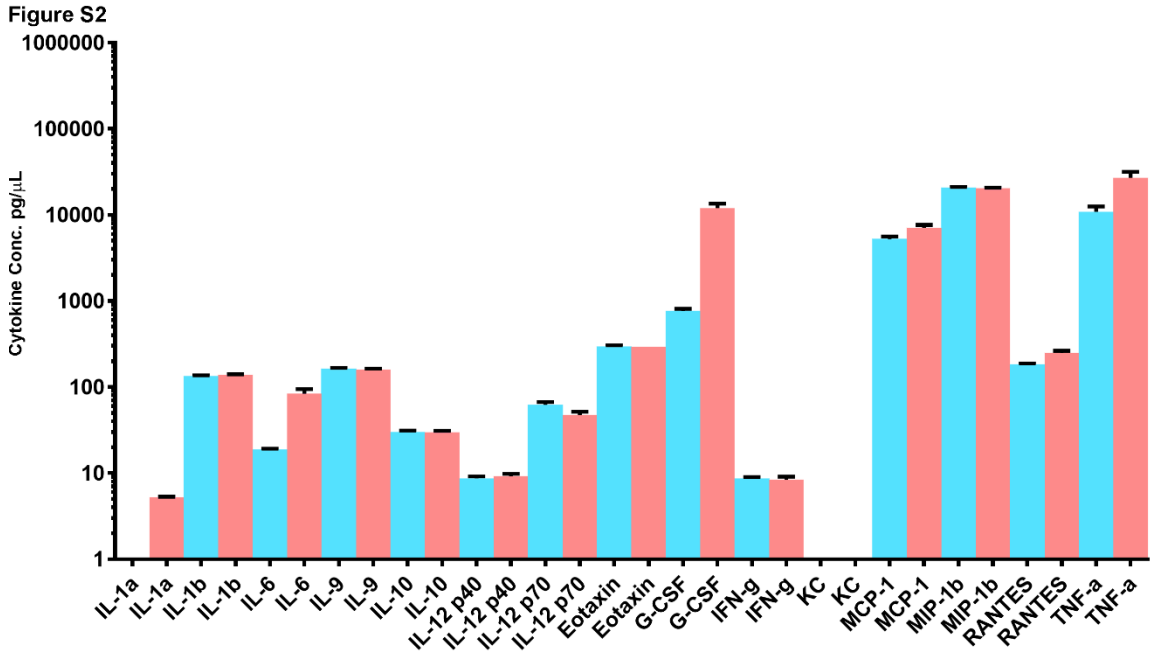

Figure S3. Effect of NSC EVs on RAW 264.7 Cytokine Profile, Related to Figure 5. A) Quantification of cytokine concentration in RAW 264.7 cell media from control (blue) and NSC EVs (red) treatments. Data show mean values (± standard error of the mean (SEM), n=6 from 3 independent samples read in duplicate).

CHAPTER FOUR

A CRE-Recombinase-Inducible and Fluorescent Extracellular Vesicle Transgenic Model System Labels Neural Stem Cells in the Subventricular Zone of Neonatal Mice

ABSTRACT

Extracellular vesicles (EVs) are primarily membrane-derived vesicles that transfer encapsulated RNA and protein from donor to recipient cells. EV content is dependent upon its cell of origin and thus each EV generated contains a unique molecular signature. Tetraspanin proteins, namely CD9, CD63, and CD81, have been used to identify and characterize EVs. EV marker proteins fused to fluorescent tags have been utilized to study EVs *in vitro* and *in vivo*. Recently, transgenic model systems have been generated to better understand EV implication in development. Subventricular zone (SVZ) neural stem cells (NSCs) reside at the lateral-ventricular interface of neonatal and adult mice. SVZ NSCs generate multiple cells types including olfactory bulb neurons and astrocytes. Moreover, SVZ NSCs interact with EV containing cerebrospinal fluid (CSF) throughout life. In the present study, a CRE-recombinase inducible expression plasmid where CD9 was fused to GFP derived from the copepod *Pontellina plumata (CD9-GFP)* was used to identify CD9-GFP-positive EVs in SVZ NSCs. Electroporation of CRE-recombinase into CD9-GFP mice resulted in expression of CD9-GFP in a subset of cells within the SVZ. Moreover, CD9-GFP expression was

detected in Nestin-positive cells in the SVZ. These results demonstrate the utility of CD9-GFP mice to study EVs *in vivo*. Future studies using CD9-GFP mice can be accomplished in a cell-type and time specific manner.

INTRODUCTION

Extracellular vesicles (EVs) are nanometer-sized vesicles that carry molecular cargo, such as miRNA, mRNA, and protein (Théry et al. 2006; Raposo and Stoorvogel 2013; Maas, Breakefield, and Weaver 2017; Morton and Feliciano 2016). Molecular cargo of EVs is dependent on its cell of origin. In glioma stem cells, exosome-associated miRNA was reflective of the cellular transcriptome (Wei et al. 2017). Furthermore, in a study regarding alcoholic hepatitis, inflammatory miRNAs found in exosomes isolated from rodent and human sera were enriched in alcohol-treated conditions (Momen-Heravi et al. 2015). Validation of exosome miRNA between species and treatment conditions demonstrated the potential use of exosomes as a diagnostic biomarker tool. Three types of EVs have been described: exomeres, exosomes, and microvesicles ((Théry, Zitvogel, and Amigorena 2002; Zhang et al. 2018; Maas, Breakefield, and Weaver 2017). Exomeres are comprised of metabolic, translational, and coagulation regulating proteins, and unlike other EVs, are not derived from the cell membrane (Zhang et al. 2018). Exosomes are generated when multivesicular bodies (MVBs) fuse with the cell membrane and release vesicles, called exosomes, into the extracellular space (Théry, Zitvogel, and Amigorena 2002; Maas, Breakefield, and Weaver 2017). Conversely, microvesicles are produced through the outward budding and

scission of the cell membrane (Raposo and Stoorvogel 2013; Maas, Breakefield, and Weaver 2017). EVs modulate intercellular communication through the transfer of RNA and proteins from donor to recipient cells (Cocucci and Meldolesi 2015; Théry, Zitvogel, and Amigorena 2002; Raposo and Stoorvogel 2013). For instance, EVs released from primary cultures of neonatal subventricular zone (SVZ) neural stem cells (NSCs) play an immuno-modulatory role in NSC-microglia communication (Morton et al. 2018). Furthermore, other studies have identified the release of particles during neurogenesis whose targets and functions are unknown (Das and Storey 2014; Dubreuil et al. 2007). EVs are found in most biological fluids including cerebrospinal fluid (CSF), but again, the sources, targets, and functions remain elusive (Feliciano et al. 2014; Tietje et al. 2014).

CD9 is a tetraspanin protein enriched in fractions of EVs (Raposo and Stoorvogel 2013). First discovered in hematopoietic cells derived from acute lymphoblastic leukemia, CD9 is one of the most commonly identified EVs marker proteins along with CD63 and CD81 (Kersey et al. 1981; Cocucci and Meldolesi 2015; Raposo and Stoorvogel 2013; Maas, Breakefield, and Weaver 2017; Théry, Zitvogel, and Amigorena 2002). It was later discovered that CD9 was released into the CSF of acute lymphoblastic leukemia patients (Komada et al. 1990). Of note, EV marker proteins are beneficial in identifying and studying EVs. However, EV marker proteins can distinguish similar and unique EV populations (Booth et al. 2006; Fang et al. 2007; Bobrie et al. 2012; Crescitelli et al. 2013). Interestingly, not all EVs are CD9-positive (Crescitelli et al. 2013). Similarly, through

immunohistochemistry, one study demonstrated the lack over overlap between CD9-positive and CD63-positive staining in cells (Bobrie et al. 2012). CD9 and CD63 are enriched in MVBs and are found throughout the secretory pathway including the cell membrane and lysosomes (Andreu and Yáñez-Mó 2014). Additional studies have also identified CD9 in EV fractions isolated from rodent and human CSF (Feliciano et al. 2014; Tietje et al. 2014). Studies involving the nervous system and nervous system development have identified the expression of CD9 in SVZ NSCs (Morton et al. 2018; Cossetti et al. 2014; Llorens-Bobadilla et al. 2015).

Fluorescently tagged EV marker proteins have historically been used to study EVs. CD9 fused to copepod GFP (CD9-GFP) has recently been used to label EVs *in vitro* and *in vivo* (Morton et al. 2018). In lower-order model systems, such as *Drosophila*, CD63-GFP has been used in studies of bone morphogenic protein (BMP), Hedgehog, Wingless, and Wnt signaling (Corrigan et al. 2014; Gross et al. 2012; Panáková et al. 2005). CD63-GFP has also been implicated in exosome studies in mammalian cells, including mouse, rat, and human cells (Yoshimura et al. 2016; Melo et al. 2015; Kosaka et al. 2013). One study reported the unidirectional transfer of miRNA from T cells to antigen presenting cells through exosomes tagged with CD63-GFP (Mittelbrunn et al. 2011). Transgenic model systems have also been implicated in the study of EVs and EV marker proteins. Early studies of transgenic CD9 mice discovered that knockout animals were infertile (Miyado et al. 2000). To investigate the role CD9 plays in reproduction,

researchers generated a CD9-eGFP mouse (Miyado et al. 2008). It was discovered that CD9-containing vesicles released from oocytes are required for proper egg-sperm fusion (Miyado et al. 2008). More recently tag-GFP from copepods was fused to human CD63 and used to create transgenic rats (Yoshimura et al. 2016). CD63-GFP expression is controlled via a constitutively activate CAG promoter. CD63-GFP was located within perinuclear regions within cells and could be detected in EVs fractions. Interestingly, transgenic animals were found to have shortened lifespans (4-6 months). To study CD63-GFP expression in neuronal cells, *CD63-GFP* was inserted downstream of a *Sox2* promoter (Yoshimura et al. 2018). *Sox2* driven CD63-GFP expression successfully labeled neuronal cells during embryonic development, and unlike CAG driven CD63-GFP, Sox2-CD63-GFP animals did not have shortened lifespans. These studies demonstrate the utility of transgenic animals to label and track EVs *in vivo* in a cell-type specific manner.

The SVZ is one of two neurogenic regions in the postnatal brain (Taverna, Götz, and Huttner 2014; Lim and Alvarez-Buylla 2016; Bjornsson et al. 2015). The SVZ is comprised of multiple cell types including neural-derived cells, such as astrocytes and ependyma, and non-neural-derived cells, such as microglia, endothelial cells, and pericytes (Bjornsson et al. 2015). Cells in the adult SVZ are arranged in a pinwheel structure surrounding NSCs (Mirzadeh et al. 2008). What developmental factors contribute to the generation of this architecture is only partially understood. Due to their abundance in the CSF, some studies point to

EVs as a source of neuro-developmental factors (Bátiz et al. 2016). Indeed, in a study conducted on rat embryonic CSF (eCSF) EVs, it was demonstrated that isolated eCSF EVs have a proliferative effect in mixed cultures of NPCs (Feliciano et al. 2014). Moreover, Coultier and colleagues demonstrated that mutations in the exosome biogenesis pathway resulted in microcephaly in patients via a reduction in Sonic Hedgehog (SHH) signaling (Coulter et al. 2018).

To study EV release from SVZ NSCs *in vivo*, a CRE-recombinase inducible transgenic mouse to label and track EVs was generated. CD9 was fused to the copepod GFP (CD9-GFP) and inserted downstream of a stop sequence flanked by loxP excision sites. The transgene backbone was modeled of off previous studies (Sasaki et al. 2006; Zambrowicz et al. 1997). 48 hrs following electroporation of CRE-recombinase into postnatal day 0 (P0) transgenic mice, CD9-GFP could be detected in Nestin-positive cells in the SVZ. This study demonstrates the potential of Nestin-positive SVZ NSCs to release EVs. Furthermore, the generation of this *in vivo* EV labeling system will allow for studies of EVs to be carried out in a spatial- and temporal-specific manner.

RESULTS

Generation and Validation of Transgenic CD9-GFP Mouse

To study EVs *in vivo,* a CRE inducible CD9-GFP plasmid using the backbone of a previously described targeting plasmid was generated (Sasaki et al. 2006; Zambrowicz et al. 1997) **(Fig. 1A).** Since cytomegalovirus (CMV) promoters

do not stimulate robust transcription of their downstream gene in the nervous system, CD9-GFP was placed into a plasmid containing a pCAGGs promoter used in the generation of transgenic mice (Gray et al. 2011). Previous reports have identified the utility of pCAGGs-CD9-GFP plasmids in studies of EVs (Morton et al. 2018). The new plasmid contains targeting arms for homologous recombination and insertion of CD9-GFP into the mouse Rosa26 locus and a neomycin resistance (NeoR) cassette for selection. Moreover, upstream of CD9-GFP is a stop sequence flanked by loxP sites. The configuration of the targeting plasmid therefore allows insertion of an inducible CD9-GFP that is activated by CRE mediated recombination.

In trial experiments, mouse neuroblastoma Neuro 2A (N2A) cells robustly express CD9-GFP when co-transfected with CAG-CRE (**Fig. 1B-C**). The mouse was generated through electroporation of the targeting plasmid into mouse embryonic stem cells (ESCs). Positive clones were screened for neomycin resistance. Long-range PCR was performed to validate proper insertion of the transgene into the Rosa26 locus. In-house long-range PCR was utilized to genotype CD9-GFP mice (**Fig. 1D-E)**. Transgenic mice containing CD9-GFP were crossed with inducible *Tomato*$^{+/+}$ mice to generate *CD9-GFP*$^{+/-}$*; Tomato*$^{+/-}$ mice. Tomato is only expressed with the addition of CRE-recombinase, similar to CD9-GFP. Thus, cells carrying CD9-GFP and Tomato should express both transgenes with the addition of CRE-recombinase.

To test whether *CD9-GFP*$^{+/-}$*; Tomato*$^{+/-}$ expression can be induced, CAG-CRE and was electroporated into the neonatal SVZ of P0 CD9-GFP mice. Within 48 hrs, CD9-GFP and Tomato expression was detected in cells surrounding the lateral ventricle (**Fig. 1F-H**)**,** CD9-GFP expression was surprisingly lower in abundance than anticipated. In contrast to Tomato expression, CD9-GFP expression could only be detected in a subset of Tomato positive (**Fig. 1F**) and Tomato negative cells (**Fig. 1G-H**). Taken together, these results indicate *CD9-GFP* mice were generated, and in the presence of CRE-recombinase, CD9-GFP could be detected in cells within the SVZ of postnatal mice.

CD9-GFP Labels Nestin+ cells in perinatal SVZ

Tissue collected from *CD9-GFP* mice electroporated with CAG-CRE were subjected to immunohistochemistry to study cell types expressing the transgene. It was found that CD9-GFP particles colocalized with Nestin-positive cells in the SVZ **(Fig. 2A)**. Given that CD9-GFP was localized to the apical side of the soma, contiguous with the lateral ventricle **(Fig. 2A)**, it is likely that SVZ NSCs release EVs containing CD9-GFP into the CSF of postnatal mice.

DISCUSSION

In the present study, we have reported use of a transgenic inducible system to label and track EVs *in vivo.* EVs are released from most, if not all, cell types (Raposo and Stoorvogel 2013; Théry, Zitvogel, and Amigorena 2002). Furthermore, their abundance in biological fluids, including CSF, blood, and urine,

renders difficulty for researchers when identifying their sources, targets, and functions. Historically, EVs have been isolated from media collected from *in vitro* cell cultures, labeled using lipophilic dyes, and then transplanted either *in vivo* or *in vitro*; however, these techniques are limited in that over-abundance of transplanted EVs may result in super-physiological responses (Morton, Neckles, and Feliciano 2018). Additionally, transfection and electroporation techniques have also been implemented to label and track EVs. However, due to plasmid dilution over time, these techniques make tracking labeled cells more challenging in lengthier studies (Lacar et al. 2010). Generating a genetic *in vivo* model to label and track EVs to circumvent issues observed with other labeling techniques will be paramount to future studies of EVs.

In the nervous system, many cells types release EVs during development and throughout life (Morton and Feliciano 2016). Recent studies have reported the release of EVs from primary cultures of SVZ NSCs in both adult and neonatal cultures (Cossetti et al. 2014; Morton et al. 2018), but evidence of *in vivo* release is lacking. In this study, a transgenic model system, in which *CD9-GFP* was inserted downstream of a stop sequence flanked by loxP sites and driven by a pCAGGs promoter, was used to identify EVs in SVZ NSCs. Studies in N2A cultures demonstrated that co-transfection of *CAG-CRE* and *CD9-GFP* DNA plasmids resulted in robust CD9-GFP expression. Transgenic CD9-GFP mice were crossed with CRE-recombinase inducible *Tomato*$^{+/+}$ to generate *CD9-GFP*$^{+/-}$*; Tomato*$^{+/-}$

mice. CAG-CRE was electroporated into P0 *CD9-GFP*$^{+/-}$*; Tomato*$^{+/-}$ mice. CD9-GFP was expressed in Nestin-positive SVZ NSCs.

Previous reports have used other EV marker proteins to label and track EVs *in vivo*. However, it is important to note that different EV marker proteins label different populations of EVs, thus the ability to label and track specific marker proteins may only provide insight into a small subset of EVs (Wei et al. 2017; Momen-Heravi et al. 2015). Recently, Yoshimira and colleagues generated a transgenic rat model in which CD63 was fused to copepod GFP and inserted downstream of a CAG promoter (Yoshimura et al. 2016). Unlike the mouse model system described here, the CD63-GFP is continuously expressed in the rat model from conception to adulthood. The constitutive expression has many pros and cons. For example, during embryonic development where outside perturbances, such as electricity or tamoxifen, can cause lethality, constitutive expression of the CD63-GFP transgene allows for EV detection without any threat to embryonic development. Although, CD63-GFP transgenic rats were shown to have a reduction in life expectancy. However, since CD63-GFP is expressed in all cell types during all stages of development and adulthood, sources, targets, and functions are difficult to decipher. To circumvent the ubiquitous expression of CD63-GFP, Yoshimira and colleagues published another manuscript in which CD63-GFP was expressed under the control of the *Sox2* promoter (Yoshimura et al. 2018). *Sox2* regulated expressed of CD63-GFP was used to label and track neuronal CD63-positive EVs during development. This approach demonstrated

specific expression of CD63-GFP and allowed for better tracking of fluorescently labeled EVs released from *Sox2* cell lineages during embryonic neurogenesis.

Conversely, the model described here offers the ability to temporally and spatially regulate the expression of the transgene, thus allowing for studies of individual cell types or cell populations during development. For instance, Nestin is a marker protein enriched in NSCs in the developing brain. To study the release of CD9-GFP from NSCs, transgenic mice carrying CRE-recombinase driven by a Nestin specific promotor (*Nestin-CRE*$^{+/+}$) could be crossed with *CD9-GFP*$^{+/+}$ mice. *Nestin-CRE*$^{+/-}$*; CD9-GFP*$^{+/-}$ mice would offer researchers spatial control over CD9-GFP expression. Additionally, to temporally regulate *CD9-GFP* expression, *Nestin-CRE-ER*^{T2} mice could be used. *ER*^{T2} is a modified estrogen receptor (Feil, Valtcheva, and Feil 2009). In the presence of Tamoxifen, the receptor dimerizes and translocates into the nucleus, and in the case of Nestin-CRE-ER^{T2}, the translocation of Cre-ER^{T2} into the nucleus would result in CD9-GFP expression in Nestin-expressing cells. The integration of Nestin-CRE-ER^{T2} would allow for a temporal regulation of CD9-GFP expression.

The growing understanding of EV biology is only limited by the tools available. As the understanding of processes and functions expands, scientific tools must do the same. It is our hope that with the generation of better tools and scientific collaboration, the scientific community will only continue to move forward.

METHODS

Mice

Research protocols were approved by the Clemson University Institutional Animal Care and Use Committee and NIH Guide for the Care and Use of Laboratory Animals. For neonatal electroporations, pregnant CD-1 were provided by Charles River Laboratories. Tomato mice (B6.Cg-Gt(ROSA)26Sortm9(CAG-td-Tomato) Hze/J; Stock No: 007909 Ai9) were obtained from Jackson Laboratories and crossed to CD9-GFP mice.

Cell Culture, Transfections and Immunocytochemistry

N2A mouse neuroblastoma cells (American Type Culture Collection CCL-131) were maintained in tissue culture-treated polystyrene multi-well plates or flasks (Falcon; BD Biosciences Discovery Labware) in a 37°C incubator with 5% CO_2. When N2A cells reached 80-90% confluence, they were passaged onto circular coverslips in six-well plates 24 hours prior to transfection. Cells were then transfected with PolyJet (SignaGen Laboratories) with DNA vectors (CD9-GFP and CAG-CRE) according to manufacturer's recommendations. 48 hours post-transfection, cells were fixed using 4% paraformaldehyde in 1x DMEM by replacing half of the media three consecutive times for 10 minutes. Coverslips were washed three times for 5 minutes in wash buffer (PBS, 0.1% Tween-20) and incubated with 1 nM TO-PRO-3 Iodide (Topro) in DMSO (Life Technologies) for 10 minutes. Coverslips were then washed in wash buffer five times for a total of 45 minutes

and then 1X PBS and mounted in ProLong Gold antifade reagent (Life Technologies) and imaged at described.

Postnatal Electroporations

Electroporations were performed on CD1 mice as previously described with minor modifications (Feliciano, Lafourcade, and Bordey 2013). Briefly, high purity DNA (OD 260/280 > 1.80) high concentration (1-10 µg/µl) DNA was prepared in sterile PBS and preloaded into a 10 cm fire polished borosilicate glass capillary tubes (O.D.: 1.5 mm, I.D.:1.10 mm) pulled with a Sutter Instrument Company Model P-97 glass pipette puller. Forceps were used to manually break tips prior to use. 10 µg/µL DNA solution containing *CAG-CRE* was combined with 0.1% weight/volume fast green solution and diluted in 1x dPBS. CD9-GFP Postnatal day 0-1 pups were placed onto a Petri dish and placed on wet ice. DNA mixture was loaded into the pulled glass and injected into the left lateral ventricle. Pups were electroporated with a BTX ECM 830 Square Wave Pulse generator and Tweezertrodes (Harvard Apparatus) using 5 square pulses, 50 msec/pulse at 100 volts, with 950 msec intervals. The negative electrode on the contralateral hemisphere ventral lateral to the pup's snout was used to direct DNA into the SVZ and electrodes were swept from dorsal to lateral positions using ~25° angle intervals. Pups were placed onto a heating pad for 5 min until recovered and placed back into cages.

Transgenic mice

Transgenic mice were generated through the assistance of the Yale Transgenic Core Facility and Charles River. Human CD9 fused to copepod GFP (CD9-GFP) was inserted into a modified version of pROSA26-1 containing *loxP*-flanked *neoR*-stop cassette as previously described (Sasaki et al. 2006). Mouse embryonic stem cells were electroporated, cultured and selected for using neomycin resistance. Long range PCR was used to identify positive clones for GFP and proper insertion into the Rosa26 locus. F1 mice were crossed with *Tomato*$^{+/+}$ mice ((B6.Cg-Gt(ROSA)26Sortm9(CAG-td-Tomato) Hze/ice) to generate double transgenic strains.

Slice Preparation and immunohistochemistry

Brains were fixed in 4% paraformaldehyde in 1X PBS for 4 hours at room temperature. Brains were rinsed overnight in 1X PBS at 4°C. 3% low-melt agar was used to mount brains for sectioning. Brains were sliced into 200 µm coronal sections on a Leica VTS1000 vibratome. Sections were incubated for 1 hour in blocking buffer (2% Bovine Serum Albumin, 0.1% Triton-X, 0.1% Tween20 and 1X PBS) at room temperature. Following blocking, sections were rinsed three times with wash buffer (0.1% Tween 20 and 1X PBS) at room temperature, and incubated with antibody solution (2% Bovine Serum Albumin, 0.1% Tween20 and 1X PBS) overnight at 4ºC along with Nestin (1:500, Novus, Catalog # NB100-1604) primary antibody. Following incubation, slices were washed in wash buffer three times for 5 min each time at room temperature. Sections were subsequently

incubated with secondary antibodies conjugated to Alexa Fluor-647 (1:500, Life Technologies) for 1 hr at room temperature. Sections were then rinsed in wash buffer five times for 10 min each at room temperature. Sections were mounted in ProLong Anti-fade mounting reagent (ThermoFischer Scientific).

Imaging

Images were taken on a Leica TCS SPE spectral confocal microscope using 63x oil immersion lense. Images were processed using Leica Application Suite X software (Leica Microsystems) to render confocal sections into 2D and 3D Z-stacks.

Long-range PCR

Long range PCR was performed according to manufacturer's instructions. In brief, toe snips were collected from transgenic mice. DNA was isolated using DNeasy Blood and Tissue kit (Qiagen, Catalog #69506) . Tissues were place in Buffer ATL and Proteinase K solution and incubated at 56ºC until tissues were completely digested. Buffer AL was added to the samples and vortexed for 15 s. Ethanol (95%) pre-chilled at -20ºC was added and the samples were again vortexed. Samples were transferred into a DNeasy Mini spin column with collection tube and centrifuged at 6,000 x g for 1 min. Flow through was discarded. Samples were washed with Buffer AW1 and again centrifuged for 1 min at 6,000 x g. Flow through was discarded. Samples were then washed with Buffer AW2 and centrifuged at

14,000 x g for 5min. Flow through was discarded. Buffer AE was added to DNeasy Mini spin column and incubated at room temperature for 5 min. Samples were centrifuged at 6,000 x g for 1 min. Samples were subjected to nanodrop technology to determine DNA concentrations.

Samples were then subjected to Long Range PCR. Using a Long Range PCR Kit a reaction mix was made as follows: 1x LongRange PCR Buffer with Mg^{2+}, 500 µM of 10 mM dNTP mix, 1x Q-solution, 0.4 µM forward and reverse LongRange primers (see Table 1.), 2 units per 50 µL reaction of LongRange PCR Enzyme Mix and nuclease free water to bring each reaction to 50 µL total volume (Qiagen, Catalog #206403). 1.5 µL of DNA sample was added to each reaction. Thermocycler protocol was programmed following manufacturer's instructions. 1.5% agarose gel was made using 1x TAE buffer. SYBR safe DNA gel stain (Thermo Fisher, Catalog #S33102) was added to get to visualize DNA. Samples were loading with BlueJuice gel loading buffer (Thermo Fisher, Catalog #10816015) and run at 104 V for 20 min.

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Figures:

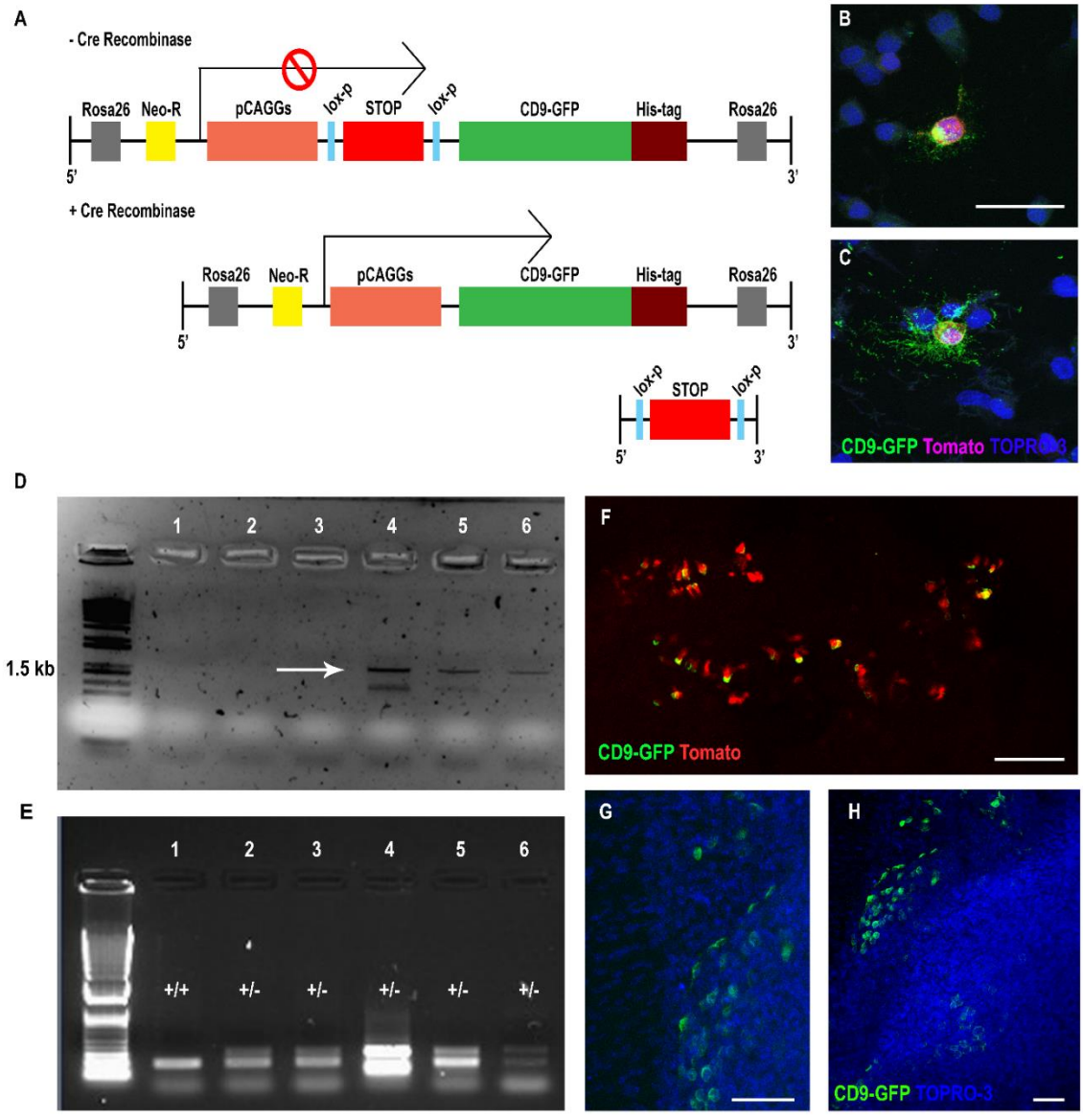

Figure 1. Generation of Cre-Inducible CD9-GFP Mice. A) Schematic diagram of Cre-inducible CD9-GFP targeting plasmid. B-C) Transfections of N2A cells with CD9-GFP (green), Tomato (red) and CAG-CRE. Nuclei were counterstained with TOPRO-3 (blue) Scale bar 10 µm. D) Long-rage PCR of ESC clones using a 5' end primers. Lanes 4-6 depict long-range PCR product expected in CD9-GFP positive

mice. Arrow = 1.5 kb. E) Genotyping PCR of *CD9-GFP*$^{+/-;}$ *Tomato*$^{+/-}$ mice. Wild type = 297 bp. Tomato heterozygote = 297 bp and 196 bp. Tomato homozygote = 196 bp. F) 20x image of brain section from *CD9-GFP*$^{+/-}$*;Tomato*$^{+/-}$ mice 48 hrs post-electroporation with CAG-CRE. CD9-GFP (green) is observed in a subset of Tomato-positive (red) cells. Scale bar 50 µm. G-H) *CD9-GFP*$^{+/-;}$ *Tomato*$^{+/-}$ mice 48 hrs after electroporation with CAG-CRE. CD9-GFP (green) is detected in perinuclear regions in cells within the SVZ. DNA is counterstained with TOPRO-3 (blue). Scale bar 50 µm.

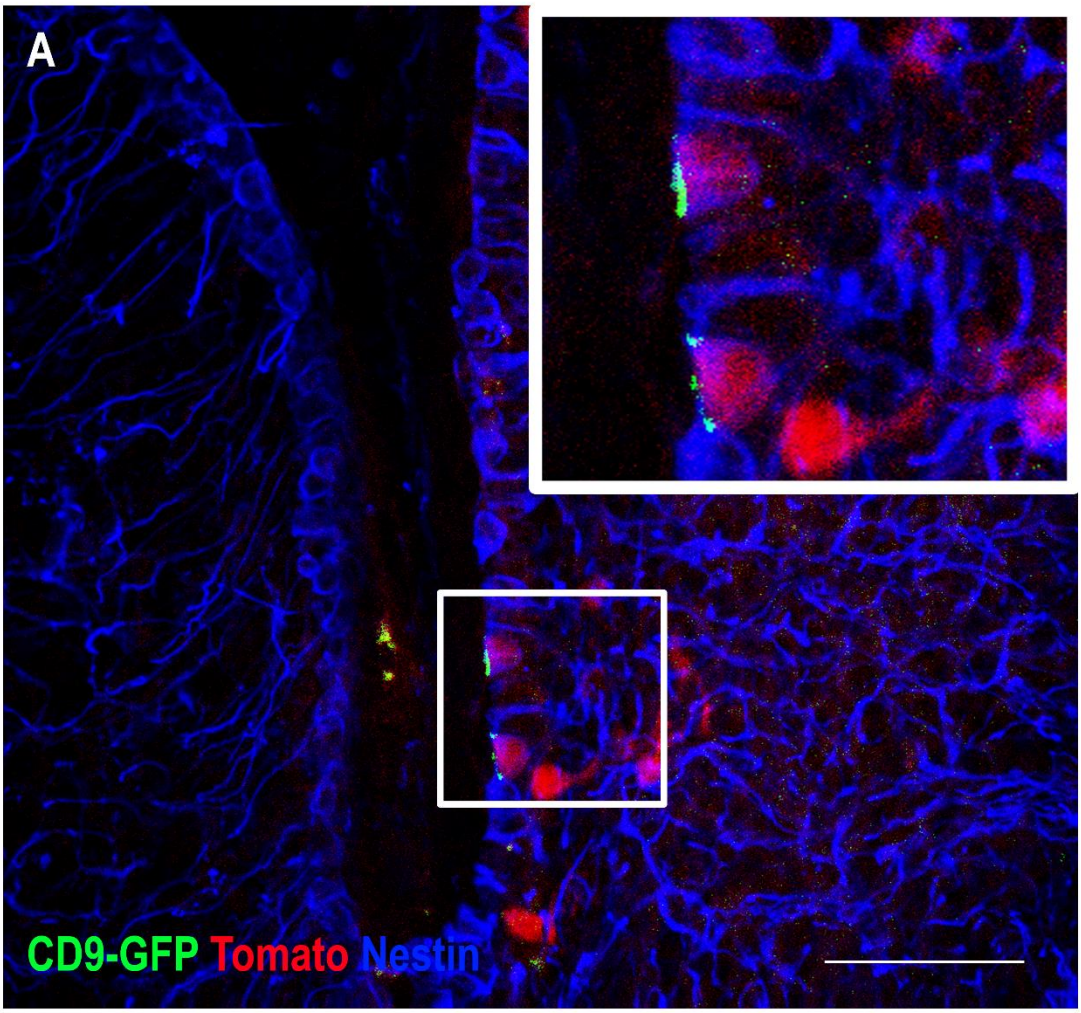

Figure 2. CD9-GFP Labels Nestin-positive cells in perinatal SVZ. A) CD9-GFP (green) particles are found within Nestin-positive (blue) and Tomato-positive (red) cells in the SVZ of postnatal day 2 (P2) mice following electroporation with CAG-CRE. Scale Bar 50µm.

TABLES:

PRIMER	SEQUENCE	Size
5' End	F = CGCCTAAAGAAGAGGCTGTG	1.5 kb
	R = TCATCAAGGAAACCCTGGAC	
3' End	F = AACAAGCACTGTCCTGTCCTCA	4.9 kb
	R = TAGTTGCCAGCCATCTGTTGTT	
Tomato	F = CTGTTCCTGTACGGCATGG	196 kb
	R = GGCATTAAAGCAGCGTATCC	
Wild Type	F = CCGAAAATCTGTGGGAAGTC	296 kb
	R = AAGGGAGCTGCAGTGGAGTA	

Table 1. Primers Used for Long Range PCR and Genotyping

CONCLUSION

Previous studies in our lab found the presence of extracellular vesicles (EVs) in the cerebrospinal fluid (CSF) of embryonic and adult mammals (Feliciano et al. 2014). EV abundance was found to under-go age-dependent declines, which suggested to us that EVs have both a developmental and homeostatic role (Tietje et al. 2014). Additionally, EVs isolated from the CSF of rodent embryos had a proliferative effect when used to treat neuroprogenitor cells *in vitro* (Feliciano et al. 2014). Moreover, many of the contents found within EVs isolated from CSF were found to be conserved between rodent and human embryos (Feliciano et al. 2014). These finding prompted us to ask what are the sources, targets, and functions of EVs. Due to the availability of tools to study neural stem cells (NSCs) both *in vivo* and *in vitro* and the lack of documentation of EV release from NSCs, I chose to focus this dissertation on NSCs. Subventricular zone (SVZ) NSCs line the lateral ventricle and interact with the CSF during early postnatal development. Thus, SVZ NSCs may be a source of CSF EVs. In Chapter 2, I developed a protocol to study the release of primary SVZ NSC EVs. Currently, there is no standard for EV isolation. Many studies have implored the use of ultracentrifugation to isolate EVs and a subsequent clean-up step to isolate exosomes. Due to issues with reproducibility (Livshits et al. 2015; Bobrie et al. 2012), ultracentrifugation alone was not sufficient to isolate and study EVs. Using a sucrose density gradient, I isolated exosomes from primary SVZ NSC media. Sucrose density gradients can cause vesicle rupture and EV fusion during (Taylor and Shah 2015; Théry et al.

2006; Linares et al. 2015), but are one of the most widely used methods for exosome isolation. Then, by labeling EVs or exosomes with the lipophilic dye, DiI, I was able to visualize transplanted vesicles in early postnatal brains. In Chapter 3, I implemented this protocol to study the effects of primary SVZ NSC EVs *in vivo*. DiI labeled cells were primarily Iba1-positive microglia. DiI labeled microglia were found to have a rounded morphology. Small RNA sequencing uncovered miRNA families, such as the Let-7 family, were enriched within primary SVZ NSC EVs. When transfected into miRNA-depleted EVs, Let-7 could induce the microglia morphological shift from a stellate to a rounded morphology. Moreover, microglia morphology was partially rescued when EVs were subjected to UV treatment prior to transplantation. NSC EV treated microglia also underwent a transcriptional change. This alteration coincided with a change in the cytokine profile of treated vs untreated microglia. Interestingly, when media condition by either treated or untreated microglia was injected into the lateral ventricles of neonatal mice, the EV-treated media caused a reduction in the number of dividing NSCs within the SVZ. These data demonstrate a negative feedback loop in which NSCs release EVs that are taken up by microglia in the SVZ. Microglia then undergo a transcriptional change which in turn alters their cytokine prolife. This change results in the reduction of dividing NSCs, although the mechanism by which this occurs has yet to be uncovered. Finally, in Chapter 4 we implore the use of a transgenic model system to study the production and released of EVs from SVZ NSCs *in vivo*. Through the postnatal electroporation of Cre-Recombinase, we

discovered that Nestin-positive cells lining the lateral ventricle co-localized with CD9-GFP-positive particles. Although it remains to be determined the frequency and abundance of EV release from SVZ NSCs, I propose that SVZ NSCs release EVs *in vivo*.

Early Postnatal SVZ NSCs Release Extracellular Vesicles

My focus while conducting these studies was to determine if neonatal SVZ NSCs release EVs. Previous reports have discovered the released of EVs from primary cultures of adult SVZ NSCs (Cossetti et al. 2014; Llorens-Bobadilla et al. 2015), but whether or not neonatal SVZ . Here we provide evidence to support the release of EVs from neonatal SVZ NSCs. Western blots of dorsal forebrain extracts showed an upregulation of tetraspanin CD9, an EV marker protein, beginning at postnatal day 2 (P2) and increasing exponentially into adulthood. Additionally, Nestin expressing cells in the SVZ stained positive for EV marker protein CD9. Electroporation of CD9-GFP into P0 SVZ NSCs showed a bipolar distribution in the apical and basal projecting processes in Nestin-positive cells 48 hrs later. GFP was also detected in perinuclear regions within the soma of Nestin-positive cells. In some cases, GFP was located outside of cells, suggestive of release. However, since SVZ NSCs generate multiple cells types in the SVZ, cortex, and olfactory bulbs, it was inconclusive as to which cell types released CD9-GFP positive particles. Therefore, I dissected and placed SVZ NSCs in culture. Approximately 92% of cells in the primary cultures stained positive for Nestin. Endogenous CD9 was detected in Nestin-positive cells exhibiting a bipolar morphology, similar to *in*

vivo phenotypes. Western blot confirmed the presence of Nestin-positive cells in primary SVZ cultures. Culture media was subjected to EV isolation via ultracentrifugation. EV pellets produced following an 18 hr centrifugation at 100,000 x g were subjected to western blotting and electron microscopy. CD63 was detected in both primary SVZ NSC cell lysates and isolated EVs. Additionally, EVs were enriched for CD9 and Alix when compared to cell lysates. Electron microscopy images showed the presence of multiple types of EVs, including microvesicles, exosomes, and exomeres, within the isolated EV pellet. Finally, since primary SVZ NSC cultures were not purely NSCs, further validation of *in vivo* EV release was required. Transgenic mice expressing CD9-GFP under control of Cre-mediated recombination of *loxP* sites flanking a stop codon were electroporated with CAG-CRE. CD9-GFP was detected in Nestin-positive cells exhibiting an SVZ NSC morphology in P2 brain sections. Furthermore, CD9-GFP was predominately localized to the apical surface which directly interfaces the CSF. Taken together, these results suggest that SVZ NSCs release EVs.

Final Concluding Thoughts

The field of EV biology has rapidly expanded over the last decade, and while many studies have led to profound discoveries, there is still much left to uncover. Specifically during nervous system development, studies have demonstrated the importance of factors within the CSF to control NSC proliferation (Lehtinen et al. 2011). When added to cultures of neural progenitor cells, EVs isolated from rodent CSF had a proliferative effect (Feliciano et al. 2014). Other studies have identified

the generation of particles during neurogenesis with unknown developmental implications (Das and Storey 2014; Dubreuil et al. 2007). Recently it was discovered that genetic loss of exosome release resulted in microcephaly in patients, suggesting that EVs play an important neurodevelopmental role (Coulter et al. 2018).

My dissertation focuses on the release of EVs from neonatal SVZ NSCs (Morton, Neckles, and Feliciano 2018; Morton et al. 2018). We demonstrated the regulatory effects of SVZ NSC EVs on microglia. While the results are provocative, it is important to state that transplantation experiments involving EVs raises the concentration of EVs exponentially. Therefore, the data obtained may only offer insight into the super-physiological response of microglia to SVZ NSC EVs. However, the data does suggest a developmental role in which SVZ NSC EVs act as a microglia morphogen within the SVZ. The early migration of microglia to the neonatal SVZ might then be a result of EVs released from SVZ NSCs. Upon arrival into the SVZ, microglia possibly regulate SVZ NSC proliferation and allow for the sufficient production of olfactory bulb neurons and other NSC-derived cell types. These data support the release of EVs from SVZ NSCs *in vitro.* Using our transgenic model system, we induced CD9-GFP expression in neonatal SVZ NSCs through electroporation of Cre recombinase. We found a low number of SVZ NSCs that expressed CD9-GFP. Of the cells expressing CD9-GFP, it was discovered that CD9-GFP localized to the apical side of Nestin-positive cells in the SVZ. It was surprising to see such a low number of NSCs expressing CD9-GFP;

however, this could simply be due to inefficient incorporation of the *CD9-GFP* transgene into the Rosa26 locus. Since the *Tomato* transgene also occupies the Rosa26 locus, there is the possibility that only one transgene is expressed in one cell at any given time. The likelihood of both *Tomato* and *CD9-GFP* expression, although possible, is very low. Since experiments were conducted on *Tomato*$^{+/-}$*;CD9-GFP* mice, it would be important to re-visit this issue with a *CD9-GFP*-positive *Tomato*$^{-/-}$ animal. Finally, while the generation of this transgenic model system that allows for the tracking of EVs *in vivo*, it is important to note that not all EVs carry CD9 and thus CD9-GFP will not label all EVs. Other model systems utilizing other EV marker proteins will be required to study all populations of EVs *in vivo*.

My dissertation demonstrates the release of EVs from SVZ NSCs and uncovers a neuro-developmental role for these nanometer sized messengers. Future studies will be required to further validate these findings *in vivo*. Knock-down and over-expression experiments will be needed to prove the necessity and sufficiency of NSC EV release and uptake *in vivo*. While the field of EV biology is relatively new, it has already altered our understanding of intercellular communication. Further advances to scientific tools will allow for more precise and in-depth characterization of these lucrative vesicles.

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