NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

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BSc in Biochemistry

PARACRINE REGULATION OF THE NEUROGENIC NICHE BY NEURAL STEM CELLS

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

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“Don’t aim for perfection. Evolution, and life, only happen through mistakes.”

(Matt Haig, in “The Humans”).

ABSTRACT

Studies on neural stem cells (NSCs) largely focus on their differentiation into neurons in the adult brain, a process known as adult neurogenesis. However, new evidence suggests NSCs also regulate their surroundings through paracrine signaling. Their location in the brain, close to the vasculature and the cerebrospinal fluid, places them in an ideal position to receive extrinsic cues from the environment and relay them to the members of the neurogenic niches. Here, we aimed to explore how metabolic regulators and insults influence the NSC conditioned media (CM) and its effects on three components of the niches: differentiating NSCs, microglia, and endothelial cells.

NSCs were pre-conditioned with metabolic regulators—tauroursodeoxycholic acid, propionate, and an exercise-mimicking cocktail of growth factors—and the resulting secretomes (mitCMs) seemed to increase neuronal differentiation. NSCs pre-conditioned with CM from injured cells released a secretome (boosted CM) which seemed to promote neuronal differentiation and microglial phagocytosis. Contrastingly, the secretome of NSCs pre-conditioned with serum of depressed mice (uCMS CM) decreased neuronal differentiation and microglial phagocytosis. No alterations in blood vessel formation by endothelial cells exposed to these CMs were detected. To understand how CMs trigger these changes, we analyzed their composition. MitCMs and boosted CM seemed to be enriched in metabolites involved in neuroprotection and metabolic remodeling, such as lactate and 3-methyl-2-oxovalerate. Boosted CM also appeared to be enriched in miRNAs involved in neurogenesis and microglial modulation, such as miRNA-21, miRNA-125b, and miRNA-424.

Summarily, our findings highlight a new role for the paracrine regulation of the neurogenic niche by NSCs. In the context of increased metabolic activity, but also of injury, NSCs appear to release a neuroprotective secretome, modulating differentiating NSCs and microglia towards a neurogenesis-favoring phenotype. However, depression-associated factors induce the release of a neurogenesis-suppressing secretome, as it appears to decrease neuronal differentiation and microglial phagocytosis.

Keywords: Neural stem cells; Neurogenesis; Neurogenic niches; Paracrine regulation; Secretome

Resumo

A neurogénese adulta, em que células estaminais neurais (NSCs) se diferenciam em neurónios, é um processo fundamental para o funcionamento adequado do cérebro. Novos estudos sugerem que, para além da sua capacidade de diferenciação neuronal, as NSCs conseguem regular a sua vizinhança através de sinalização parácrina. A sua localização no cérebro, perto da vasculatura e do líquido cefalorraquidiano, é ideal para a receção de sinais sistémicos. Neste trabalho, procurámos explorar o impacto de reguladores metabólicos e insultos no meio condicionado (CM) de NSCs e o seu efeito em três componentes dos nichos neurogénicos: NSCs em diferenciação, microglia, e células endoteliais.

As NSCs foram pré-condicionadas com reguladores metabólicos—ácido tauroursodesoxicólico, propionato, e um *cocktail* de fatores de crescimento elevados durante o exercício—e os resultantes secretomas (mitCMs) pareceram aumentar a diferenciação neuronal. NSCs pré-condicionadas com o CM de células danificadas libertaram um secretoma (boosted CM) que aparentou promover diferenciação neuronal e a capacidade fagocítica da microglia, enquanto que o secretoma de NSCs pré-condicionadas com soro de murganhos deprimidos (uCMS CM) teve o efeito oposto. Não foram detetadas alterações na formação de vasos sanguíneos por células endoteliais expostas a estes CMs. Para clarificar potenciais mecanismos de ação, a composição dos CMs foi analisada. Foram detetados níveis aparentemente elevados de metabolitos neuroprotetores e envolvidos em remodelação metabólica (lactato e 3-metil-2-oxovalerato) nos mitCMs e boosted CM. Adicionalmente, foram detetados níveis elevados de miRNAs envolvidos na neurogénese e modulação de microglia (miRNA-21, miRNA-125b, miRNA-424) no boosted CM.

Em resumo, estes resultados realçam um novo papel para as NSCs na regulação parácrina dos nichos neurogénicos. Em situações de maior atividade metabólica, mas também de dano, as NSCs aparentam libertar um secretoma neuroregenerativo, promovendo diferenciação neuronal e fagocitose por microglia. Por outro lado, sinais no soro de murganhos deprimidos parecem suprimir as propriedades neuroregenerativas do secretoma.

Palavas chave: Células estaminais neurais; Neurogénese; Nichos neurogénicos; Regulação parácrina; Secretoma

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ABBREVIATIONS

6-OHDA	6-Hydroxydopamine.
ASCL1	Achaete-scute homolog 1.
ATP	Adenosine 5'-triphosphate.
BDNF	Brain-derived neurotrophic factor.
bFGF	Basic fibroblast growth factor.
BrdU	Bromodeoxyuridine.
cAMP	Cyclic adenosine monophosphate.
CM	Conditioned media.
CoA	Coenzyme A.
CPT1	Carnitine palmitoyltransferase 1.
CR	Calretinin.
CSF	Cerebrospinal fluid.
DCX	Doublecortin.
DNA	Deoxyribonucleic acid.
Drp1	Dynamin-related protein 1.
EGF	Epidermal growth factor.
FAO	Fatty acid oxidation.
FASN	Fatty acid synthase.
GC-MS	Gas chromatography–mass spectrometry.
GFAP	Glial fibrillary acidic protein.
GFP	Green fluorescent protein.
GPR	G protein-coupled receptor.
HPA	Hypothalamic-pituitary-adrenal.
HUVEC	Human umbilical vein endothelial cells.
IGF-1	Insulin-like growth factor 1.
IGFBP	Insulin-like growth factor-binding protein.
iPSC	Induced pluripotent stem cells.
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry.
MFGE8	Milk fat globule epidermal growth factor 8.
Mfn1/2	Mitofusin 1/2.
miRNA	Micro RNA.

MPP+	1-Methyl-4-phenylpyridinium.
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
NeuN	Neuronal nuclear protein.
NO	Nitric oxide.
NSC	Neural stem cell.
NSE	Neuron-specific enolase.
NS-TGFP	Tau-GFP mouse NSC cell line.
OB	Olfactory bulb.
Opa1	Optic atrophy 1.
OXPHOS	Oxidative phosphorylation.
p-CREB	Phosphorylated cAMP-response element binding protein.
PSA-NCAM	Polysialylated-neural cell adhesion molecule.
RMS	Rostral migratory stream.
RNA	Ribonucleic acid.
ROI	Region of interest.
ROS	Reactive oxygen species.
SCCI	Single-cell calcium imaging.
SDNSF	Stem cell-derived neural stem/progenitor cell supporting factor.
SEM	Standard error of the mean.
SGZ	Subgranular zone.
STAT3	Signal transducer and activator of transcription 3.
SVZ	Subventricular zone.
TA	Transit-amplifying.
TUDCA	Tauroursodeoxycholic acid.
uCMS	Unpredictable chronic mild stress.
VEGF	Vascular endothelial growth factor.
WHO	World health organization.

1

INTRODUCTION

1.1 Neural Stem Cells

1.1.1 Introduction to Stem Cells

Most cells in the human body are highly specialized: adipocytes store energy, lymphocytes protect against foreign invaders, neurons transmit information, and so on (Alberts, Bruce; Heald, Rebecca; Johnson, Alexander; Morgan, David; Raff, Martin; Roberts, K; Walter, 2022). But the body also retains pools of unspecialized cells—the stem cells (Zakrzewski, Dobrzyński, Szymonowicz, & Rybak, 2019). They are classically defined as undifferentiated cells which can self-renew and differentiate into specialized cells, and they exist across many tissues and stages of development (Kolios & Moodley, 2013).

Stem cells have varying potentials to differentiate into specialized cells (Alison, Poulsom, Forbes, & Wright, 2002). Totipotent cells have the highest potency, as they can generate embryonic and extra-embryonic tissues (Alison et al., 2002). Pluripotent cells can differentiate into any cell belonging to embryonic tissues (Alison et al., 2002). Multipotent cells have a more restricted potential, as they can only differentiate into cell types belonging to the same lineage (Alison et al., 2002). Finally, unipotent cells lie at the bottom of potency: they only differentiate into one cell type (Alison et al., 2002). According to this classification, the human body arises from a totipotent cell (Zakrzewski et al., 2019). When a sperm fuses with an egg, it generates a cell capable of forming embryonic and extra-embryonic tissues: the zygote (Gerri, Menchero, Mahadevaiah, Turner, & Niakan, 2020). After approximately four days and multiple cell divisions, the zygote transforms into a structure called the blastocyst (Gerri et al., 2020). The inner cell mass of the blastocyst becomes pluripotent, losing the ability to differentiate into extra-embryonic tissues (Zakrzewski et al., 2019). These embryonic stem cells generate the three germ layers—endoderm, mesoderm, and ectoderm—from which all tissues and organs develop (Kolios & Moodley, 2013).

The adult body retains some stem cells in most organs, with the notable exception of the heart (Alison et al., 2002). In comparison with their embryonic counterparts, adult stem cells generally have

lower potential; most are multipotent and can only differentiate into cells belonging to the organ where they reside (Dulak, Szade, Szade, Nowak, & Józkowicz, 2015). They are instrumental in maintaining tissue homeostasis and regeneration following injury, providing a steady supply of healthy new cells (Dulak et al., 2015). While they can proliferate and generate differentiated cells, adult stem cells often remain in a non-proliferative or "quiescent" state (Morales & Mira, 2019). Quiescence is a defining feature of adult stem cells, key in preserving their function and identity. In this state, cells are arrested either in the G0 or G2 phase of the cell cycle; this arrest is reversible, meaning that quiescent cells can re-enter the cell cycle and begin proliferating (Urbán, Blomfield, & Guillemot, 2019). This low-activity state has multiple advantages: it prevents unnecessary consumption of resources, protects the cells' proliferation potential, and prevents damage to DNA, proteins, and organelles (Urbán et al., 2019). As a result, quiescent adult stem cells can last through long periods of time, ensuring homeostasis and regeneration throughout adulthood (Morales & Mira, 2019).

Upon activation, stem cells must self-renew; otherwise, the stem cell pool would quickly become depleted and the tissue would lose its regenerative potential (Alison et al., 2002). There are two forms of stem cell division that ensure self-renewal: asymmetric and symmetric (Shenghui, Nakada, & Morrison, 2009). Asymmetric divisions result in one replacement stem cell and one transit-amplifying cell, which further proliferates and differentiates (Shenghui et al., 2009). Symmetric divisions result in either two replacement stem cells or two transit-amplifying cells, and for each stem cell undergoing symmetric division to transit-amplifying cells, another divides into replacement stem cells (Shenghui et al., 2009). The balance between stem cell quiescence and proliferation is very delicate: if proliferation is insufficient, regeneration is compromised; if proliferation is excessive, tumours can develop (Alison et al., 2002).

Stem cells' characteristics make them invaluable throughout a vast range of biological and therapeutic applications (Zakrzewski et al., 2019). Embryonic stem cells in culture are often used to study the early stages of human development, and adult stem cells can be used to replicate tissues and organs *in vitro* (Bai, 2020; Drubin & Hyman, 2017). Furthermore, their ability to proliferate and differentiate into specialized cell types makes them very attractive for regenerative medicine (Mahla, 2016). Currently, over 6,000 clinical trials involving stem cells are registered in the WHO International Clinical Trials Registry, studying the therapeutic viability of using stem cells in the treatment of a wide range of diseases, from diabetes to neurodegenerative disorders. Since embryonic stem cells are associated with ethical controversies due to their origin, efforts have been made to find viable alternatives (de Miguel-Beriain, 2015). In this regard, the generation of induced pluripotent stem cells (iPSCs) represented a great breakthrough (Takahashi et al., 2007). By exposing differentiated cells to a cocktail of transcription factors—the "Yamanaka factors"—scientists were able to dedifferentiate them, regaining pluripotency (Takahashi et al., 2007). Since iPSCs can be generated from adult differentiated cells, they avert the ethical concerns of embryonic stem cells, while retaining their pluripotency (de Miguel-Beriain, 2015). Furthermore, they are very promising in the field of personalized therapy: a patient's own cells can be used to generate iPSCs, avoiding issues of immune incompatibility during transplantation (Chun, Byun, & Lee, 2011).

1.1.2 Introduction to Neural Stem Cells

During the early stages of stem cell research, the scientific community believed stem cells resided only in the most plastic tissues, such as the skin (Gage & Temple, 2013). Therefore, the discovery of neural stem cells (NSCs) in the adult mammalian brain by Altman, Smart, and Leblond in the 1960s was met with widespread scepticism (Altman, 1963; Gage & Temple, 2013; Smart & Leblond, 1961). Yet, in the following years several studies confirmed their findings in different animal models, and today the existence of NSCs in the mammalian adult brain is generally accepted (Gage & Temple, 2013). The discovery of adult NSCs was also the first challenge to the notion of the "post-mitotic brain", which postulated that no new neurons are added to the mammalian brain during adulthood (Ramon y Cajal, 1928; Temple, 2001).

NSCs are a subset of stem cells residing in the nervous system, capable of self-renewing and generating neurons, astrocytes, and oligodendrocytes (Temple, 1989). During embryonic development, the population of NSCs is called radial glia, due to their long radial processes and astrocyte-like morphology (Barry, Pakan, & McDermott, 2014). Radial glial cells proliferate during embryogenesis, forming the central nervous system (X. Zhao & Moore, 2018). In a first wave, radial glial cells proliferate and differentiate into neurons—a process known as neurogenesis (Okano & Temple, 2009). After the first wave of neurogenesis, radial glia generate astrocytes and oligodendrocytes (Okano & Temple, 2009). Adult NSCs aren't mere leftovers of developmental radial glia (Morales & Mira, 2019). They are set aside specifically and have important differences, namely a longer cell cycle, quiescence, and a different transcriptomic program (Morales & Mira, 2019). The tripotency of adult NSCs *in vivo* has been raised into question in recent years, as new evidence suggests that they are a heterogenous population and specific subsets are responsible for generating either neurons, astrocytes, or oligodendrocytes (Obernier & Alvarez-Buylla, 2019).

1.1.3 The Neurogenic Niches

Given their importance and special requirements, stem cells reside in dynamic specialized microenvironments that support their activity: the stem cell niches (Singh, Yadav, Tabassum, Bajpeyee, & Verma, 2019). Niches are constituted by a specific population of heterogenous cells, a specialized extracellular matrix, and multiple signalling molecules, all of which work in tandem to ensure proper stem cell behaviour (Ferraro, Celso, & Scadden, 2010). Stem cells continuously interact with the various members of the niche, which regulate stemness and differentiation (Singh et al., 2019).

In the adult mammalian brain, pools of NSCs exist in at least two regions: the subventricular zone of the lateral ventricles and the subgranular zone in the hippocampal dentate gyrus (**Figure 1.1**) (Andreotti et al., 2019). These neurogenic niches contain different populations of NSCs but share some important features (Fuentealba, Obernier, & Alvarez-Buylla, 2012). Both niches are more vascularized than other regions of the brain, the vasculature is more permeable, and the blood flow is slower (Andreotti et al., 2019). These characteristics increase the contact between adult NSCs and blood-derived signals, suggesting that systemic signalling can be important in regulating their behaviour and fate.

Furthermore, much like the rest of the brain, the neurogenic niches are rich in astrocytes (Schneider, Karpf, & Beckervordersandforth, 2019). Accumulating data show that astrocytes are powerful regulators of NSCs and their progeny, through the release of growth factors and cytokines (Schneider et al., 2019).

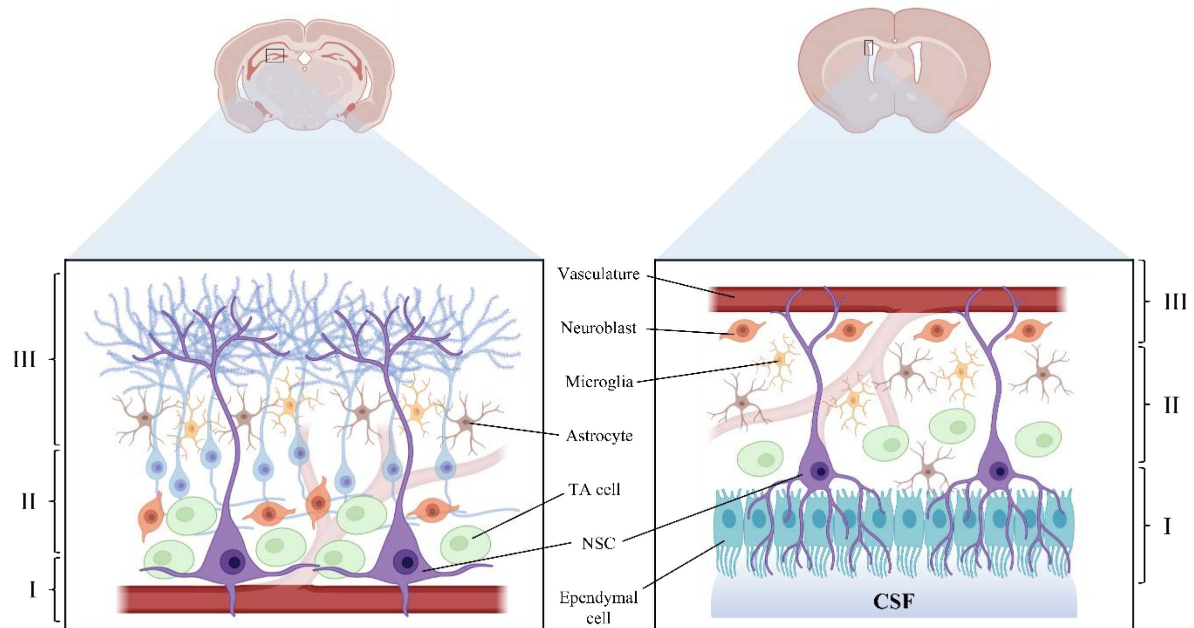

Figure 1.1 – The adult mammalian neurogenic niches. (**A**) Cellular composition of the SGZ niche. In the proximal domain I, NSCs contact with the vasculature and neighbouring NSCs. In the intermediate domain II, NSCs contact with their progeny and mature neurons. In the distal domain III, NSCs extend a highly branched arborization, interacting with neurons and glial cells. (**B**) Cellular composition of the SVZ niche. In the proximal domain I, NSCs contact with the CSF and ependymal cells. In the intermediate domain II, NSCs contact with glial cells, neurons, and their progeny. In the distal domain, NSCs contact with the vasculature. (Created with BioRender.com). CSF, cerebrospinal fluid; NSC, neural stem cell; SGZ, subgranular zone; SVZ, subventricular zone; TA, transit-amplifying.

The main subpopulation of NSCs in the subventricular zone are Type B cells (Bjornsson, Apostolopoulou, Tian, & Temple, 2015). They are uniquely positioned to receive systemic signals, bound on one side by the surface of the cerebrospinal fluid-filled ventricles and on the other by a planar vascular plexus, extending processes that contact directly with the cerebrospinal fluid and the vasculature (Mirzadeh, Merkle, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2008). Both the cerebrospinal fluid and blood are reservoirs of signalling molecules—growth factors, hormones, metabolites—that can influence the members of the niche, according to the body's physiological or pathological state (Andreotti et al., 2019). Type B cells span three domains in the subventricular zone: proximal, intermediate, and distal. In the proximal domain, B cells extend a primary cilium surrounded by ependymal cells in a "pinwheel-like" structure, contacting with the cerebrospinal fluid (Mirzadeh et al., 2008); in this domain, soluble factors from the cerebrospinal fluid and signalling molecules from neighbouring

ependymal cells regulate NSC behaviour (Buddensiek et al., 2010; Lim et al., 2000; Ramírez-Castillejo et al., 2006). In the intermediate domain, B cells interact with their progeny, glial cells, and neuronal terminals, all of which support and regulate NSC activity (Baker, Baker, & Hagg, 2004; Fuentealba et al., 2012; Walton et al., 2006). Curiously, despite lacking synapses, NSCs present receptors to various neurotransmitters, indicating that neuronal activity may affect NSC behaviour (Berg, Belnoue, Song, & Simon, 2013). Finally, in the distal domain, B cells extend a long basal process ending in a specialized end-food that contacts with the vasculature (Q. Shen et al., 2008). Soluble factors from the blood and signalling molecules from the endothelial cells that line blood vessels regulate NSCs in this domain (Lacar, Herman, Hartman, Hyder, & Bordey, 2012; Q. Shen et al., 2004).

The main subpopulation of NSCs in the subgranular zone—a thin band located between the hilus of the dentate gyrus and the granular zone of the hippocampus—are Type 1 cells (Fuentealba et al., 2012). In this niche, Type 1 cells contact with the vasculature, but not with the cerebrospinal fluid (Palmer, Willhoite, & Gage, 2000). Much like the subventricular zone, this niche may be divided into three domains: proximal, intermediate, and distal (Fuentealba et al., 2012). In the proximal domain, Type 1 cells contact with blood vessels through a primary cilium and with neighbouring Type 1 cells through lateral processes (Fuentealba et al., 2012). Within this domain, soluble factors from the blood, as well as signalling molecules from endothelial cells and neighbouring NSCs act on Type 1 cells (Q. Shen et al., 2004; H. Toda et al., 2003). The cell body resides in the intermediate domain, where a main shaft traverses the granule cell layer, contacting transit-amplifying NSCs and mature granule neurons (Seri, García-Verdugo, Collado-Morente, McEwen, & Alvarez-Buylla, 2004). Type 1 cells' progeny remains closer to the origin than Type B cells' progeny, suggesting that progeny-mediated regulation of NSCs may play a more important role in the subgranular zone (Fuentealba et al., 2012). Finally, the distal domain is highly branched and contacts with neuronal processes, synapses, astrocytes, and microglia of the inner molecular layer, all of which have been shown to regulate NSC behaviour (Fuentealba et al., 2012; Sierra et al., 2010; Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005).

Recent studies have found evidence of adult NSCs in other regions, such as the hypothalamus, striatum, amygdala, *substantia nigra*, and cortex (Bernier, Bedard, Vinet, Levesque, & Parent, 2002; Evans et al., 2002; Magavi, Leavitt, & Macklis, 2000; Parent, Cicchetti, & Beach, 1995; M. Zhao et al., 2003). While critics suggest that the presence of dividing cells in these regions is merely a result of NSCs migrating from the classic neurogenic niches, these findings indicate that the presence of NSCs in the adult brain might be more widespread than previously thought (Jurkowski et al., 2020).

1.1.4 NSC Metabolism

Cellular metabolism is the source of energy and anabolic precursors of all cells, and it plays a big role in their identity, fate, and survival (Zhu & Thompson, 2019). Since stem cells possess unique features, such as long-term quiescence and self-renewal capacity, they require a distinct metabolic program (Shyh-Chang, Daley, & Cantley, 2013). Rather than being a passive response to the cells' energetic requirements, accumulating evidence suggests metabolism actively drives stem cell fate (Ly, Lynch, & Ryall, 2020).

The primary sources of ATP—the “energy currency” of cells—are glycolysis and oxidative phosphorylation (Ly et al., 2020). Glycolysis is a rapid process that converts glucose into two molecules of pyruvate in a series of stepwise reactions yielding two molecules of ATP. Pyruvate is subsequently converted to lactate and secreted by cells, to prevent intracellular acidification. Alternatively, in the presence of sufficient oxygen, pyruvate can fuel oxidative phosphorylation. Pyruvate is converted into acetyl-CoA (may also be derived from fatty acid oxidation), which enters the tricarboxylic acid pathway in the mitochondria. This cyclic reaction results in the decarboxylation of acetyl-CoA and production of the high-energy electron carriers $NADH/H^+$ and $FADH_2$, as well as CO_2. Then, the electron transport chain, a series of protein complexes inserted into the inner mitochondrial membrane, uses $NADH/H^+$ and $FADH_2$ to fuel a chemiosmotic proton gradient to produce ATP. The metabolism of sugars through oxidative phosphorylation can generate up to fifteen times more ATP than glycolysis, but it also generates more reactive oxygen species (ROS) as by-products. Normally, molecular oxygen acts as the electron acceptor of the electron transport chain, being reduced to H_2O. However, a small fraction of the oxygen is reduced partially, generating ROS: the superoxide anion ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2) (Brand, 2016). These molecules, due to the presence of unstable electrons, are highly reactive and capable of interacting with most biomolecules and cell structures, often damaging them and compromising their function (Krumova & Cosa, 2016).

Quiescent adult stem cells produce energy mainly through glycolysis, since they have lower energy requirements and oxidative phosphorylation increases ROS, which can damage DNA and compromise their long-term survival and function (Shyh-Chang et al., 2013; Suda, Takubo, & Semenza, 2011). Paradoxically, highly proliferating cells, including activated NSCs, also rely primarily on glycolysis (Candelario, Shuttleworth, & Cunningham, 2013; Ly et al., 2020). Despite its low energy yield, studies have shown that when glucose is abundant, glycolytic ATP production rates can surpass oxidative phosphorylation (Tsogtbaatar, Landin, Minter-Dykhouse, & Folmes, 2020). Furthermore, glycolysis is interlinked with various side pathways, which use intermediate glycolytic metabolites to generate biomass through alternative carbon routing (Lunt & Vander Heiden, 2011). The pentose phosphate pathway uses glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate to generate precursors for the biosynthesis of nucleotides and amino acids (Stincone et al., 2015). The serine synthesis pathway uses 3-phosphoglycerate to generate serine, which can be further used to synthesize membrane lipids, nucleotides, and the antioxidant glutathione (M. Yang & Vousden, 2016). A steady supply of these anabolic precursors helps build new membranes, strands of DNA, and proteins, facilitating proliferation (Ly et al., 2020).

Due to the increased efficiency of oxidative phosphorylation, differentiated cells often prefer it as their main source of energy (Ly et al., 2020). Accordingly, NSCs undergo a “metabolic shift” during differentiation into neurons, from glycolysis to oxidative phosphorylation (**Figure 1.2**) (Zheng et al., 2016). This change isn’t just a passive response to the cells’ new metabolic needs: the metabolic shift towards oxidative phosphorylation drives stem cell differentiation (Shyh-Chang & Ng, 2017). As previously mentioned, oxidative phosphorylation increases ROS production. Despite their classical representation as nefarious agents, studies show that, in small concentrations, they play an important role in

cellular signalling (D'Autréaux & Toledano, 2007). In NSCs, ROS activate a nuclear transcriptional programme that promotes differentiation (Khacho, Harris, & Slack, 2019). Accordingly, deleting FOXO genes—part of the cell's antioxidant system—leads to excessive ROS levels, causing uncontrolled NSC proliferation and differentiation (Renault et al., 2009). The tricarboxylic acid pathway is also an important source of intermediate metabolites that regulate stem cell fate through epigenetic modifications (Ly et al., 2020). Acetyl-CoA can donate its acetyl group to lysine residues on the tails of histones, a family of proteins which associate with DNA, helping condensate it into chromatin (Wellen et al., 2009). Histone acetylation generally enhances chromatic accessibility, thereby increasing gene transcription (Struhl, 1998). Curiously, α-ketoglutarate promotes DNA and histone demethylation (Okabe et al., 2020; Qiyuan Yang et al., 2016). DNA demethylation generally increases gene transcription (Wolffe, Jones, & Wade, 1999), whereas histone demethylation can increase it or decrease it (Jenuwein & Allis, 2001). While the role of epigenetic modification by metabolites in neuronal differentiation remains relatively unexplored, these findings suggest that they play a complex part in regulating stem cell fate.

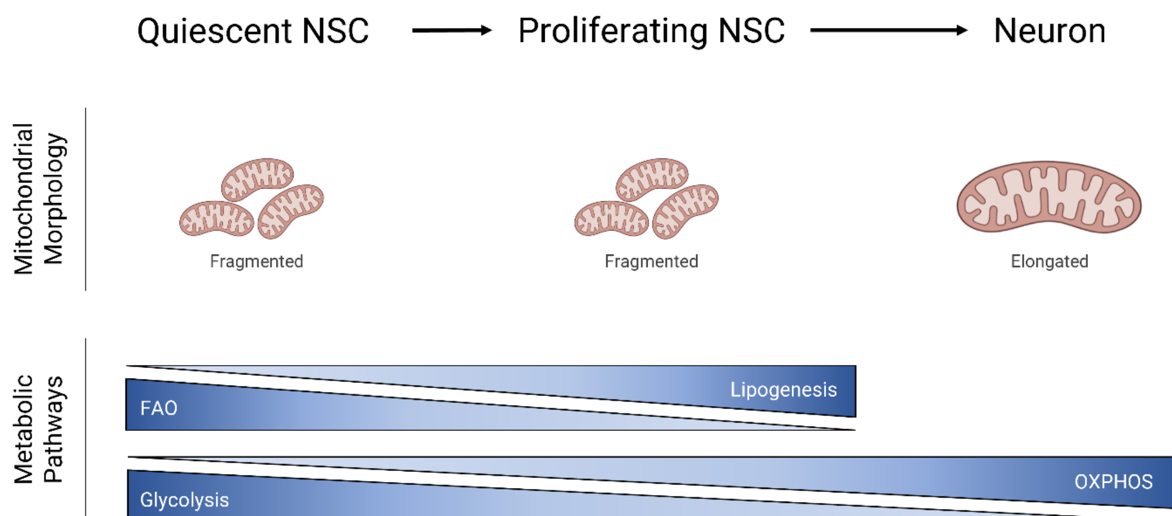

Figure 1.2 – Metabolism and mitochondrial morphology during NSC differentiation. Quiescent NSCs rely on glycolysis and fatty acid oxidation for the production of energy and present a fragmented mitochondrial network. During proliferation, NSCs become more dependent on lipogenesis to fuel the production of new membranes. During neuronal differentiation, NSCs suffer a metabolic shift towards oxidative phosphorylation and present a fused mitochondrial network, which support the energy requirements of mature neurons. (Created with BioRender.com). FAO, fatty acid oxidation; NSC, neural stem cell; OXPHOS, oxidative phosphorylation.

Lipid metabolism also plays an active role in NSC fate (Knobloch, 2017). Lipids belong to a vast class of biomolecules, broadly defined as any organic compounds insoluble in water but soluble in organic solvents (Nelson & Cox, 2017). As such, they play diverse roles, from structuring membranes to energy storage and signalling (Beenakkers, Van der Horst, & Van Marrewijk, 1981; Irvine, 2003; RAISON, 1980). Fatty acids are the building blocks of most lipids, and when internalized by cells they can be used for anabolic or catabolic purposes (De Carvalho & Caramujo, 2018). If the cells require energy, fatty acids undergo degradation by fatty acid oxidation (Houten, Violante, Ventura, & Wanders,

2016). If they require anabolic precursors for building membranes, for example, they enter lipogenesis (Ameer, Scandiuzzi, Hasnain, Kalbacher, & Zaidi, 2014). The rate-limiting step of fatty acid oxidation involves the enzyme carnitine palmitoyltransferase I (CPT1), which facilitates the transport of fatty acids into the mitochondrial matrix, where oxidation occurs (Schlaepfer & Joshi, 2020). Quiescent rodent NSCs have increased levels of CPT1 and a higher rate of fatty acid oxidation (Knobloch et al., 2017). Treating these cells with malonyl-CoA, an inhibitor of CPT1, is sufficient to trigger proliferation (Knobloch et al., 2017). Therefore, fatty acid oxidation tightly regulates quiescence in NSCs. When NSCs become activated, a shift occurs towards lipogenesis (**Figure 1.2**). Specifically, the key enzyme fatty acid synthase (FASN) is highly active in proliferating mouse NSCs (Knobloch et al., 2013). Inhibiting this enzyme results in decreased rates of lipogenesis and proliferation (Knobloch et al., 2013). This is unsurprising, as rapidly proliferating cells require a steady supply of membrane lipids to form new cells (Ly et al., 2020).

1.1.5 Mitochondrial Regulation of NSCs

Mitochondria are commonly referred to as the "powerhouses of the cell" due to their role in the production of ATP through oxidative phosphorylation (Siekevitz, 1957). However, their importance in eukaryotic cells goes beyond energy generation: mitochondria are involved in cell signalling, calcium homeostasis, anabolic precursor generation, apoptosis, and much more (McBride, Neuspiel, & Wasiak, 2006). Unlike other organelles, mitochondria are double-membraned and have their own mitochondrial DNA (mtDNA) (Nelson & Cox, 2017). Importantly, the viability of most eukaryotic cells depends on the maintenance of mitochondrial structure and function (Bock & Tait, 2020).

For a long time, mitochondria were thought to be irrelevant in stem cell populations. In fact, since stem cells mainly rely on glycolysis and show little mitochondrial respiration, it was assumed that mitochondria were not necessary for proper stem cell function (Khacho et al., 2019). This view was challenged by numerous reports that disruption of mitochondrial function results in stem cell abnormalities (Ahlqvist et al., 2012; Fox, Magness, Kujoth, Prolla, & Maeda, 2012; Norddahl et al., 2011). Today, mitochondria are known to play a central role in regulating stem cell fate and behaviour (Khacho et al., 2019). As previously mentioned, ROS and intermediate metabolites from the tricarboxylic acid cycle, generated in the mitochondria, drive transcriptional and epigenetic changes that control stemness and differentiation. The neuronal differentiation of NSCs depends on the metabolic switch from glycolysis to mitochondrial oxidative phosphorylation (Zheng et al., 2016). Disrupting mitochondrial function, particularly the electron transport chain, has been shown to decrease the generation and survival of adult-born neurons in rodents, thereby impairing adult neurogenesis (Beckervordersandforth et al., 2017). On the other hand, restoring mitochondrial function in aged mice resulted in increased rates of hippocampal neurogenesis (Beckervordersandforth et al., 2017).

Beyond mitochondrial function, mitochondrial dynamics also regulate NSC fate (Khacho et al., 2019). Rather than their traditional isolated, "bean-like" structure, mitochondria are highly dynamic and form a tubular network throughout the cell (Rafelski, 2013). This network is very sensitive to the status and requirements of the cell, constantly adapting its morphology to fit the cell's needs (Benard et al.,

2007). Mitochondrial dynamics are a combination of two processes: fusion and fission (Anzell, Maizy, Przyklenk, & Sanderson, 2018). Fusion results in the merger of the double membrane of two mitochondria, while fission results in the division of a single mitochondrion into two separate mitochondria (Anzell et al., 2018). Dysregulated mitochondrial dynamics are involved in many neurodegenerative diseases, including Parkinson's, Huntington's, and amyotrophic lateral sclerosis (Burté, Carelli, Chinnery, & Yu-Wai-Man, 2015). As such, proper cell function depends on a delicate balance between mitochondrial fusion and fission. In NSCs, the mitochondrial network suffers extensive remodelling during neuronal differentiation (Khacho & Slack, 2018). Adult NSCs have fragmented mitochondria, both during quiescence and proliferation (Beckervordersandforth et al., 2017). Mitochondrial fragmentation is important to ensure an even distribution of mitochondria between daughter cells when cells are rapidly dividing (Pangou & Sumara, 2021). With neuronal differentiation, mitochondrial fusion increases, since elongated mitochondria enhance oxidative phosphorylation and ATP output, meeting the bioenergetic demands of mature neurons (**Figure 1.2**) (Beckervordersandforth et al., 2017). Genetic manipulation has confirmed a causal relationship between mitochondrial dynamics and NSC fate. Inhibiting the mitochondrial fission factor dynamic-related protein 1 (Drp1) prevents NSC proliferation (Steib, Schäffner, Jagasia, Ebert, & Lie, 2014), while deleting the mitochondrial fusion factors mitofusin 1/2 (Mfn1/2) and optic atrophy 1 (Opa1) causes excessive proliferation and premature differentiation (Khacho et al., 2016). These changes are partially related to the increased generation of ROS by fragmented mitochondria, activating nuclear transcriptional programmes that promote differentiation and suppress self-renewal (Khacho et al., 2016).

1.1.6 *In Vitro* Models of NSCs

Defining appropriate study models is one of the first steps in scientific research, which often defines the relevance of the findings. While *in vivo* models more closely resemble the complexity of the nervous system, *in vitro* models' simplicity can be precious in studying specific pathways or interactions, which would be a daunting task *in vivo*. Furthermore, *in vitro* models are cheaper and facilitate genetic manipulation.

The simplest model is growing NSCs in monolayer culture systems (Conti & Cattaneo, 2010). Despite being less physiologically relevant, this model is easy to maintain and remains mostly pure (Conti & Cattaneo, 2010). NSCs grown in monolayer are easy to observe, maintain, and manipulate (Conti & Cattaneo, 2010). Neurospheres are a popular alternative; they are free-floating aggregates of neural progenitors, each potentially derived from a single NSC (Brewer & Torricelli, 2007). They are heterogenous mixes of cells, usually with differentiated cells at the core (Campos, 2004; Suslov, Kukekov, Ignatova, & Steindler, 2002). Their three-dimensional nature is more resemblant of the neurogenic niches than monolayers, but the complexity of this model presents several difficulties (Jensen & Parmar, 2006). Neurospheres have limited neurogenic competence, as they mostly generate glial cells (Conti & Cattaneo, 2010). As such, they may not be the most appropriate model to study neuronal differentiation. Furthermore, since they are in suspension, monitoring their morphological properties is harder (Jensen & Parmar, 2006). Finally, their sensitivity to culturing methods makes it hard to compare

data from different groups (Jensen & Parmar, 2006). Brain organoids are an exciting new prospect, as scientists can generate "mini-brains" on a petri dish (Chiaradia & Lancaster, 2020). Human pluripotent stem cells are used to generate three-dimensional organoids whose architecture and cell composition closely resemble the fetal human brain (Shou, Liang, Xu, & Li, 2020). These models are very promising, as they most closely resemble their *in vivo* counterparts (Shou et al., 2020). Replicating the three-dimensional structure is particularly important, as the interactions cells establish with their neighbours in the brain are central to its function (Qian Yang, Hong, Zhao, Song, & Ming, 2022). However, the costs and difficulty associated are much higher than the alternatives. Furthermore, due to their fetal identity, brain organoids are not as suitable to study NSC function in the adult brain (Qian Yang et al., 2022).

1.2 Adult Neurogenesis

1.2.1 The Discovery of Adult Neurogenesis

For a long time, the scientific community believed that no new neurons were added in the adult mammalian brain (Kumar, Pareek, Faiq, Ghosh, & Kumari, 2019). Representative of this view, Santiago Ramón y Cajal, a leading figure in neuroscience of his time, stated in 1913 that "In the adult… the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated." (Ramon y Cajal, 1928). Since scientists observed that the brain's structure remained fixed soon after birth, and considering its complexity, they found it inconceivable that new neurons could be added during adulthood (Kumar et al., 2019).

The first significant evidence for adult neurogenesis was possible thanks to the introduction of [^{3}H]-thymidine autoradiography (Kumar et al., 2019). [^{3}H]-thymidine is incorporated into the DNA of dividing cells, labelling newly divided cells (Duque & Rakic, 2011). Using this technique, in 1963 Altman found adult-born neurons in the rat brain in the dentate gyrus and olfactory bulb (Altman, 1963). However, due to the prevailing views of the time, his findings were met with scepticism and were largely ignored, despite their significance. As late as 1970, an authoritative neuroscience textbook still stated that "there is no convincing evidence of neuron production in the brains of adult mammals." (M. Jacobson, 1969). Over time, an accumulating body of evidence confirmed Altman's findings regarding adult neurogenesis in a variety of model organisms, including primates (Kumar et al., 2019). The first evidence of adult neurogenesis in the human brain appeared in 1998, when Eriksson et al examined the brains of cancer patients injected with bromodeoxyuridine (BrdU) for diagnostic purposes (Eriksson et al., 1998). BrdU is a thymidine analogue which, much like [^{3}H]-thymidine, is incorporated into the DNA of rapidly diving cells (Duque & Rakic, 2011). As such, it can be used to label newly divided cells through immunohistochemistry. In the dentate gyrus, the authors identified multiple BrdU-positive cells which co-localized with the neuronal markers neuronal nuclear protein (NeuN), calbindin, or neuron specific enolase (NSE) (Eriksson et al., 1998). These findings were revolutionary, as they suggested for the first time that adult-born neurons can be found in the human brain.

Five decades after its postulation, adult neurogenesis remains a controversial subject (Kempermann et al., 2018). Studies presenting conflicting evidence are constantly being published, raising the question of whether adult neurogenesis occurs in humans after all. In 2018, in a space of two weeks, two papers were published with contradictory results. Boldrini et al found evidence of lifelong neurogenesis in humans (Boldrini et al., 2018), while Sorrells et al concluded that neurogenesis drops to undetectable amounts after childhood (Sorrells et al., 2018). The conflicting results can be attributed to differences in methodologies, study design, and interpretation (Kempermann et al., 2018). Critics of Sorrells' study point to the post-mortem interval as well as the use of samples from epilepsy patients as factors of concern (Kempermann et al., 2018), while critics of Boldrini's suggest that using markers such as BrdU and doublecortin (DCX) can lead to incorrect interpretation, since BrdU may also label dying cells and DCX may label some glial cells (Sorrells et al., 2018). These points of disagreement call for a standardization of procedures when studying adult neurogenesis, as well as a revaluation of the markers used to label NSCs and adult-born neurons. However, the current consensus is that adult neurogenesis occurs in the human brain, even if at low levels, in the subventricular zone of the lateral ventricles and in the subgranular zone of the hippocampal dentate gyrus (Kempermann et al., 2018; Y. Zhou et al., 2022).

1.2.2 Mechanisms of Adult Neurogenesis

Adult neurogenesis is an intricate, highly regulated process, beginning with the activation of NSCs and ending in the integration of mature adult-born neurons into functional neuronal circuits (H. Liu & Song, 2016). Considering the lack of studies on the mechanisms of adult neurogenesis in humans and the similarities shared between rodent and human neurogenesis, this section will focus on the mechanisms of adult neurogenesis in mice. Since the NSC population and supporting microenvironment are different in the two neurogenic niches, so are the mechanisms of neurogenesis.

Hippocampal neurogenesis is comprised of 4 sequential steps: (i) the precursor cell phase; (ii) the early survival phase; (iii) the post-mitotic phase; and (iv) the late maturation phase (**Figure 1.3**) (Kempermann, Song, & Gage, 2015). In the precursor cell phase, type 1 NSCs are activated and divide asymmetrically to generate type 2 transit-amplifying cells (Kempermann et al., 2015). Type 1 cells express the undifferentiated neural progenitor marker nestin and the astrocytic marker glial fibrillary acidic protein (GFAP) (Filippov et al., 2003; Seri, Garcıa-Verdugo, McEwen, & Alvarez-Buylla, 2001). Type 2 cells are highly proliferative and can be identified by the expression of the transcription factor Tbr2 (Hodge et al., 2008). They further differentiate into type 3 neuroblasts, which keep proliferating and migrate into the granule cell layer (Kempermann et al., 2015). Type 3 cells express the immature neuronal marker DCX and polysialylated neuronal cell adhesion molecule (PSA-NCAM) but lose the expression of nestin (Filippov et al., 2003; Kempermann et al., 2015). Within three days of cell division, this cell population can increase up to five times, and neuroblasts begin the final stage of differentiation into neurons, which express neuronal markers such as NeuN and calretinin (CR) (Brandt et al., 2003; Kempermann et al., 2015). The early survival phase begins with the exit from the cell cycle: the number of adult-born neurons rapidly declines over the following four days, due to widespread apoptosis (Biebl,

Cooper, Winkler, & Kuhn, 2000). In the post-mitotic phase, the surviving adult-born neurons are incorporated into the existing neuronal circuitry, establishing functional connections and growing dendritic projections (Kempermann et al., 2015). The late maturation phase is less characterized; during this stage, adult-born neurons become morphologically and electrophysiologically identical to the existing neurons, thus finishing their functional integration into the hippocampal circuits (Kempermann et al., 2015). Typically, adult-born neurons in the dentate gyrus become glutamatergic granule neurons (Ambrogini et al., 2004; van Praag et al., 2002).

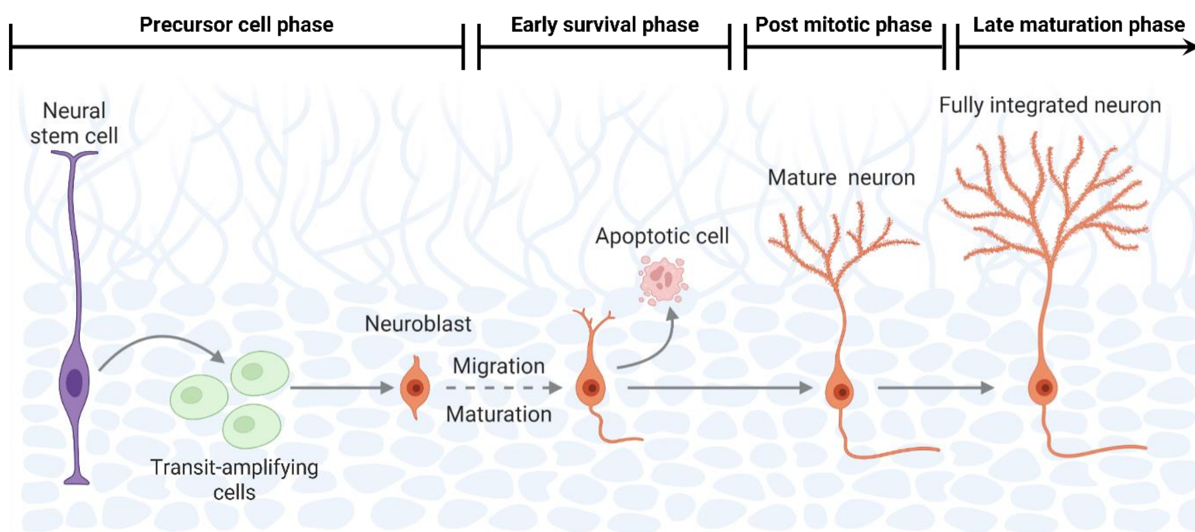

Figure 1.3 – Adult neurogenesis in the SGZ. During the precursor cell phase, activated NSCs divide asymmetrically to generate TA cells. TA cells proliferate and give rise to neuroblasts, which keep proliferating and migrate to the granule cell layer, where they begin maturation into granule neurons. In the early survival phase, most maturing neurons die, and the remaining neurons are integrated into the neuronal circuitry during the post-mitotic phase. In the late maturation phase, adult-born neurons become morphologically and electrophysiologically identical to pre-existing neurons. (Created with BioRender.com). NSC, neural stem cell; SGZ, subgranular zone; TA, transit-amplifying.

Subventricular neurogenesis can be divided into three developmental stages: (i) the precursor cell phase; (ii) the migratory phase; and (iii) the maturation phase (**Figure 1.4**) (Ming & Song, 2011). During the precursor cell phase, type B NSCs are activated and divide asymmetrically to generate type C transit-amplifying cells (Ming & Song, 2011). Type B cells express nestin and GFAP, while type C cells express nestin and Achaete-scute homolog 1 (ASCL1) (Fiona Doetsch, Garcıa-Verdugo, & Alvarez-Buylla, 1997; Ponti, Obernier, & Alvarez-Buylla, 2013). Type C cells proliferate and differentiate into type A neuroblasts, which express DCX and PSA-NCAM (Fiona Doetsch et al., 1997). During the migratory phase, neuroblasts travel through the rostral migratory stream towards the granule cell layer of the olfactory bulb (F Doetsch & Alvarez-Buylla, 1996). There, the surviving neuroblasts enter the maturation phase. They are incorporated into the local circuits, typically differentiating into GABAergic granule neurons that establish connections with mitral and tufted cells (Ming & Song, 2011).

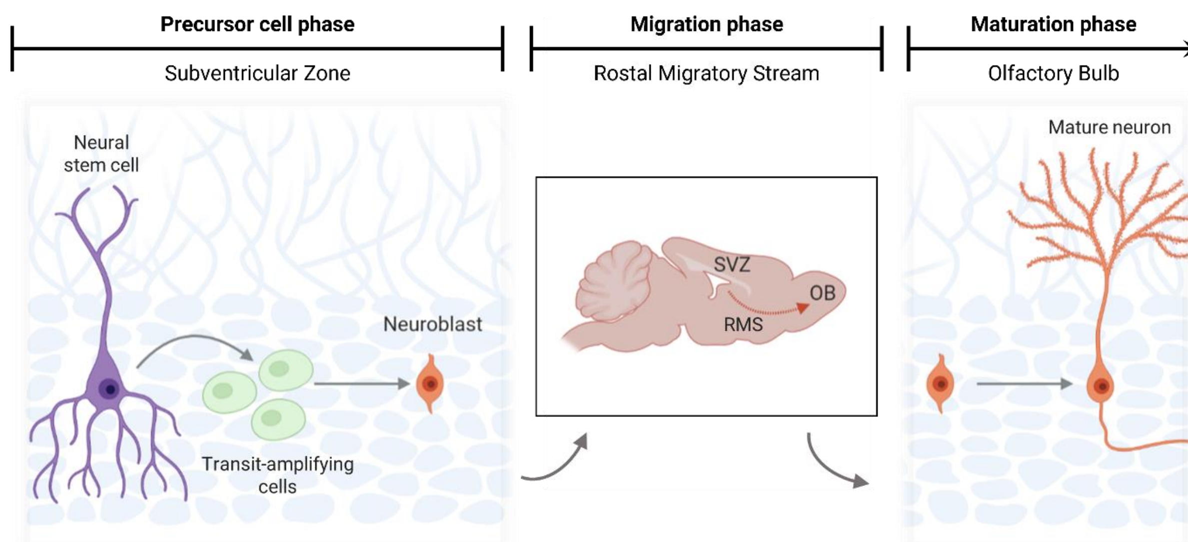

Figure 1.4 – Adult neurogenesis in the SVZ. During the precursor cell phase, activated NSCs generate TA cells, which proliferate and give rise to neuroblasts. During the migration phase, neuroblasts migrate along the RMS towards the OB. In the OB, neuroblasts enter the maturation phase, generating mature neurons. (Created with BioRender.com). NSC, neural stem cell; OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone; TA; transit-amplifying.

1.2.3 Regulation by the Neurogenic Niche

The members of the neurogenic niche are deeply interconnected, supporting each other and communicating through secreted factors and direct cell-cell interactions (Bjornsson et al., 2015). A growing body of evidence suggests that successful adult neurogenesis depends on the concerted action of all members of the niche.

Microglia are important components of the subventricular and subgranular zones, constantly surveilling the local environment for potential threats (Bjornsson et al., 2015). During adult neurogenesis, most immature neurons undergo apoptosis, resulting in an accumulation of cell debris which can be damaging to the brain (Biebl et al., 2000). In the hippocampus, microglia phagocytize apoptotic immature neurons, contributing to their clearance (Sierra, Abiega, Shahraz, & Neumann, 2013). Suppressing microglial phagocytosis has been shown to decrease adult hippocampal neurogenesis in mice, illustrating its importance (Diaz-Aparicio et al., 2020). Beyond phagocytosis, microglial cells also seem to regulate NSC fate and behaviour through the release of soluble factors. In 2003, Aarum et al demonstrated that the conditioned media from cultured microglia attracted NSCs and promoted neuronal differentiation (Aarum, Sandberg, Haeberlein, & Persson, 2003). As such, it appears that microglia secrete factors which direct NSC migration and regulate differentiation. This regulation, however, depends on the activation state of microglia. *In vitro* studies often use the bacterial endotoxin lipopolysaccharide to mimic infection by Gram-negative bacteria, which causes acute excessive activation of microglia (Ye et al., 2020). The conditioned media of lipopolysaccharide-activated microglia has been shown to reduce NSC survival and prevent neuronal differentiation (Osman et al., 2019). Acutely activated microglia release multiple pro-inflammatory cytokines, including interleukin-6, interferon-γ, and tumour necrosis factor-

α (Lynch, 2009), which have been shown to supress neurogenesis and may mediate the negative effects of activated microglia's conditioned media (Cacci, Claasen, & Kokaia, 2005; Gómez, Medina, Baena, Planas, & Pozas, 2015; Vallieres, Campbell, Gage, & Sawchenko, 2002). However, further *in vivo* studies need to be performed to clarify the relationship between microglia and NSCs in the neurogenic niches.

Both neurogenic niches are deeply irrigated and the vasculature is a potent regulator of adult neurogenesis (Bjornsson et al., 2015). In the adult subventricular zone, the blood vessels of the vascular plexus function as a migratory scaffold, guiding neuroblasts along the rostral migratory stream into the olfactory bulb (Bovetti et al., 2007). Curiously, most NSCs reside closer to the blood vessels than other cell types (Palmer et al., 2000). The subgranular zone harbours a more dynamic vascular bed, where angiogenesis—the formation of new blood vessels—can occur (Palmer et al., 2000). Angiogenesis is spatially and temporally associated with neurogenesis, with both processes being regulated by the vascular endothelial growth factor (VEGF) (Ferrara, 2005; Jin et al., 2002; Palmer et al., 2000). A study in songbirds revealed that angiogenesis and neurogenesis regulate each other bidirectionally (Chen, Ye, & Goldman, 2013). In the songbird brain, testosterone upregulates VEGF and VEGF receptor 2 in neurons and astrocytes, respectively, increasing angiogenesis. In turn, the newly generated blood vessels release brain-derived neurotrophic factor (BDNF), which promotes the recruitment and migration of newly born neurons. Despite the differences between the songbird and the mammalian adult brain, VEGF and BDNF seem to have similar effects, suggesting that this relationship may also occur in the mammalian brain. Endothelial cells, which line the blood vessels, regulate NSC fate and behaviour through secreted factors and direct contact. *In vitro* studies show that NSCs exposed to the conditioned media of endothelial cells or in co-culture have increased rates of proliferation and differentiation (Q. Shen et al., 2004). Various soluble factors, namely VEGF, pigment epithelium-derived factor, and betacellulin, seem to mediate these effects (Gómez-Gaviro et al., 2012; Ramírez-Castillejo et al., 2006; Sun, Zhou, Ma, & Yang, 2010). Importantly, the secretory profile of endothelial cells changes according to different stimuli (Burghoff & Schrader, 2011), suggesting that endothelial cells may modulate adult neurogenesis differently in pathological situations.

Astrocytes are the most common cell type in the neurogenic niches and perform several important functions, including neuronal metabolic support, ion homeostasis regulation, and phagocytosis (Cassé, Richetin, & Toni, 2018). They are also important secretory cells, releasing a wide array of neurotransmitters, neuromodulators, hormones, and trophic factors which regulate the behaviour of other cell types (Schneider et al., 2019). One of the first studies that identified a link between adult neurogenesis and astrocytes showed that the conditioned media of astrocytes from the adult hippocampus increased NSC proliferation and neuronal differentiation *in vitro* (H. Song, Stevens, & Gage, 2002). Curiously, astrocytes isolated from the spinal cord failed to recapitulate these effects, indicating that the hippocampal niche houses a specific astrocyte population capable of regulating neurogenesis (H. Song et al., 2002). Further studies suggested adenosine 5′-triphosphate, basic fibroblast growth factor, thrombospondin-1, and D-serine as possible mediators of astrocyte-induced neurogenesis (X. Cao et al., 2013; Kirby et al., 2013; Lu & Kipnis, 2010; Sébastien Sultan et al., 2015). As in microglia and endothelial cells,

pathological states may alter the secretory profile of astrocytes. In mice, West Nile virus infection impairs hippocampal function and adult neurogenesis (Garber et al., 2018). A recent study identified astrocytes, rather than microglia, as the main source of interleukin-1 in West Nile virus-infected mice. In this animal model, the authors identified interleukin-1 as a suppressor of adult neurogenesis (Garber et al., 2018). Chronic production of the pro-inflammatory interleukin-6 by astrocytes, as seen in some neurodegenerative disorders and infections, also reduces NSC proliferation and neuronal differentiation in the mouse hippocampus (Vallieres et al., 2002). As such, the impact of astrocytes on adult neurogenesis depends on the context and their activation state.

1.2.4 Regulation of NSCs by Lifestyle Choices

NSCs' location in the adult brain, close to the cerebrospinal fluid-filled ventricles and the vasculature, places them in an ideal position to receive cues from the external environment (Bjornsson et al., 2015). Accumulating evidence shows that adult neurogenesis is finely regulated by lifestyle choices, including diet, exercise, and cognitive stimulation.

Caloric restriction is usually defined as a 20-40% reduction in energy intake and is one of the most well-established lifestyle modifications known to improve general health (Trepanowski, Canale, Marshall, Kabir, & Bloomer, 2011). It became particularly popular after scientists demonstrated that caloric restriction alone was sufficient to dramatically increase the lifespan of several model organisms, including rodents and *Caenorhabditis elegans* (Lakowski & Hekimi, 1998; McCay, Crowell, & Maynard, 1935). Such observations showed that caloric restriction activated several protective pathways which promoted systemic health benefits (Masoro, 2005). Since then, several studies identified benefits of caloric restriction in brain health, namely by promoting adult neurogenesis (Levenson & Rich, 2007). In 2002, Lee et al showed that restricting calories for 4 weeks in mice increased the number of adult-born neurons in the dentate gyrus (Jaewon Lee, Seroogy, & Mattson, 2002). A follow-up study revealed that caloric restriction promotes adult neurogenesis by upregulating BDNF (Jaewon Lee, Duan, & Mattson, 2002). First, the authors identified increased levels of BDNF in the circulation and neurons of mice after caloric restriction. Then, they generated heterozygous BDNF knockout mice (BDNF +/-) and observed that caloric restriction did not improve the survival of new neurons as much as in the wild-type group. Later studies revealed that overexpression of BDNF is sufficient to enhance hippocampal adult neurogenesis, further implicating this neurotrophic factor in caloric restriction-mediated neurogenesis (Scharfman et al., 2005). In fact, caloric restriction also increases the levels of acyl-ghrelin, a hormone known to cross the blood-brain barrier and mediate neuroprotective effects (Cummings et al., 2001; Jiao et al., 2017). Since NSCs express ghrelin receptors, scientists sought to discover whether it is involved in promoting adult neurogenesis (Moon, Kim, Hwang, & Park, 2009). Ghrelin knockout and wild-type mice were maintained in caloric restriction or control conditions for three months (Kim et al., 2015). Once again, caloric restriction improved the survival of neuroblasts in the dentate gyrus of wild type mice. However, this effect was not observed in ghrelin knockout mice. Further studies showed that acute injection of ghrelin in mice increases NSC proliferation in the hippocampus, implicating this hormone in caloric restriction-mediated neurogenesis (Moon et al., 2009).

A subtype of caloric restriction that has garnered popularity in recent years is fasting. Fasting regimens involve long periods of time when no calories are consumed, followed by short intervals during which energy intake is allowed (Patterson & Sears, 2017). Fasting's health benefits seem to go beyond caloric restriction, activating different protective pathways (De Cabo & Mattson, 2019). A recent study compared the effects of fasting and caloric restriction in adult neurogenesis (Dias et al., 2021). Adult mice were divided into three groups: control, 10% caloric restriction, and every-other-day feeding (also resulting in 10% caloric restriction). After three months, mice in the fasting regimen exhibited the highest number of dividing neuroblasts, surpassing the effects of caloric restriction. While specific mechanisms remain to be explored, fasting-induced neurogenesis may be attributed to increased levels of Notch, BDNF, cAMP response element-binding protein (p-CREB), as well as upregulation of longevity genes such as Klotho, which was found to be increased in fasting mice, but not in calorie-restricted mice (Baik, Rajeev, Fann, Jo, & Arumugam, 2020; Dias et al., 2021).

The impact of high-fat diets in adult neurogenesis is more controversial. Diets rich in saturated fats and sugars are very damaging health-wise; they cause obesity, diabetes, inflammation, neurodegeneration, and decreased cognitive abilities (Duan et al., 2018; Freeman, Haley-Zitlin, Rosenberger, & Granholm, 2014). Accordingly, some studies show that high-fat diets have a negative effect on adult neurogenesis in rodents. Park et al found that adult mice fed a high-fat diet for seven weeks had less adult-born neurons in the hippocampus than mice in control conditions (Park et al., 2010). This reduction was associated with decreased levels of BDNF and increased levels of malondialdehyde, a marker of oxidative stress. Robison et al found a similar decrease in proliferating NSCs in the hippocampus, but only in female mice (Robison et al., 2020). Curiously, a recent study found increased NSC proliferation in the subventricular zone of mice fed a high-fat, choline deficient diet for 14 weeks (Ribeiro et al., 2020). This increase is short-lived, as mice maintained in this diet for 24 weeks had decreased NSC proliferation. The authors suggest that the initial increase in neurogenesis is detrimental: the high-fat diet triggered excessive proliferation and premature neuronal differentiation, leading to increased oxidative stress and exhaustion of the NSC pool with impairment of long-term neurogenesis.

The specific composition of the diet, beyond overall energy intake, also affects adult neurogenesis. Polyunsaturated fatty acids are found in vegetables and certain animal foods, such as salmon, and are considered "healthy fats", with particularly beneficial effects on cardiovascular health (Calder, 2006). Supplementing the diet of rodents with various polyunsaturated fatty acids increases hippocampal volume, as well as NSC proliferation and neuronal differentiation in the dentate gyrus (C. He, Qu, Cui, Wang, & Kang, 2009; Kawakita, Hashimoto, & Shido, 2006). These changes are associated with increased levels of BDNF, which may mediate polyunsaturated fatty acid-induced neurogenesis (Venna et al., 2009). Polyphenols are an additional dietary compound with many health benefits (Rasouli, Farzaei, & Khodarahmi, 2017). They are a broad range of organic molecules found in plants, particularly berries, whole grains, and spices (Rasouli et al., 2017). Resveratrol is a polyphenol enriched in the skin of red grapes, responsible for many health benefits associated with red wine consumption (Frémont, 2000). Injecting adult rats with resveratrol increases serum BDNF concentration and hippocampal neurogenesis (Kodali et al., 2015; Y.-N. Zhao et al., 2013). Curcumin is another popular polyphenol, present

in the spice turmeric, which is known for its anti-inflammatory and antioxidant properties (Sharma, Gescher, & Steward, 2005). Dong et al reported that curcumin supplementation enhances neurogenesis in the dentate gyrus of aged rats, by promoting the expression of neuroprotective genes such as NeuroD1 and Wnt2 (Dong et al., 2012).

Besides dietary habits, regular physical exercise is also a key determinant in long-term health, with a growing body of evidence showing an inverse relationship between exercise and diseases such as type II diabetes, cardiovascular-related diseases, cancer, and mental disorders (Ruegsegger & Booth, 2018). In rodents, the relationship between physical exercise and adult neurogenesis is well-established (Van Praag, 2008). Mice given free access to running wheels show higher levels of adult-born neurons in the hippocampus, and studies suggest this increase is intensity-dependent (Ma et al., 2017; van Praag, Christie, Sejnowski, & Gage, 1999). Indeed, physical exercise increases the expression of several neurotrophic factors in the hippocampus, mainly VEGF, insulin-like growth factor 1 (IGF-1), and BDNF (Q. Ding, Vaynman, Akhavan, Ying, & Gomez-Pinilla, 2006; Y.-H. Ding et al., 2006) and individual administration of these growth factors in rodents is enough to enhance adult hippocampal neurogenesis, while inhibiting their expression blocks exercise-induced neurogenesis (Åberg, Åberg, Hedbäcker, Oscarsson, & Eriksson, 2000; Fabel et al., 2003; Y. Li et al., 2008; Trejo, Carro, & Torres-Aleman, 2001). Therefore, these three neurotrophic factors seem to work in concert to promote neurogenesis in response to physical exercise.

The hippocampus plays an important role in cognitive processes, such as learning and memory formation (Jarrard, 1993). Curiously, cognitive stimulation seems to promote adult neurogenesis, possibly as an adaptive response (Clemenson, Deng, & Gage, 2015). Housing rodents in enriched environments—large spaces with toys, tunnels, bedding and running wheels—stimulates spatial exploration and social behaviour (Clemenson et al., 2015). Several studies also found that these conditions improve the generation and long-term survivability of adult-born neurons in the hippocampus (Kempermann, Kuhn, & Gage, 1997; Olson, Eadie, Ernst, & Christie, 2006). Since rodents in enriched environments have free access to running wheels, increased rates of neurogenesis could be attributed to physical exercise, and not to cognitive stimulation. To test this hypothesis, mice were housed in enriched environments lacking running wheels (Birch, McGarry, & Kelly, 2013). Strikingly, enriched mice also displayed higher hippocampal neurogenesis, despite the lack of physical exercise. The classic neurogenesis-promoting trophic factors BDNF and VEGF were found to be necessary for the environmental induction of neurogenesis (L. Cao et al., 2004; Rossi et al., 2006). Attempting to study the effects of an enriched environment in humans is a difficult task: beyond the typical technical challenges of studying brain-related changes in humans, most people already live in what can be considered an "enriched environment" with constant stimulation. However, some studies have found a correlation between spatial exploration and increased hippocampal volume in humans, which could be a result of adult neurogenesis (Clemenson et al., 2015). London taxi drivers are great study subjects in this regard, as they must learn the complex and irregular layout of London's labyrinthic streets. Two studies examined changes in the brain structure of London taxi drivers during training and found that hippocampal grey matter increased throughout the training (Maguire et al., 2000; Woollett & Maguire, 2011). This increase was correlated with time spent

as a taxi driver and spatial memory of the London streets. These findings suggest that continuous cognitive stimulation can be an effective method to promote neurogenesis in both animal models and humans.

1.2.5 The Functional Significance of Adult Neurogenesis

The conservation of adult neurogenesis in mammals must serve an important purpose, considering the energetic cost of maintaining a NSC population and generating new neurons. Initially, the scientific community assumed that, much like in other stem cell populations, NSCs were a regenerative source for new neurons, to counteract neuronal loss in situations of injury (Bond, Ming, & Song, 2015). However, most studies found the rate of adult neurogenesis to be too low to compensate for injury-induced neuronal loss (Bond et al., 2015). Accumulating evidence indicates that the primary function of adult-born neurons is to confer an additional layer of plasticity to the brain (Christian, Song, & Ming, 2014). During the first weeks after generation, adult-born neurons have distinct properties from developmentally-born neurons, enabling them to perform special functions. They are hyper-excitable and have a lower threshold for long-term potentiation (a plasticity mechanism that strengthens synaptic connections in frequently activated neurons) (Ge, Sailor, Ming, & Song, 2008). These features allow them to make unique contributions to information processing in the brain (Christian et al., 2014).

Most studies investigating the functional significance of adult-born neurons focus on hippocampal adult neurogenesis, due to the important role played by the hippocampus in cognitive processes such as learning, memory formation, and mood regulation (Jarrard, 1993). The dentate gyrus is responsible for pattern separation, a process through which overlapping inputs are transformed into less similar outputs in the brain (Leutgeb, Leutgeb, Moser, & Moser, 2007). At the behavioural level, pattern separation helps distinguish similar events and environments (T. Toda, Parylak, Linker, & Gage, 2019). Since the dentate gyrus is the primary site of adult hippocampal neurogenesis, it was hypothesized that adult-born neurons participate in pattern separation. Eliminating adult neurogenesis through irradiation in mice impairs their ability to distinguish stimuli with little spatial separation, but not widely-separated stimuli (Clelland et al., 2009). Conversely, increasing adult neurogenesis through selective deletion of the proapoptotic gene Bax enhances their ability to discriminate overlapping contexts (Sahay et al., 2011). These findings suggest that adult-born neurons mediate pattern separation in the dentate gyrus. Curiously, adult hippocampal neurogenesis also seems to be involved in forgetting memories. Akers et al trained mice in hippocampus-dependent tasks, after which mice were divided in sedentary or running groups (Akers et al., 2014). They found that running mice, which displayed higher levels of neurogenesis, showed poorer memory of the tasks learned prior to the running regimen. Using a transgenic system to supress running-induced neurogenesis prevented memory deficits in running mice, establishing a causal relationship between adult neurogenesis and forgetting. Although this might seem detrimental, forgetting is an important part of neuronal plasticity: since the brain's storage capacity is limited, old unused memories must be eliminated to make space for new ones (Davis & Zhong, 2017).

Regarding mood regulation, adult neurogenesis appears to buffer the stress response. Stressful situations activate the hypothalamus-pituitary-adrenal (HPA) axis, triggering the production of

glucocorticoid hormones (Smith & Vale, 2006). The hippocampus negatively regulates the HPA axis, preventing unnecessary extreme responses (L. Jacobson & Sapolsky, 1991). Strikingly, supressing adult neurogenesis in mice leads to increased levels of circulating glucocorticoids upon exposure to mild stressors and greater stress responses in behavioural tests (Schloesser, Manji, & Martinowich, 2009). These results suggest that adult-born neurons play an important role in hippocampus-mediated stress buffering.

In comparison with hippocampal neurogenesis, the functional significance of adult neurogenesis in the subventricular zone remains relatively unexplored. Since neuroblasts from the subventricular zone migrate to the olfactory bulb in rodents, most studies have focused on the role of adult subventricular neurogenesis in olfaction, yielding contradictory results (Lazarini & Lledo, 2011). Some studies have found that specific inhibition of adult subventricular neurogenesis impairs long-term olfactory memory, while others have found no significant differences (Breton-Provencher, Lemasson, Peralta, & Saghatelyan, 2009; Imayoshi et al., 2008; S Sultan et al., 2010). Further studies on this subject need to be conducted to clarify the relationship between adult subventricular neurogenesis and olfaction. Curiously, adult subventricular neurogenesis may regulate sexual behaviour (Bedos, Portillo, & Paredes, 2018). Many animal species, including rodents, release sex pheromones—odours which trigger behavioural reactions in the opposite sex, bringing males and females together for the purpose of mating (Tirindelli, Dibattista, Pifferi, & Menini, 2009). Several studies have found that exposure to pheromones of the opposite sex promote NSC proliferation in the subventricular zone of mice (Larsen, Kokay, & Grattan, 2008; Oboti et al., 2009; Schellino et al., 2016). Importantly, suppressing neurogenesis prevents female mice from showing pheromone-induced behaviours, portraying the importance of adult subventricular neurogenesis in pheromone-mediated sexual attraction (Mak et al., 2007). The existence of human pheromones is a heavily debated topic (Wyatt, 2015); as such, these findings may not translate to a regulation of sexual behaviour by adult subventricular neurogenesis in humans.

Considering the many benefits associated with adult neurogenesis, one may wonder why evolution has selected against it; while lower vertebrates can regenerate entire regions of the brain, adult neurogenesis in adult mammals is much lower and restricted to specific locations (Kempermann, 2016). To answer this question, the functional disadvantages of adult neurogenesis must be considered. As vertebrates become more evolved, their neuronal circuitry becomes more complex. Advanced reasoning and memory storage rely on the maintenance of these complex circuits; therefore, adding a large number of new neurons to the brain would disrupt many already-established connections, with potentially catastrophic results in terms of cognition (Kempermann, Wiskott, & Gage, 2004). The low rates of adult neurogenesis in higher vertebrates seem to be a compromise between its long-term benefits and preserving the existing neuronal circuitry. Recent studies suggest a rate of 0.004% of new neurons added daily to the human brain (Spalding et al., 2013). While this rate is insufficient to compensate for massive injury-related neuronal loss, over the course of a decade it translates to nearly 15% of new neurons (Snyder, 2019). Therefore, adult neurogenesis seems to be key in promoting long-term brain health, rather than short-term regeneration.

1.2.6 Adult Neurogenesis in Disease

Taking into consideration the functional significance of adult neurogenesis in cognition and behaviour, the scientific community began to question whether dysfunctional adult neurogenesis may be involved in the pathogenesis of neuronal diseases. Neurodegenerative disorders, such as Parkinson's, Alzheimer's, and Huntington's, involve a gradual loss of specific neuronal populations (Gorman, 2008). As such, dysfunctional adult neurogenesis may aggravate the effects of neuronal loss, since less adult-born neurons are being added to the brain over the long term. Neuropsychiatric disorders affect several hippocampal functions: mood regulation, cognition, and behaviour (Yun, Reynolds, Masiulis, & Eisch, 2016). Can adult hippocampal neurogenesis be involved in the development of these symptoms?

The role of adult neurogenesis appears to vary according to the neurodegenerative disorder. Parkinson's disease—the most common movement disorder—involves massive loss of dopaminergic neurons in the *substantia nigra* (Winner & Winkler, 2015). Patients often suffer from olfactory and cognitive deficits during the early stages of the disease, suggesting a potential role for dysregulated adult neurogenesis in its pathogenesis (Carlesimo et al., 2012; Tolosa, Gaig, Santamaría, & Compta, 2009). Studies in mice often use the neurotoxic compounds 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to cause dopaminergic loss and mimic Parkinson's disease (Blandini & Armentero, 2012). Suzuki et al reported a decrease in adult-born neurons in the subgranular zone of mice treated with 6-OHDA (Suzuki et al., 2010). Winner et al also reported a decrease in total levels of adult neurogenesis in the subventricular zone, but observed a selective increase of adult-born neurons committed to dopaminergic fate in the glomerular layer of the olfactory bulb (Winner et al., 2006). Thus, while 6-OHDA neurotoxicity appears to impair global adult neurogenesis, it also seems to promote selective dopaminergic neuron generation, possibly as a regenerative mechanism. Alzheimer's disease is the most common type of dementia and, much like Parkinson's, it involves olfactory and cognitive deficits during the early stages (Winner & Winkler, 2015). Thus far, a consensus hasn't been reached regarding how Alzheimer's affects adult neurogenesis. Some studies report increased levels of adult neurogenesis in the subventricular zone, while others report decreased levels in both neurogenic niches (Lazarov & Marr, 2010; Marlatt & Lucassen, 2010). This variability can be attributed to the many different types of animal models of Alzheimer's disease (Drummond & Wisniewski, 2017). Since animal models often fail to accurately replicate the features of human diseases, it is useful to consider studies involving human samples. A recent study by Terreros-Roncal et al analysed the contribution of adult hippocampal neurogenesis to multiple neurodegenerative disorders using post-mortem human samples (Terreros-Roncal et al., 2021). In contrast with previous findings in animal models, they identified higher numbers of NSCs in the dentate gyrus of patients with amyotrophic lateral sclerosis, Huntington's disease, and Parkinson's disease. While the number of immature neurons also increased, the authors detected alterations in their morphological maturation across all neurodegenerative diseases. These findings suggest that, in humans, adult neurogenesis increases in multiple neurodegenerative disorders, possibly as a compensatory mechanism. However, due to alterations in the neurogenic niche, the adult-born neurons fail to complete maturation and integrate neuronal circuits.

Since the hippocampus is involved in mood regulation, the role of adult hippocampal neurogenesis in psychiatric disorders has become a topic of interest (Yun et al., 2016). Major depressive disorder is the second most common psychiatric disorder and a leading cause of disability worldwide (Malhi & Mann, 2018). Due to its complexity and variable symptomatology, it is impossible to identify a single root cause (Malhi & Mann, 2018). Some scientists defend that, in some cases, major depressive disorder can be caused by stress-induced reductions in hippocampal neurogenesis (Eisch & Petrik, 2012; Jacobs, van Praag, & Gage, 2000). The first studies to support this link showed that patients with major depression present reduced hippocampal volume, a reduction which correlates with the duration of the disease (Kempton et al., 2011; Sheline, Wang, Gado, Csernansky, & Vannier, 1996). Further studies revealed that NSC proliferation decreases in the dentate gyrus of patients with major depression, as well as with schizophrenia, anxiety, and drug addiction (Kang, Wen, Song, Christian, & Ming, 2016). Interestingly, antidepressants have been shown to promote adult hippocampal neurogenesis (Malberg, Eisch, Nestler, & Duman, 2000). When neurogenesis is suppressed through genetic or radiological methods, the behavioural improvements associated with antidepressant administration are prevented (Santarelli et al., 2003). Therefore, antidepressants exert positive effects on mood and behaviour through increased hippocampal neurogenesis. These findings further suggest a causal link between adult hippocampal neurogenesis and major depressive disorder.

1.3 The NSC Secretome

1.3.1 Introduction to the Secretome

Stem cells release a large number of factors into the extracellular space—growth factors, cytokines, angiogenic factors, microRNAs (miRNAs), among others—collectively defined as the "stem cell secretome" (Daneshmandi et al., 2020). The therapeutic potential of the stem cell secretome is being increasingly recognized, as numerous studies demonstrate its ability to modulate inflammation, angiogenesis, immune response, among many other important events (Daneshmandi et al., 2020). Broadly, the components of the secretome can be classified as soluble factors (ex.: growth factors and cytokines) and extracellular vesicles (exosomes, microvesicles, apoptotic bodies) (Daneshmandi et al., 2020). While the composition of the stem cell secretome varies according to the stem cell type and their microenvironment, it is generally known to have therapeutic effects, preventing cell death and promoting regeneration (Daneshmandi et al., 2020).

Stem cell transplantation is a popular approach in regenerative medicine, whereby healthy stem cells are inserted into a patient's damaged tissue to promote regeneration (Trounson & McDonald, 2015). Peculiarly, researchers found that stem cell transplantation often had regenerative effects even when stem cells failed to differentiate and integrate into the tissue (Drago et al., 2013). This led to the formulation of the "paracrine hypothesis": stem cell transplantation's beneficial effects may not be due to differentiation and cell replacement, but to the release of beneficial factors that promote regeneration (Drago et al., 2013). This hypothesis is very attractive, since stem cell transplants face many problems.

Immunocompatibility and transmission of infections are always a source of concern when transplanting foreign cells to a host, and uncontrolled stem cell proliferation can lead to tumorigenicity (Laurencin & McClinton, 2020). Isolating the therapeutic factors of the stem cell secretome and using them to treat patients would avert these complications (Daneshmandi et al., 2020). Furthermore, using the secretome rather than stem cell transplants offers many logistical advantages, namely reduced costs, increased scalability, availability, and longer shelf-life (Daneshmandi et al., 2020).

Thus, following standardized, well-established protocols is important when isolating secretomes, to increase reproducibility and avoid contaminations (Daneshmandi et al., 2020). Typically, stem cells are cultured in serum-containing medium, usually until 70-80% confluency. At this stage, pre-conditioning may be performed, to improve the therapeutic properties. This usually involves exposing the cells to chemical or physical stimuli, which trigger the release of beneficial factors. Prior to collection, cells are washed extensively to remove any remnants of serum proteins that may contaminate or interfere with the detection of low levels of cell-produced proteins. The conditioned medium, containing the secretome, is collected and centrifuged to remove dead cells or cell debris. Finally, if the secretome is going to be analysed, proteins and extracellular vesicles can be concentrated through centrifugation or lyophilization protocols, since their concentration in the conditioned medium is usually lower than most techniques' detection capacity.

1.3.2 Soluble Factors in the NSC Secretome

The soluble fraction of the stem cell secretome is comprised of a large number of secreted proteins, which are actively transported out of the cell (Kelly, 1985). According to their function, secreted proteins may be considered growth factors, angiogenic factors, cytokines, chemokines, among others (Daneshmandi et al., 2020). They may also be classified according to where they act: autocrine signalling proteins act on the secretory cell itself, paracrine signalling proteins act on neighbouring cells, and endocrine signalling proteins are usually secreted into the bloodstream, acting far away from the tissue of origin (Alberts, Bruce; Heald, Rebecca; Johnson, Alexander; Morgan, David; Raff, Martin; Roberts, K; Walter, 2022). Characterizing the soluble factors of the NSC secretome is therefore a key step in understanding its properties.

Interestingly, the NSC secretome is particularly rich in neurotrophic factors. Several studies have identified BDNF, VEGF, epidermal growth factor, among many others in the secretome of human and rodent NSCs (Q. Zhang, Li, An, Fan, & Cao, 2020). A recent study has performed an extensive analysis of the mouse NSC secretome *in vitro*, using two proteomics approaches: a protein antibody-based array and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Denninger et al., 2020). These approaches are useful when studying the expression of a vast number of proteins. *In vitro*, the conditioned media of hippocampal NSC cultures was collected and probed for over 300 growth factors, chemokines, cytokines, adipokines, angiogenic factors, proteases, soluble receptors, and soluble adhesion molecules using the antibody array. In accordance with previous findings, VEGF had the third highest signal intensity, indicating its presence in high amounts in the NSC secretome. Through the secretion of VEGF, NSCs may regulate the surrounding vasculature, increasing blood vessel formation and

permeability (Ferrara, 2005). In fact, VEGF's previously described role in promoting neurogenesis indicates that NSCs may also regulate their own behaviour in an autocrine manner (Jin et al., 2002). Additionally, many members of the insulin-like growth factor binding protein (IGFBP) family were among the top 50 signals. IGFBPs are carrier proteins, which bind circulating IGF-1 and lengthen its half-life (Hwa, Oh, & Rosenfeld, 1999). In NSCs, IGFBPs have been shown to supress differentiation, thereby promoting NSC maintenance (H. S. G. Kalluri & Dempsey, 2011; F. Shen, Song, Liu, Zhang, & Wei Song, 2019). The existence of proteins in the secretome of NSCs with opposing effects on adult neurogenesis suggests that NSC fate depends on a delicate balance between these factors. When NSCs are exposed to specific stimuli, the secretome's composition may rapidly change, shifting the balance either towards the suppression or enhancement of adult neurogenesis. Various anti-inflammatory (interleukin-10, interferon-β) and pro-inflammatory (interleukin-6, interleukin-3, interferon-γ) cytokines were also detected, suggesting a role for NSCs in regulating microglial behaviour. Strikingly, analysing the secretome through LC-MS/MS did not yield the same results. While some high intensity signals were common across both methodologies (particularly IGFBPs), most of the top 50 proteins were different. These discrepancies highlight the need to further validate the expression of proteins of interest through western blotting.

1.3.3 Extracellular Vesicles in the NSC Secretome

NSCs also release to the extracellular space lipid bilayer-delimited nanoparticles: the extracellular vesicles. Exosomes are an important class of extracellular vesicles, ranging in size from 40 to 160 nm and derived from multivesicular endosomes within the cell (R. Kalluri & LeBleu, 2020). They can transport valuable biological factors, including proteins, metabolites, nucleic acids, and even functional mitochondria (Amari & Germain, 2021; R. Kalluri & LeBleu, 2020). In comparison with soluble factors, the cargo of exosomes is more protected against degradation by proteases and nucleases in the extracellular space (R. Kalluri & LeBleu, 2020). Furthermore, some exosomes have proteins in their membrane which interact with receptors in target cells to mediate fusion, thereby increasing the specificity of the delivery (J. He et al., 2022). These properties make exosomes an important part of the secretome with remarkable therapeutic capabilities.

Exosomes are particularly important in the transport of miRNAs, a class of short, non-coding RNAs that regulate gene expression post-transcriptionally, either by messenger RNA degradation or translational repression (J. Zhang et al., 2015). Mounting evidence suggests that miRNAs play a central role in regulating important cellular events, such as differentiation, proliferation, and apoptosis (Huang et al., 2011). Using an ultracentrifugation protocol and next-generation sequencing, Stevanato et al characterized the miRNA content of exomes derived from human NSCs *in vitro* (Stevanato, Thanabalasundaram, Vysokov, & Sinden, 2016). They found 113 miRNAs in the exomes, out of which hsa-miR-1246, hsa-miR-4488, hsa-miR-4508, hsa-miR-4492 and hsa-miR-4516 were the most enriched. Some of these miRNAs, notably miR-1246, have been implicated in cell proliferation (X. J. Li, Ren, Tang, & Yu, 2017), suggesting a role for exossomal miRNAs in the therapeutic properties of the NSC secretome. Notably, exosomes can also be involved in "mitochondrial transfer", a process through

which functional mitochondria can be exchanged between cells (Amari & Germain, 2021; D. Liu et al., 2021). Since dysfunctional mitochondria compromise cell viability and tissue function, mitochondrial transfer is an important mechanism in guaranteeing tissue homeostasis (D. Liu et al., 2021). While mitochondrial transfer between mammalian cells was first observed to occur via tunnelling nanotubes, studies have shown that exosomes can also carry functional mitochondria to target cells (Amari & Germain, 2021; D. Liu et al., 2021). Recently, Peruzzotti-Jametti et al demonstrated that NSC-derived extracellular vesicles traffic functional mitochondria capable of integrating the mitochondrial network in target cells (Peruzzotti-Jametti et al., 2021). They first isolated extracellular vesicles from the secretome of mouse NSCs *in vitro* and performed a proteomic analysis. Surprisingly, extracellular vesicles were highly enriched in mitochondrial proteins. Subsequent morphological analysis by cryogenic electron microscopy revealed intact mitochondria inside the extracellular vesicles. Using NSCs that constitutively express the mitochondrial MitoDsRed fluorescent reported, the authors further showed that NSCs can transfer mitochondria through extracellular vesicles to fibroblasts and macrophages. These results are particularly meaningful, considering the relevance of mitochondria in NSC fate. Since neuronal differentiation requires a metabolic shift towards mitochondrial oxidative phosphorylation, mitochondrial transfer between the NSC pool may play a determinant role in ensuring adequate adult neurogenesis.

1.3.4 Therapeutic Properties of the NSC Secretome

In view of the various beneficial factors present in the NSC secretome, several studies have sought to test the effects of the NSC secretome in neurodegenerative disorders (Q. Zhang et al., 2020). Conventionally, this is performed either by collecting the conditioned media of cultured NSCs or by isolating extracellular vesicles from said conditioned media, and subsequent injection into animal disease models (Q. Zhang et al., 2020).

The NSC secretome seems to exert neuroprotective effects in various animal models of neurodegenerative disorders. Chronic administration of conditioned media from human NSCs in a spinal cord injury rat model resulted in axonal regeneration, neuronal preservation, and locomotor recovery (Liang et al., 2014). NSC transplantation studies also support the therapeutic properties of the NSC secretome in spinal cord injury. As an example, researchers transplanted human NSCs into the injury epicentre of rats with spinal cord injury and observed a rapid improvement in motor function (Romanyuk et al., 2015). Analysing the injured tissue revealed that, following transplantation, the expression of human neurotrophic factors (nerve growth factor, fibroblast growth factor 8, glial cell line-derived neurotrophic factor) increased, while the expression of rat neurotrophic factors suffered no alteration. This indicates that transplanted NSCs promote recovery from spinal cord injury through the secretion of neurotrophic factors. In a rat model of stroke, human NSC transplantation resulted in enhanced functional recovery (Amemori et al., 2015). Further analysis revealed that few NSCs migrated into the infarcted tissues and exhibited poor survival. On the other hand, neuronal survival and angiogenesis increased, while astrogliosis and microglial infiltration decreased. Therefore, the authors suggest that the therapeutic properties of NSC transplantation in this model were due to the release of a neurorestorative secretome.

Administration of conditioned media from NSCs also yields positive results in animal models of Parkinson's disease. Ni et al showed that concentrated NSC-derived conditioned media improves motor function and reduces dopaminergic neuronal loss in a Parkinson's disease rat model (Ni et al., 2022). A study by Mendes-Pinheiro et al confirmed these findings, further showing that conditioned media injection was more beneficial than NSC transplantation (Mendes-Pinheiro et al., 2018). As the authors suggest, further studies need to be conducted to determine which factors in the secretome mediate these beneficial effects. Collectively, these studies show that NSCs are capable of modulating neurons, microglia, astrocytes, and endothelial cells through their secretome, thereby promoting regeneration and recovery in animal models of neurodegeneration.

Recent studies are also beginning to explore the effects of NSC-derived extracellular vesicles in animal disease models. Webb et al have shown, in both rodent and porcine models of stroke, that extracellular vesicles derived from human NSCs efficiently target infarcted tissues when injected intravenously (Webb, Kaiser, Jurgielewicz, et al., 2018; Webb, Kaiser, Scoville, et al., 2018). This treatment results in improved tissue preservation, motor function, and episodic memory. Quantitative flow cytometry analysis of blood samples also revealed that treatment with extracellular vesicles promoted macrophage polarization towards an anti-inflammatory phenotype, increased the number of regulatory T cells, and decreased pro-inflammatory Th17. Therefore, NSC-derived extracellular vesicles seem to induce a reparative systemic immune response, reducing inflammation and promoting recovery. In a transgenic mouse model of Alzheimer's disease, extracellular vesicles derived from human NSCs also seem to downregulate the systemic inflammatory response, as portrayed by the decrease in pro-inflammatory cytokines (interferon-γ and interleukin-17) and increase in anti-inflammatory cytokines (interleukin-10) in the spleen of treated mice (Apodaca et al., 2021). Furthermore, extracellular vesicle treatment reduced the number of amyloid β aggregates—which disrupt cell function and are involved in Alzheimer's pathology—in the amygdala and medial prefrontal cortex. This effect may be related to the upregulation of autophagy by NSC-derived extracellular vesicles, as reported in a rat model of spinal cord injury (Rong et al., 2019). Lastly, autophagy is a conserved cellular process through which cells sequester intracellular components, such as organelles and proteins, and deliver them to lysosomes for degradation and recycling (Glick, Barth, & Macleod, 2010). Rong et al has reported increased levels of the autophagy markers beclin-1 and LC3B in the infarcted tissue of spinal cord injury rats treated with mouse NSC-derived extracellular vesicles (Rong et al., 2019). Further *in vitro* experiments in primary spinal cord neurons confirmed that extracellular vesicles increase autophagosome formation and revealed that co-treatment with the autophagy inhibitor 3-methyladenine prevented the anti-apoptotic effects of extracellular vesicles. As such, autophagy induction appears to be involved in the beneficial effects of extracellular vesicles. Considering the large number of neurodegenerative disorders linked with the formation of toxic insoluble protein aggregates, the ability of NSC-derived extracellular vesicles to upregulate autophagy may prove to be very promising.

1.3.5 Adult Neurogenesis and the NSC Secretome

As explained in the subsection 1.2.3. of this introduction, successful adult neurogenesis depends on the concerted action of all members of the neurogenic niche. Microglia phagocyte apoptotic new-born neurons and the vasculature directs the migration of neuroblasts (Bovetti et al., 2007; Sierra et al., 2013). Furthermore, all members secrete factors that regulate NSC fate and behaviour (Aarum et al., 2003; Q. Shen et al., 2004; H. Song et al., 2002). Studies show that the NSC secretome modulates the behaviour of different cell types of the neurogenic niche, possibly orchestrating adult neurogenesis (**Figure 1.5**).

Mosher et al tested the influence of the NSC secretome in microglial behaviour *in vitro* (Mosher et al., 2012). They exposed both primary mouse microglia and the BV2 microglia cell line to conditioned media from primary mouse NSCs. The exposure resulted in increased microglial proliferation, migration, and phagocytosis. Since VEGF was identified in the NSC-derived conditioned media, the authors tested the role of this factor in modulating microglial behaviour. Curiously, treating microglia with VEGF was sufficient to induce proliferation, migration, and phagocytosis, while removing VEGF from the NSC-derived conditioned media prevented these effects. Therefore, the NSC secretome seems to activate microglia through the secretion of VEGF. These results have important implications in the context of adult neurogenesis. In fact, NSCs' ability to recruit microglia with enhanced phagocytic capacity may be key in ensuring adequate removal of apoptotic new-born neurons, thus facilitating adult neurogenesis. On the other hand, the NSC secretome can suppress neuroinflammation in situations of injury. Zhou et al showed that treatment with human NSC secretome reduces systemic and neuroinflammation in LPS-treated mice, as exemplified by reduced gliosis and increased levels of anti-inflammatory cytokines (Jiqin Zhou et al., 2022). Further *in vitro* experiments revealed that the NSC secretome promotes polarization of LPS-exposed BV2 microglia from the M1 proinflammatory phenotype to the M2 anti-inflammatory phenotype. As such, the NSC secretome appears to modulate microglia in a context-dependent manner. In physiological conditions, NSCs seem to release factors which recruit and activate microglia, thereby promoting the clearance of cell debris from the neurogenic niche and facilitating neurogenesis. In pathological conditions, NSCs seem to release factors which suppress microglial activation, thereby preventing exacerbated neuroinflammation and damage to the brain.

Li et al have studied the effects of NSCs on endothelial cells and blood vessel formation *in vitro* (Q. Li, Ford, Lavik, & Madri, 2006). After co-culturing mouse NSCs and endothelial cells separated by a porous membrane, they observed robust vascular tube formation and maintenance. Curiously, endothelial cell proliferation was unaffected, suggesting that the new blood vessels were formed as a result of the reorganization of pre-existing endothelial cells, a process known as morphogenesis. The authors identified BDNF, VEGF, and nitric oxide as important mediators of NSC-induced blood vessel formation. Similar effects have been observed in *in vitro* models of injury. Roitbak et al established mouse NSC and endothelial cell co-cultures exposed to transient oxygen and glucose deprivation to replicate ischemia (Roitbak, Li, & Cunningham, 2008). NSCs prevented endothelial cell death and rescued blood vessel formation, in comparison with endothelial cells in monoculture under ischemic conditions. Once again, VEGF mediated these effects, as inhibiting VEGF signalling prevented the beneficial effects of

co-culturing. Further *in vivo* experiments revealed that NSC transplantation in a mouse model of ischemia resulted in increased microvascular density, showing that NSCs can promote angiogenesis *in vivo*. Since neuroblast migration is directed by the vasculature, NSCs' ability to promote blood vessel formation can play an important role in ensuring the correct delivery of adult-born neurons to their target sites. Furthermore, the reciprocal positive signalling established between NSCs and endothelial cells may be a powerful driving force during adult neurogenesis.

NSCs also regulate their own fate and behaviour through autocrine signalling. Many factors are involved in the autocrine regulation of NSCs, including the classic neurotrophic factors (VEGF, IGF-1, BDNF), as well as some new players (Willis, Nicaise, Peruzzotti-Jametti, & Pluchino, 2020). Toda et al identified a new protein in rodent and human NSCs called stem-cell derived NSC supporting factor (SDNSF) (H. Toda et al., 2003). *In vivo*, SDNSF was found to be upregulated in the rat hippocampus under ischemia-induced adult neurogenesis. *In vitro*, SDNSF increased the viability of NSCs under basic fibroblast growth factor (bFGF) deprivation but had no effect on proliferation. Thus, NSCs appear to protect themselves through the release of SDNSF. Zhou et al observed increased levels of milk fat globule-epidermal growth factor 8 (MFGE8) in quiescent NSCs of the mouse hippocampus (Y. Zhou et al., 2018). Interestingly, conditional deletion of MFGE8 in NSCs led to excessive activation and consequent depletion of the NSC pool, impairing long-term adult neurogenesis. As such, NSC-secreted MFGE8 seems to maintain NSCs in a quiescent state. Despite remaining relatively unexplored, there is direct evidence that the NSC secretome can stimulate adult neurogenesis under specific contexts. In a mouse model of stroke, administration of concentrated conditioned media from mouse NSCs increased the number of BrdU+/DCX+ and BrdU+/NeuN+ positive cells around the lesion site 28 days post-stroke (Doeppner et al., 2017). Thus, the secretome of NSCs may promote adult neurogenesis after stroke to promote functional recovery. In a mouse model of Alzheimer's disease, administration of conditioned media from mouse NSCs had similar effects (Hijroudi et al., 2022). Treated mice displayed enhanced functional recovery and the number of BrdU+/Nestin+ and BrdU/NeuN+ cells in the subgranular zone of the hippocampus increased. Cumulatively, these studies show that NSCs can modulate adult neurogenesis in the injured brain through the release of a neuroregenerative secretome.

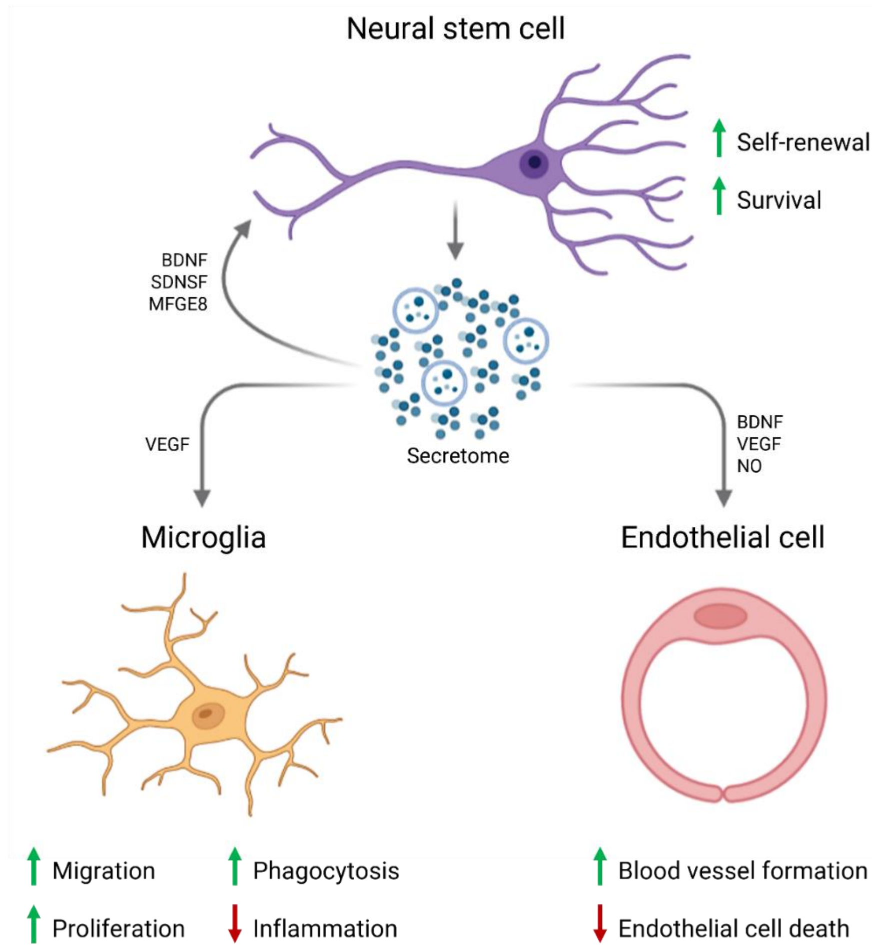

Figure 1.5 – The NSC secretome regulates the neurogenic niche. The NSC secretome has been shown to protect NSCs, enhance the protective properties of microglia, and stimulate blood vessel formation through several growth factors. (Created with BioRender.com). BDNF, brain-derived neurotrophic factor; MFGE8, milk fat globule-epidermal growth factor 8; NO, nitric oxide; NSC, neural stem cell; SDNSF, stem-cell derived neural stem cell supporting factor; VEGF, vascular endothelial growth factor.

2

MOTIVATION AND AIMS

Since its discovery in mammals, adult neurogenesis has been implicated in a wide variety of cognitive processes, ranging from memory formation to mood regulation (Oomen, Bekinschtein, Kent, Saksida, & Bussey, 2014). Therefore, the generation of new neurons in the adult brain is an essential part of appropriate brain functioning and overall health. Importantly, lifestyle choices, such as dieting and exercise, as well as neurodegenerative and neuropsychiatric disorders can modulate adult neurogenesis (Levenson & Rich, 2007; Winner & Winkler, 2015; Yau, Gil-Mohapel, Christie, & So, 2014). These effects appear to be mediated by systemic signals, which cross the blood-brain barrier and reach the brain through the circulation and cerebrospinal fluid. However, the cellular and molecular mechanisms behind the modulation of adult neurogenesis by these systemic signals remain largely elusive.

Recently, a new role for NSCs beyond neuronal differentiation has emerged: the regulation of its surroundings through paracrine signalling (Willis et al., 2020). Numerous studies show that NSCs are potent regulators of their microenvironment, releasing a secretome which regulates the behaviour of its neighbours, particularly microglia, endothelial cells, and even other NSCs (Mosher et al., 2012; Y. Zhou et al., 2018). Significantly, NSCs are uniquely positioned in the brain, at the interface between the neurogenic niches and systemic signalling. Their close association with the vasculature and the cerebrospinal fluid-filled ventricles means that NSCs are the first cell types to sense changes in systemic signals, making them prime candidates for relaying this information to its neighbours (Bjornsson et al., 2015). Thus far, no studies have explored the impact of systemic cues on the paracrine regulation of the neurogenic niche by NSCs. Here, we posit that NSCs orchestrate adult neurogenesis in response to changes in systemic signalling, modulating the activity of the various members of the niche and ensuring they work in tandem to provide an appropriate response.

To explore this hypothesis, we used an *in vitro* system previously established by our group. The tau-GFP mouse NSC (NS-TGFP) cell line was pre-conditioned with compounds that mimic systemic signals that NSCs may receive from the blood and cerebrospinal fluid in both favourable and unfavourable conditions. To replicate injury *in vitro*, cells were pre-conditioned with MPP^+, a neurotoxic compound that causes oxidative stress (Nicklas, Vyas, & Heikkila, 1985). The conditioned media (CM) produced by these injured NSCs was named injury CM (injCM). Previous studies from our group have revealed that NSCs treated with injCM, representing healthy NSCs receiving danger signals from

neighbouring injured NSCs, release a neuroprotective conditioned media, named boosted CM. To replicate changes in systemic signalling in the context of depression, cells were treated with the serum of mice exposed to the unpredictable chronic mild stress (uCMS) protocol, which causes depressive-like behaviour (Nollet, 2021). In the context of favorable conditions, cells were treated with well-established metabolic regulators, known to enhance mitochondrial activity and metabolism in NSCs. Particularly, we used TUDCA, propionate, and a cocktail of growth factors (BDNF, IGF-1, and VEGF) which are upregulated after physical exercise *in vivo* (Maass et al., 2016; Ribeiro et al., 2020; Soares et al., 2018).

Previous studies from our group showed that both negative (boosted CM and uCMS CM) and positive (TUDCA CM, propionate CM, exercise CM; collectively named mitCMs) pre-conditioning improve the therapeutic properties of the NSC secretome (unpublished data). In fact, these CMs were capable of rescuing H_2O_2-induced cell death of NSCs undergoing neuronal differentiation. Furthermore, boosted CM was shown to induce oxidative phosphorylation in recipient differentiating NSCs. Since a metabolic shift towards oxidative phosphorylation is a crucial event during neuronal differentiation, these preliminary results suggest that pre-conditioned NSCs can release a neurogenesis-boosting secretome. Since successful adult neurogenesis depends on the concerted action of the multiple members of the neurogenic niche, in this study we aimed to test and compare the effects of pre-conditioned CMs on different cell types, particularly:

i. On the neuronal differentiation rate of NSCs
ii. On the phagocytic capacity of microglia
iii. On the formation of new blood vessels by endothelial cells

Furthermore, to identify potential mechanisms behind the action of pre-conditioned CMs, we sought to characterize their composition in terms of metabolites and miRNAs. While often overlooked, these molecules are powerful signaling factors, capable of regulating cell fate, behavior, and metabolism.

Therefore, this study represents an important first step in unveiling a new role for NSCs in the adult brain. Rather than simply differentiating into new neurons, they may actively orchestrate neurogenesis through paracrine signaling according to the body's supplies. A deeper understanding of the regulation of adult neurogenesis has great therapeutic potential, considering the important role played by this process in multiple neurodegenerative and neuropsychiatric diseases. Moreover, characterizing the composition of neurogenesis-boosting secretomes will help identify beneficial factors, contributing to the development of scalable, cell-free therapies.

3

MATERIALS AND METHODS

3.1 Ethics Statement

The NS-TGFP cell line used in this study was obtained from Dr. Smith's Laboratory, University of Cambridge, Cambridge, UK, and provided by Dr. Henrique, University of Lisbon, Lisbon, Portugal. The Animal Ethical Committee at the Faculty of Pharmacy, University of Lisbon, Portugal waived the need for approval.

3.2 Cell Line Handling and Maintenance

The NS-TGFP cell line was derived from 14.5-days post-coitum mouse fetal forebrain, as previously described (Pratt, Sharp, Nichols, Price, & Mason, 2000; Silva, Chambers, Pollard, & Smith, 2006). Forebrain progenitor cells in the fetus are quiescent and similar to post-natal NSCs, representing a good model of adult NSCs. *In vitro*, these cells continuously expand by symmetrical division and are capable of tri-potential differentiation into neurons, astrocytes, or oligodendrocytes (Glaser, Pollard, Smith, & Brüstle, 2007; Pollard, Conti, Sun, Goffredo, & Smith, 2006).

NSCs were grown in monolayer and maintained in self-renewal conditions in DMEM/F-12 + GlutaMAX medium (Gibco, Thermo Fisher Scientific, Inc., USA) in 75 cm^2 tissue culture-treated flasks (Falcon, Corning Inc., NY, USA) at 37ºC in a humified atmosphere of 5% CO_2. The self-renewal medium was supplemented with penicillin-streptomycin (Gibco, Thermo Fisher Scientific Inc., USA), N-2 supplement (Gibco, Thermo Fisher Scientific Inc., USA), epidermal growth factor (EGF; PeproTech EC, UK) and basic fibroblast growth factor (bFGF; PeproTech EC, UK) (see **table 3.1** for further details).

Cell culture was renewed every 2-3 days when 80% confluence was reached, to prevent degeneration and death due to nutrient depletion and contact inhibition. The depleted media was removed and 1.5 ml of StemPro Accutase Cell Dissociation Reagent (Gibco, Thermo Fisher Scientific, Inc.) was added to gently detach cells from the flask. After 3 min of incubation, DMEM/F-12 + GlutaMAX was added to dilute the accutase and further dissociate cells from the flask. One third of the cell suspension was collected and centrifuged at 500 *g* for 5 min at room temperature. The supernatant was discarded

and the cell pellet was resuspended in freshly prepared self-renewal medium, which was then transferred to a new 75 cm^2 flask.

Table 3.1 – Resumed details of culture media composition used to expand mouse NSCs.

Self-renewal Media % (V/V)	**Differentiating Growth Media % (V/V)**
DMEM/F-12 + GlutaMAX	DMEM/F-12 + GlutaMAX
1% Penicillin-streptomycin	1% Penicillin-streptomycin
1% N-2	0.5% N-2
20 ng/ml bFGF	10 ng/ml bFGF
20 ng/ml EGF	1% B-27

3.3 Secretome Production

NSCs were plated at a density of $1x10^5$ cells/cm^2 in 60 mm tissue culture-treated dishes (Falcon, Corning Inc., NY, USA). 24 hours (h) later, cells were pre-conditioned with one of the treatments represented in **Table 3.2**. 10 h later, the cell media was completely replaced with fresh self-renewal media to remove the treatment compounds. 14 h later, the conditioned media (CM), containing the cells' secretome, was collected, centrifuged at 500 *g* for 5 minutes (min) at room temperature to remove cell debris, and stored at -80ºC for future use (**Figure 3.1**).

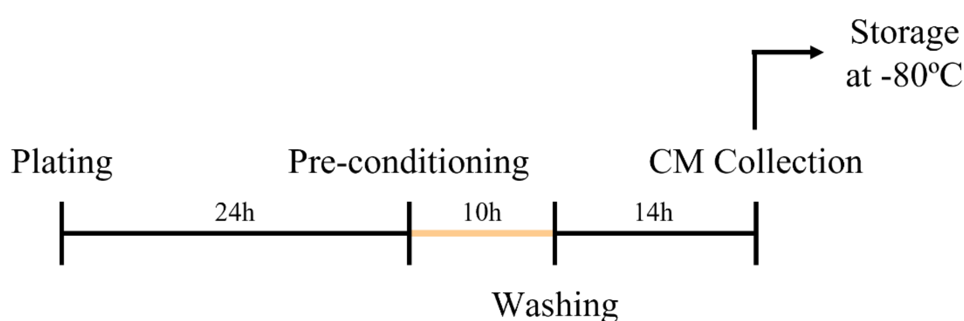

Figure 3.1 – Schematic step-by-step representation of the conditioned media preparation protocol.

To mimic NSC injury *in vitro*, cells were treated with MPP^+ (D048-000; MERCK), a neurotoxic compound that inhibits the complex I of the electron transport chain and causes oxidative stress (Nicklas et al., 1985). The conditioned media produced by these injured NSCs was named injury CM (injCM). The conditioned media of untreated cells was named untreated CM and used as a control for injCM. Previous studies from our group have revealed that NSCs treated with injCM, representing healthy NSCs receiving transient danger signals from neighboring injured NSCs, release a neuroprotective conditioned media, named boosted CM. Cells were also treated with untreated CM, and the resulting conditioned media was named CTRL CM and used as a control for boosted CM.

NSCs were also treated with well-established metabolic regulators: tauroursodeoxycholic acid (TUDCA; Sigma-Aldrich Corp.), propionate (P1880; Sigma-Aldrich Corp), and a cocktail of growth factors comprising BDNF (kindly provided by Regeneron), IGF-1 (I3769; Sigma-Aldrich Corp), and VEGF (AF-100-20; PeproTech) that mimics the effects of exercise *in vitro*. These treatments promote mitochondrial activity in NSCs and represent signals that NSCs may receive in favorable conditions, such as healthy dieting and physical exercise. The resulting conditioned media were named TUDCA CM, propionate CM, and exercise CM, respectively. Collectively, they were named mitCMs. Untreated CM was used as the control condition.

Finally, NSCs were treated with the serum of mice with depressive-like behavior, to mimic signals that NSCs may receive from the vasculature in the event of depression. The unpredictable chronic mild stress (uCMS) paradigm was used to mimic depressive-like behavior in mice and mice serums were kindly provided by Dr. Sara Xapelli, iMM, University of Lisbon, Lisbon, Portugal. The CM of NSCs treated with uCMS serum was named uCMS CM. As a control for uCMS CM, cells were treated with the serum of healthy mice and the resulting healthy CM was collected.

Table 3.2 – Pre-conditioning conditions used to produce CMs.

Conditioned Media	**Treatment**
Untreated CM	No treatment
injCM	50 µM MPP^+
Control CM	25% untreated CM
Boosted CM	25% injCM
TUDCA CM	100 µM TUDCA
Propionate CM	1 mM propionate
Exercise CM	30 ng/ml VEGF, IGF-1, and BDNF
Healthy CM	1% serum from healthy mice
uCMS CM	1% serum from uCMS mice

3.4 Single-Cell Calcium Imaging

NSCs were plated at a density of $1x10^5$ cells/cm^2 in tissue culture-treated µ-slide 8 well chamber slides (80826; Ibidi) and maintained in self-renewal condition. After 24 h, self-renewal media was completely replaced with differentiating growth media to induce neuronal differentiation (**Table 3.1**). After 24 h, 25% of the differentiating growth media was replaced with CMs. MitCMs, boosted CM, and uCMS CM were used in this experiment, as well as their respective controls. After 24 h, single-cell calcium imaging was performed. This experiment evaluates the neuronal differentiation of NSCs, according to variations in intracellular calcium concentration ($[Ca^{2+}]_i$) in single cells following exposure to 50 mM KCl and 100 mM histamine. KCl depolarization causes an increase in $[Ca^{2+}]_i$ in neurons, while stimulation with histamine leads to an increase in $[Ca^{2+}]_i$ in stem/progenitor cells. To measure $[Ca^{2+}]_i$ variations, the ratiometric dye Fura-2/AM was used. Fura-2/AM can be excited at two wavelengths: 340 nm

for calcium-bound and 380 nm for unbound, and it emits at 510 nm. Therefore, the 340/380 fluorescence ratio provides a relative measure of $[Ca^{2+}]_i$.

For single-cell calcium imaging, cells were washed with Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM MgCl2, 1 mM CaCl2, 6 mM glucose, 10 mM HEPES, pH 7.4) and loaded with 5 μM Fura-2/AM (F1221; Invitrogen) in Krebs solution for 45 min. Cells were washed again with Krebs solution and the slide was mounted on an inverted microscope with epifluorescent optics (Axiovert 135TV, Zeiss) equipped with a xenon lamp and band-pass filters of 340 and 380 nm wavelengths. Image pairs were obtained for 5 min every 10 seconds (s) by exciting the preparations at 340 and 380 nm. Excitation wavelengths were changed through a high-speed wavelength switcher, Lambda DG-4 (Sutter Instrument, Novato, CA, United States). After this pre-stimulation period, Krebs solution was completely replaced with a high-potassium Krebs solution (containing 50 mM KCl, isosmotic substitution with NaCl) to stimulate mature neuron depolarization. Image pairs after KCl stimulation were obtained for 2 min every 1 s. High-potassium Krebs was completely replaced with Krebs solution to re-establish baseline conditions and image pairs were obtained for 3 min every 10 sec. Krebs solution was completely replaced with 100 μM histamine in Krebs solution to stimulate NSC depolarization. Image pairs were obtained for 2 min every 1 s. Finally, the histamine solution was completely replaced with Krebs solution to re-establish baseline conditions and image pair were obtained for 3 min every 10 s. Image data were recorded with a cooled CCD camera (Photometrics CoolSNAP fx) and processed and analyzed using the software MetaFluor (Universal Imaging, West Chester, PA, United States). Regions of interest were defined manually over the cell profile. KCl and histamine peaks given by the normalized ratios of fluorescence at 340/380 nm, at the proper time periods, were used to calculate the ratios of the responses.

3.5 Microglial Phagocytosis Assay

Primary mouse microglial cell culture was established by Dr. Ana Rita Vaz, in collaboration with Prof. Dora's research group at iMed.ULisboa. Microglia were incubated with 50% of different CMs and 50% of microglial media supplemented with 1% fetal bovine serum. Exercise CM, propionate CM, boosted CM, and uCMS CM were used in this experiment, as well as their respective controls. Furthermore, microglia were incubated with unconditioned microglial media or unconditioned NSC self-renewal media as negative controls. After 24 h, microglia were incubated with 0.0025% (w/w) fluorescent latex beads (L1030; Sigma-Aldrich) for 75 min at 37ºC and fixed with freshly prepared 4% (w/v) paraformaldehyde in phosphate buffer saline.

For immunostaining, microglia were permeabilized by completely covering each well with a solution of 0.2% Triton X-100 in phosphate buffer saline for 20 min at room temperature. Cells were washed with phosphate buffer saline and blocked with blocking solution (3% BSA) for 30 min at room temperature. Then, microglia were incubated with rabbit anti-Iba1 primary antibody (1:250, 019-19741, Wako) diluted in blocking solution on a moist bed at 4ºC overnight. After three washes with phosphate buffer saline, microglia were incubated with goat anti-rabbit Alexa Fluor 594 secondary antibody (1:500, A-11012, Invitrogen) diluted in blocking solution at room temperature for 2 h. Afterwards, cells

were washed three times with phosphate buffer saline and nuclei were counterstained with Hoechst 33258 dye. Cells were once more washed with PBS and mounted with one drop of PBS:glycerol (1:1).

Fluorescence was visualized under 630× magnification in an AxioSkope A1 fluorescence microscope coupled with AxioCam HR camera (Zeiss). Five random images from different microscopic fields were acquired per sample. The Fiji software was used to analyze the images. Briefly, individual microglial cells were identified as regions of interest (ROIs) in the Iba-1 (594 nm) channel. Subsequently, the fluorescence intensity of the latex beads in each cell was measured (488 nm channel). Microglial cells with high phagocytic capacity were defined as those with a relative fluorescence value superior to 16. Using the particle measurement analysis tool, we also determined three morphological parameters: area, perimeter, and Feret's diameter.

3.6 Vascularization Assay

The following experiment was performed by Dr. João Afonso, ICVS, University of Minho, Braga. Human umbilical vein endothelial cells from Passage 5 were used on two independent assays. 40 µL of Matrigel (BioCell, Inc) was plated in 96-well plates for 1 h, in the cell incubator, to allow for the gelification process to occur. Cells were resuspended in previously thawed CMs (supplemented with endothelial growth supplement) and plated in each corresponding well at a cell density of 80 000 cells/cm^2. Boosted CM and uCMS CM were used in this experiment, as well as their respective controls. A positive control group was performed in normal endothelial medium. After 16 h, the vessel-like structures formed were fixed with 4% paraformaldehyde for 45 min.

After fixation, cells on the hydrogel were permeabilized with 0.3% (w/v) PBS-Triton X for 10 min, followed by 3 washes with PBS 1x. Then, phalloidin (0.1 µg/ml) (Sigma) was added for 45 min, before doing 3 washes with PBS 1x. All pictures were taken on Olympus Widefield Inverted Microscope IX81, using the Stage Navigator tool of the CellSens software (Olympus) to do a mosaic of the vessel-like structures obtained. AngioTool64 Version 0.6a Software was used to evaluate the degree of vascularization. After background and small particles removal, an intensity and diameter threshold was applied to do a skeleton of the vessel-like structures. After this, the software automatically quantifies different parameters of the vascular organization such as vessels area, branching index, and average lacunarity.

3.7 Gas Chromatography-Mass Spectrometry

The following experiment was performed by Dr. Margarida Silva, iMed.UL, University of Lisbon, Lisbon. Aliquots of CMs containing the target metabolites were processed for the quantitative analysis of organic acids using gas chromatography coupled with mass spectrometry (GC-MS). MitCMs, boosted CM, and uCMS were used in this experiment, as well as their respective controls. The analytical system consisted of a QP2010Plus GC-MS (Shimadzu, Kyoto, Japan), comprising a GC-17 model and an automated injector (model AOC-20i). The fused silica capillary column was from

Chrompack (Varian) with 25 m x 0.25 mm (inner diameter) x 0.25 µm (film thickness). Helium was used as carrier gas and data acquisition was controlled by Lab Solutions software (v.1.02).

Firstly, the internal standard 3-phenylbutyric acid (Sigma-Aldrich Co, USA) was added to sample aliquots followed by acidification with 2 M HCl. The addition of ethoxyamine was made to stabilize ketoacids followed by an 1 h incubation at room temperature. After saturation with NaCl, metabolites were extracted twice with ethyl acetate. The organic phases were collected into a glass tube and dried with sodium sulphate anhydrous. The collected supernatants were evaporated under a gentle nitrogen stream. The dried extracts were resuspended in derivatization reagent (BSTFA/TMCS/Pyridine) (Pierce-Thermo Scientific, USA) and incubated for 1 h at 60 ºC. Final derivatives solutions were transferred to microvials and injected (1 µl) into the GC-MS through an automatic injector under the split injection mode (1/50 ratio). Chromatographic separation was achieved through a high-resolution capillary column and analytes were identified based on respective characteristic retention indexes and unequivocal mass spectra (MS), based on an in-house built MS-library, obtained with scanning acquisition mode.

3.8 miRNA Array

The Total Exosome Isolation (from cell culture media) kit (4478359; Invitrogen) was used to isolate exosomes from CMs. Boosted CM and uCMS CM, as well as their respective controls, were used in this experiment. Briefly, to each CM was added half the volume of the total exosome isolation reagent, followed by mixing until a homogenous solution was formed. After incubation at 4ºC overnight, the mixtures were centrifuged at 10000 *g* for 1 h at 4ºC. The supernatant was discarded and the pellet, containing the exosomes, was resuspended in PBS 1x.

The Total Exosome RNA and Protein Isolation kit (4478545; Invitrogen) was used to isolate RNA from the exossomal samples. To each sample was added an equal volume of 2x denaturing solution, followed by thorough mixing and incubation on ice for 5 min. An equal volume of acid-phenol:chloroform was added to each sample, followed by vortexing and centrifugation at 10000 *g* for five min at room temperature. The aqueous upper phase was then transferred to a new tube. To each sample was added 1.25x the volume of 100% ethanol. 3 µl of a miRNA spike-in (cel-miR-39-3p) were added at this point, used as an exogenous control, since the NS-TGFP cell line does not express this miRNA. Samples were vortexed and 700 µl were transferred to a filter cartridge placed on top of a collection tube. Then, they were centrifuged at 10000 *g* for 15 s. The flow-through was discarded and filters were washed once with 700 µl wash solution and with 500 µl wash solution 2/3 (centrifuged at 10000 *g* for 15 s for each wash, followed by discarding of flow-through). Filters were transferred to new collection tubes and 50 µl of nuclease-free water at 95ºC were applied to the center. They were centrifuged at 10000 *g* for 30 s to elute the RNA isolated in the filters. This step was repeated with a new volume of nuclease-free water to increase the recovery of RNA. The RNA eluates were collected and stored at -20ºC.

The TaqMan® Advanced miRNA Assay (A25576; Applied Biosystems) was used to amplify miRNAs from the purified RNA samples. All reaction mixes were prepared according to the

manufacturer's instructions. Before incubation in the thermocycler, samples were always vortexed and spun-down by centrifuging, to ensure thorough mixing. To 2 µl of each RNA sample were added 3 µl of Poly(A) reaction mix. Samples were incubated in the thermocycler for 45 min at 37ºC, 10 min at 65ºC, followed by storing at 4ºC, to perform the poly(A) tailing reaction. 10 µl of Ligation reaction mix were added and samples were further incubated in the thermocycler for 60 min at 10ºC and stored at 4ºC, to perform the adaptor ligation reaction. 15 µl of reverse transcription reaction mix, containing universal primers, were added to each sample and incubation was performed in the thermocycler for 15 min at 42ºC, 5 min at 85ºC, and stored at 4ºC, to perform cDNA synthesis. Finally, 5 µl of the reverse transcription reaction product were mixed with 45 µl of the miR-Amp reaction mix and incubation was performed in the thermocycler for 5 min at 95ºC (1 cycle), 3 s at 95ºC followed by 30 s at 60ºC (14 cycles), 10 min at 99ºC, and storage at 4ºC, to perform the miRNA amplification reaction. The miRNA amplification products were collected and stored at -20ºC.

The miRNA array was performed using the TaqMan® Advanced miRNA Human Serum/Plasma Card (A34717; Applied Biosystems). miRNA amplification products were diluted in a proportion of 1:10 with RNase-free water. Diluted miRNA, TaqMan® Advanced Master Mix (2X) and RNase-free water were mixed in a proportion of 1:2:1. Each reservoir of the array card was filled with 100 µl of the prepared PCR mix. The card was centrifuged, sealed, and loaded into the Quantstudio™ 7 Flex real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The following qPCR protocol was performed: 10 min at 92ºC (1 cycle), and 1 second at 95ºC followed by 20 s at 60ºC (40 cycles). Raw cycle threshold (Ct) values were generated using the Applied Biosystems SDS and RQ Manager software. After filtering for unreliable and undetermined miRNAs (including miRNAs with Ct values < 15 and > 38), the dataset was normalized to the cel-miR-39 exogenous control using the ΔCt method.

3.9 Statistical Analysis

Statistical analysis was performed using Student's t-test and one-way ANOVA followed by Dunnet's post-test for multiple comparisons since the data followed a normal distribution (Shapiro-Wilk test; $p > 0.05$). Results were expressed as mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed with GraphPad Prism 8.0.2 software (GraphPad Software, Inc., USA.)

4

RESULTS AND DISCUSSION

4.1 Neuronal differentiation seems to be enhanced by mitCMs and boosted CM, but repressed by uCMS CM

Adult neurogenesis is a complex, multi-step process involving the coordinated action of several cell types (Ming & Song, 2011). But fundamentally, it depends on the success of a specific event: the neuronal differentiation of NSCs. Previous data collected by our group revealed that both negatively and positively pre-conditioned NSCs release a secretome which increases the viability of differentiating NSCs under injury. Curiously, the transient negatively pre-conditioned NSCs (boosted NSCs) were shown to deliver a secretome capable of inducing oxidative phosphorylation in target cells. This last finding is particularly interesting, since a metabolic shift to oxidative phosphorylation has been shown to actively drive neuronal differentiation through the induction of a nuclear transcriptional program (Khacho et al., 2019). Therefore, we hypothesized that these pre-conditioned CMs can modulate neuronal differentiation.

To approach this hypothesis, we exposed NSCs in the early stages of neuronal differentiation to 25% of pre-conditioned CMs and evaluated neuronal differentiation through single-cell calcium imaging (SCCI). Studies have demonstrated that histamine depolarizes immature NSCs, causing an influx of Ca^{2+}, while KCl does the same in mature neurons. As such, we can use SCCI to measure the influx of Ca^{2+} in response to histamine and KCl. The histamine/KCl response ratio can subsequently be calculated to provide an estimate of the differentiation state of the NSCs. Cells with a histamine/KCl response ratio below 0.8 were considered mature neurons, while cells with a ratio above 1 were considered immature NSCs, as determined by Agasse et al (Agasse et al., 2008).

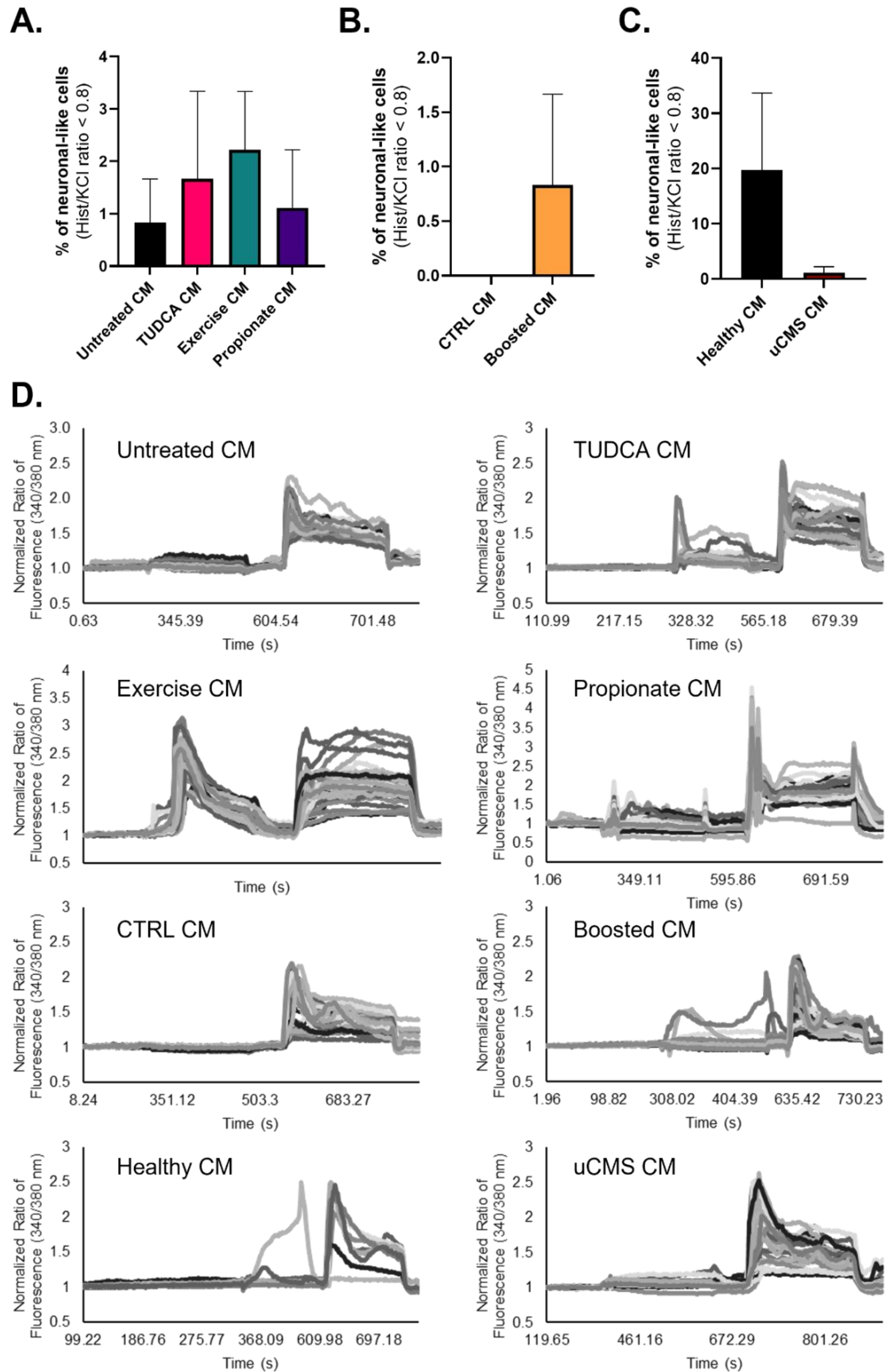

Figure 4.1 – MitCMs and Boosted CM appear to promote neuronal differentiation, while uCMS seems to decrease it. The percentage of differentiating NSCs presenting a neuronal-like response to SCCI (Hist/KCl ratio < 0.8) when exposed to (**A**) mitCMs, (**B**) boosted CM, and (**C**) uCMS CM. Data are expressed as mean ± SEM. n = 3-4. (**D**) Representative SCCI response profiles of an average of 30 cells per condition. Hist, histamine; NSCs, neural stem cells; SCCI, single cell calcium imaging.

Our results showed an apparent increase in the number of differentiated neurons in NSCs treated with mitCMs, particularly those treated with exercise CM (**Figure 4.1.A**). Interestingly, treatment with boosted CM also seemed to increase the number of differentiated neurons (**Figure 4.1.B**), while uCMS CM seemed to decrease differentiation (**Figure 4.1.C**).

Mitochondria are now recognized as central regulators of stem cell function, whose importance extends well beyond their traditional energy generation role (Khacho et al., 2019). These results showed that, in general, mitCMs seem to enhance neuronal differentiation; thus, stimulating mitochondrial activity may be an attractive strategy to improve the neuroregenerative properties of the NSC secretome. In fact, other studies have similarly identified an important link between the mitochondria and the secretome. In adipose-like stem cells, overexpressing peroxisome proliferator activated receptor λ coactivator 1α—a known promoter of mitochondrial biogenesis and activity—improved the antioxidant and anti-inflammatory properties of the secretome, which promoted liver regeneration *in vivo* (Jaeim Lee et al., 2019). Conversely, mitochondrial dysfunction induces a senescence-associated secretory phenotype in several human cell lines (Wiley et al., 2016). Therefore, we suggest that healthy mitochondria can enhance the regenerative properties of the NSC secretome, adding to the ever-expanding array of roles played by this organelle. The specific compounds used to produce mitCMs have all been linked to neurogenesis. A study revealed that treating SVZ-derived neurospheres with TUDCA caused a marked increase in proliferation and differentiation markers (Xavier, Morgado, Rodrigues, & Solá, 2014). Furthermore, intracerebroventricular administration of TUDCA in adult rats also induced NSC proliferation and early differentiation in the SVZ niche (Soares et al., 2018). Propionate has also been shown to enhance proliferation and differentiation of NSCs *in vitro* (Ribeiro et al., 2020). Our results suggest that, beyond direct stimulation of neurogenesis, these compounds can also remodel the NSC secretome, signalling to its neighbours to induce neuronal differentiation. Among the mitCMs, exercise CM had the most prominent effect on neuronal differentiation. Indeed, physical exercise is a well-established promoter of adult neurogenesis in various animal models (Phillips, 2017). In response to exercise, several growth factors increase in the circulation and in the brain, particularly IGF-1, VEGF, and BDNF (Maass et al., 2016). Here, we showed that NSCs exposed to these growth factors appear to release a secretome capable of promoting neuronal differentiation. This observation suggests an important role for the paracrine regulation of the neurogenic niche by NSCs in response to exercise. When exercise-induced neurotrophic factors contact with NSCs through the vasculature and cerebrospinal fluid, they may trigger changes in the NSC secretome, promoting the neuronal differentiation of neighbouring stem cells.

Curiously, boosted CM also seems to induce a similar effect on neuronal differentiation. In situations of brain injury, such as stroke or neurodegenerative disorders, there is often a large loss of neurons, which can severely compromise brain function (Gorman, 2008). In this regard, boosted NSCs, which receive danger signals from injured NSCs, may act in a compensatory manner, releasing a neurogenesis-promoting secretome in an attempt to replace lost neurons. Accordingly, several studies have identified increased rates of neurogenesis after stroke (Cuartero et al., 2021) and in the early stages of some neurodegenerative diseases, particularly Parkinson's and Huntington's (Bender, Fietz, Richter, & Stanojlovic, 2021; Curtis et al., 2003). Our observations suggest an important role for the paracrine

action of NSCs in response to transient situations of injury. As the diseases progress, however, the healthy NSC pool will gradually be lost, which would explain the decreased rates of neurogenesis observed in the later stages of some neurodegenerative diseases (Winner & Winkler, 2015).

In contrast, uCMS CM appears to decrease neuronal differentiation. Decreased adult neurogenesis, particularly in the hippocampus, is a recognized feature of multiple neuropsychiatric disorders (Yun et al., 2016). In major depressive disorder, some studies even suggest a correlation between disease duration and decreased neurogenesis (Yun et al., 2016). Furthermore, antidepressants upregulate adult neurogenesis and blocking this upregulation prevents the behavioural improvements associated with antidepressant medications (Santarelli et al., 2003). Therefore, there appears to be a causal relationship between adult neurogenesis and major depressive disorder. Our results support this link, since exposing NSCs to the serum of depressed mice appears to supress the neurogenic potential of their secretome. As such, the paracrine action of NSCs may help to bridge the link between major depressive disorder and decreased adult neurogenesis: as depression-associated factors in the circulation reach the brain, they contact with NSCs, potentially triggering a shift in their secretome which supresses neuronal differentiation in their surroundings.

All in all, our data corroborate the hypothesis that NSCs can modulate neurogenesis through paracrine regulation in response to systemic cues. Due to technical limitations, this experiment had to be performed at a very early stage of the differentiation protocol (48 h). Consequently, the percentage of differentiated neurons across all conditions was very small, which limited the differences induced by the pre-conditioned CMs. To confirm the trends observed here, we plan to perform SCCI at a more intermediate stage of differentiation, which would increase the number of differentiated cells and potentially emphasize the differences induced by pre-conditioned CMs.

4.2 Microglial phagocytosis appears to be suppressed by uCMS CM

Among the members of the neurogenic niches, microglia play a determining role. During hippocampal neurogenesis, microglia phagocyte apoptotic new-born neurons, preventing the accumulation of cell debris and the release of cytotoxic and immunogenic intracellular contents (Sierra et al., 2010). Furthermore, during the maturation phase microglia prune synapses, ensuring appropriate neural wiring (Ekdahl, 2012). Thus, adequate microglial phagocytosis is an essential part of successful adult neurogenesis and brain regeneration. In this regard, we questioned whether NSCs can modulate microglial phagocytosis through paracrine signalling in response to systemic cues.

To explore this question, we co-incubated microglia with green-fluorescent latex beads and 50% of pre-conditioned CMs. Subsequently, we measured the level of intracellular green fluorescence through fluorescence microscopy, which provides a relative measurement of the level of bead internalization, and consequently of the microglial phagocytic capacity. Here, we defined microglia with high phagocytic capacity as cells with an internalized relative fluorescence value superior to 16.

Surprisingly, our results showed a significant decrease in the proportion of microglia with high phagocytic capacity upon exposure to untreated CM, in comparison with exposure to unconditioned NSC self-renewal medium (**Figure 4.2.A**). Boosted CM appeared to slightly increase this proportion (**Figure 4.2.D**), while uCMS CM appeared to markedly decrease it (**Figure 4.2.E**), in comparison with their respective controls. Finally, mitCMs did not induce any effect in the proportion of microglia with high phagocytic capacity (**Figure 4.2.C**). We also analysed microglial morphological parameters (area, perimeter, and Feret's diameter), but no changes were observed across all conditions (**Figure 4.3**).

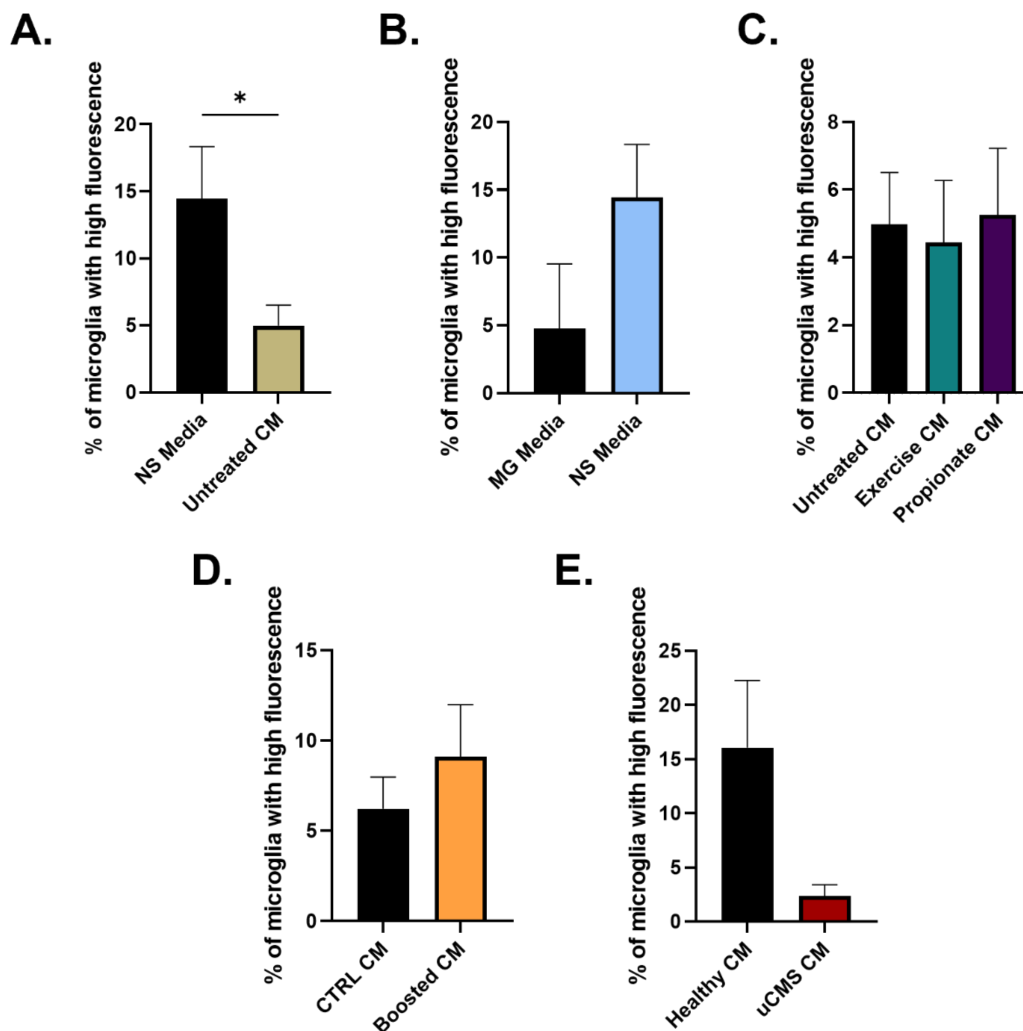

Figure 4.2 – Boosted CM seems to slightly stimulate microglial phagocytosis, while untreated CM and uCMS CM suppress it. The percentage of microglial cells with high phagocytic capacity (intracellular relative fluorescence intensity > 16) when exposed to (**A**) untreated CM, (**B**) NSC media, (**C**) mitCMs, (**D**) boosted CM, and (**E**) uCMS CM. Data are expressed as mean ± SEM. n = 3-6. *$p < 0.05$ vs. NSC media, unpaired and parametric t-test.

Our results show that the NSC secretome can decrease microglial phagocytosis, as portrayed by a significant decrease in the percentage of cells with high phagocytic capacity after exposure to untreated CM. Curiously, this contradicts the findings published in the literature. Mosher et al tested the influence of the NSC CM on primary mouse microglia and on the BV2 microglial cell line (Mosher et al., 2012).

They observed a dose-dependent increase of microglial phagocytosis in response to NSC CM in both cases. However, to the best of our knowledge, this increase was in comparison with microglia in microglial media. As such, the observed increase in microglial phagocytosis could be related to factors present in the NSC cell culture medium which are not secreted by NSCs. To test this hypothesis, we performed this experiment comparing the activity of microglia exposed to unconditioned microglial medium with those exposed to unconditioned NSC medium. Accordingly, exposure to unconditioned NSC media seemed to increase microglial phagocytic capacity, supporting our hypothesis (**Figure 4.2.B**). Therefore, our results indicate that NSCs, contrary to previous findings, have a supressing effect on microglial phagocytosis.

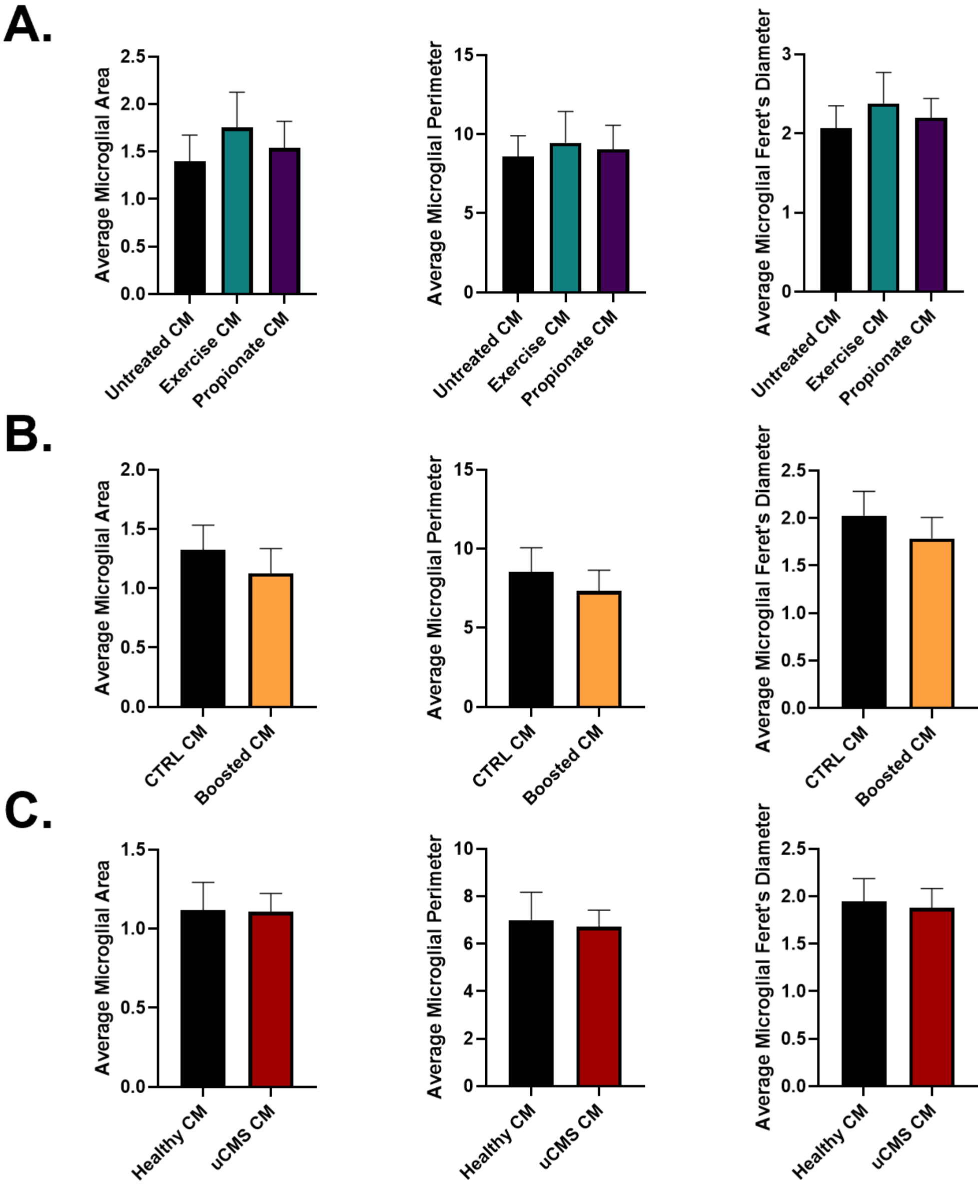

Figure 4.3 – Pre-conditioned CMs do not alter microglial morphology. Average area, perimeter, and Feret's diameter of microglial cells exposed to (**A**) mitCMs, (**B**) boosted CM, and (**C**) uCMS CM. Data are expressed as mean ± SEM. n = 3-6.

Pre-conditioning NSCs with metabolic regulators did not seem to change the phagocytosis-regulating effects of the secretome, as mitCMs had no effect on the percentage of microglia with high phagocytic capacity. This is particularly surprising in the case of exercise CM, since physical exercise is known to regulate microglial activity in the brain (Mee-inta, Zhao, & Kuo, 2019). A recent study demonstrated that a 6-week treadmill exercise protocol in mice promoted recovery after intracerebral haemorrhage, partly by increasing the number of phagocytic microglia (Kinoshita et al., 2021). Furthermore, metabolic reprogramming from glycolysis to oxidative phosphorylation enhances microglial phagocytosis (S. Song et al., 2022) and exercise has been shown to decrease glycolysis in the microglia of aged mice (Mela et al., 2020). However, the mechanisms through which physical exercise influences microglial metabolism remain unclear. As such, it is possible that NSCs are not involved in the metabolic reprogramming of microglia in response to exercise. An investigation of the metabolic profile of microglia in response to NSC CMs would greatly contribute to our understanding of its effects. Thus far, these findings suggest that the increased microglial phagocytosis induced by exercise in the brain is not mediated by the paracrine action of NSCs.

In situations of transient injury, NSCs may promote microglial phagocytosis, as suggested by the apparent increase in microglia with high phagocytic capacity exposed to boosted CM. After injury in the brain, there is often an accumulation of cell debris and apoptotic cells that can be highly toxic; thus, an effective clearance of these remains through phagocytosis is key to a healthy recovery (Galloway, Phillips, Owen, & Moore, 2019). Our results suggest that when NSCs receive danger signals, they stimulate microglial phagocytosis through paracrine factors, which may contribute to the clearance of cell debris upon injury and promote recovery. Since successful adult neurogenesis depends on microglial phagocytosis (Sierra et al., 2010), this effect may also indirectly contribute to the generation of new neurons. Accordingly, several *in vivo* studies have demonstrated a microglia-modulating effect of NSC transplantation in disease models. In a mouse model of Alzheimer's disease, human NSC transplantation decreased the levels of amyloid plaques, which resulted in improved cognitive recovery (McGinley et al., 2018). This was associated with increased recruitment of activated microglia, suggesting that NSCs promote microglial phagocytosis of the amyloid plaques, enhancing recovery. In a mouse model of corpus callosum demyelination, NSCs from the subventricular zone were recruited to the damaged areas; these cells were found to be highly enriched in MFGE8 and co-localized with a microglial cell subpopulation presenting an immunomodulatory phenotype (Brousse et al., 2021). *In vitro* experiments also revealed that MFGE8 in the NSC secretome induces microglial phagocytosis of myelin debris. Therefore, the authors suggest that NSCs from the subventricular zone promote recovery after demyelination through promotion of microglial phagocytosis, partly mediated by extracellular MFGE8. Taken together, our results corroborate the published findings in the literature, which indicate that NSCs support microglial phagocytosis after injury by paracrine-dependent mechanisms.

In the context of depression, NSCs may suppress microglial phagocytosis, since uCMS CM seems to markedly decrease the proportion of highly phagocytic microglia. Interestingly, this effect may prove to be beneficial. In a mouse model of depression (chronic social defeat stress), the density of Iba1^{+}CD68^{+} phagocytic microglia was found to be elevated in the hippocampal dentate gyrus (Han et al., 2022).

These cells displayed abnormal phagocytosis of synapses, which can disrupt brain function and contribute to the development of depression symptoms. Minocycline administration resulted in decreased microglial phagocytosis of synapses in the dentate gyrus, and consequently, normalized behavioural deficits. A different study using the uCMS paradigm to induce depressive-like behaviour in mice obtained similar results (Bassett et al., 2021). The authors observed an increase in $CD68^+$ phagocytic microglia in the hippocampal dentate gyrus, which correlated with a decrease in neurogenesis. Administration of minocycline blocked microglial phagocytosis, rescuing neurogenesis and improving behavioural symptoms. Therefore, these studies indicate that excessive microglial phagocytosis contributes to the pathology of depression through abnormal phagocytosis of synapses and suppression of adult neurogenesis. Taking these findings into consideration, our results suggest that NSCs exposed to depression-associated factors attempt to revert abnormal microglial activity, releasing a phagocytosis-suppressing and "calming" secretome.

In summary, the results of this experiment point to a role for NSCs in the regulation of microglial phagocytosis through paracrine signalling in the context of disease. Upon sensing danger signals, NSCs seem to promote phagocytosis, potentially contributing to the clearance of toxic cell debris. Contrastingly, in situations of excessive phagocytosis, such as depression, NSCs may try to supress abnormal phagocytosis, thereby preventing unnecessary elimination of healthy cells and synapses. Curiously, microglial phagocytosis is promoted by a metabolic shift from glycolysis to oxidative phosphorylation, which our group has previously shown to be induced by boosted CM in differentiating NSCs. Studying the metabolic profile of microglia exposed to these CMs could be an attractive next step in clarifying the modulation of microglial activity by the NSC secretome. The potential effects of the observed changes in microglial phagocytosis on neurogenesis also remain unclear. As such, co-culturing differentiating NSCs and microglia exposed to pre-conditioned CMs could provide valuable insights into the indirect effect of microglial modulation by NSCs on neurogenesis.

4.3 Pre-conditioned CMs have no effect on blood vessel morphogenesis

There is a strong association between brain vasculature and adult neurogenesis. In the subventricular zone, blood vessels serve as a migratory scaffold, guiding neuroblasts along the rostral migratory stream (Bovetti et al., 2007); in the subgranular zone, angiogenesis is temporally and spatially associated with neurogenesis, with both processes regulating each other bi-directionally (Chen et al., 2013; Jin et al., 2002). Since adult neurogenesis is so dependent on the supporting vasculature, we hypothesized that NSCs can regulate blood vessel formation in response to systemic cues.

To test this hypothesis, human umbilical vein endothelial cells were resuspended in pre-conditioned CMs and plated on Matrigel, a substrate composed of numerous extracellular matrix proteins and growth factors capable of promoting endothelial cell differentiation and tube formation. After an incubation period, cells were stained with phalloidin and observed through fluorescence microscopy. Several parameters of vascular organization were automatically quantified by the AngioTool software. Due to

limited resource availability, we only performed this experiment for the disease-related pre-conditioned CMs: boosted CM and uCMS CM.

In this experiment, no noticeable changes were observed in terms of vessel area, branching index, and lacunarity across all tested conditions (**Figure 4.4**).

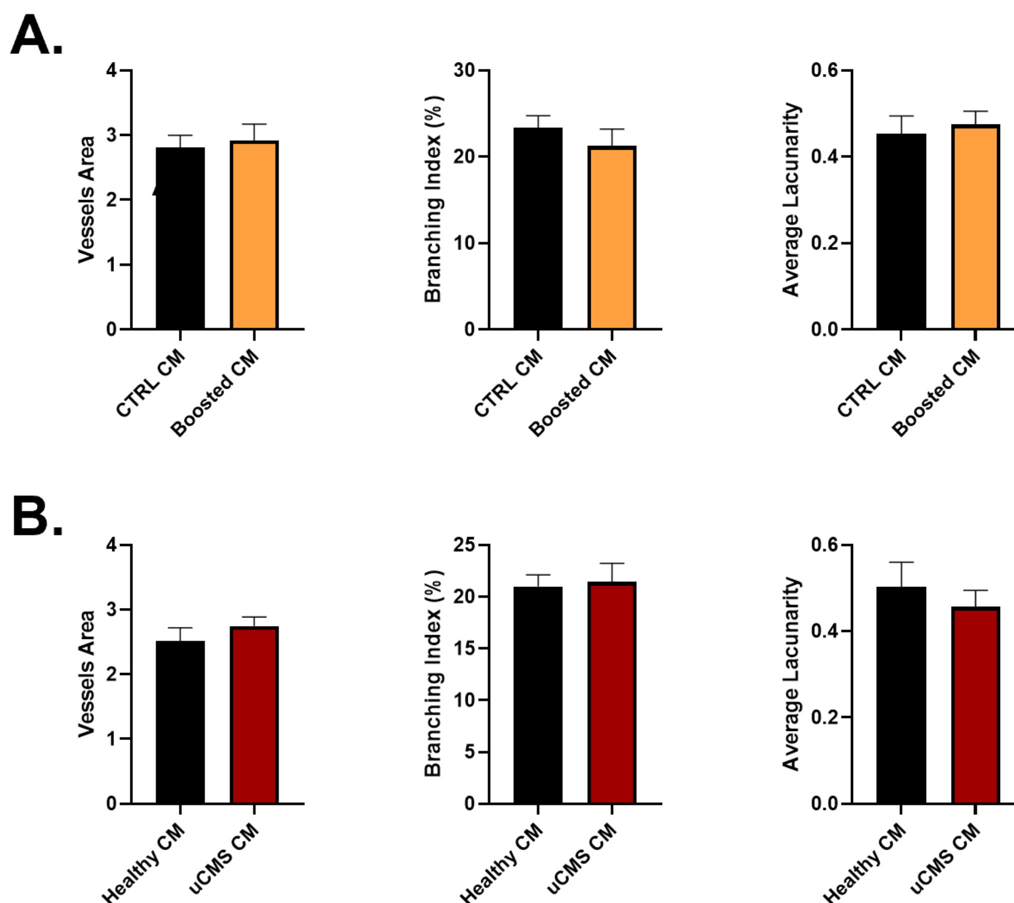

Figure 4.4 – Disease-related pre-conditioned CMs do not alter blood vessel formation or morphology. Morphological parameters (vessels area, branching index, and average lacunarity) of blood vessels formed by HUVECs in Matrigel in response to (A) boosted CM and (B) uCMS CM, automatically quantified by the AngioTool software. Data are expressed as mean ± SEM. n = 3-6. HUVECs, human umbilical vein endothelial cells.

Angiogenesis plays an important role in response to brain injury. Krupinski et al analysed the brains of patients with cerebral ischemic stroke and observed an increase in the density of microvessels in the infarcted tissue (Krupinski, Kaluza, Kumar, Kumar, & Wang, 1994). Further studies revealed that angiogenesis after stroke supports neurogenesis and enhances recovery (Ruan, Wang, ZhuGe, & Jin, 2015). Similarly, transient increases in angiogenesis have been observed in the early stages of several neurodegenerative disorders. For example, in Parkinson's disease, increased microvessel density and pro-angiogenic factors have been identified in animal models and humans (Paul & Elabi, 2022). This increase occurs in the early stages of the disease, as a compensatory response to dopaminergic cell loss; with the progression of the disease and accumulation of damage, there is an eventual regression of the vasculature. In Alzheimer's disease, angiogenesis increases as a response to impaired cerebral brain

flow (Jefferies et al., 2013). Furthermore, amyloid plaques have been shown to directly induce angiogenesis (Boscolo et al., 2007). Although this increase in vascular density represents an attempt to re-establish adequate blood flow to the brain, in later stages it becomes dysregulated and actively contributes to the accumulation of amyloid plaques, aggravating the disease (Jefferies et al., 2013). All in all, increased angiogenesis is a hallmark response to injury in the brain. Surprisingly, our results suggest that, at least in our experimental conditions, NSCs do not seem to be involved in the regulation of angiogenesis after injury. Boosted CM did not increase vessel density or change the branching of the vessels formed by endothelial cells, indicating that NSCs exposed to danger signals do not differentially regulate blood vessel formation and morphogenesis. Since the neurogenic niches are populated by a diverse population of cell types, including astrocytes and microglia, there are plenty of candidates beyond NSCs for the regulation of angiogenesis in response to injury. Furthermore, endothelial cells have been shown to directly respond to danger signals (Mai, Virtue, Shen, Wang, & Yang, 2013), thereby indicating that increased angiogenesis can be a direct response to injury by the vasculature.

Neuropsychiatric disorders have also been linked to dysregulations in angiogenesis. A fascinating study by Casas et al studied the secretome of NSCs derived from iPSCs of patients with schizophrenia (Casas et al., 2018). They found that, in comparison with NSCs derived from healthy individuals, there were decreased levels of pro-angiogenic factors. Furthermore, the secretome reduced the number of tubes and sprouts formed by endothelial cells. Therefore, NSCs can modulate blood vessel formation in the context of neuropsychiatric disorders. Regarding depression, increased levels of VEGF have been identified in the circulation of patients after acute episodes of depression (B.-H. Lee & Kim, 2012), but not in patients suffering recurring episodes (Ventriglia et al., 2009). Antidepressant use has been shown to increase both neurogenesis and angiogenesis in the human hippocampus (Boldrini et al., 2012). As such, there appears to be a therapeutic role for angiogenesis in the context of depression, which may increase in the early stages of the disease as a compensatory mechanism—potentially to support neurogenesis—but eventually becomes dysregulated with the progression of the disease. Nevertheless, since uCMS CM did not change the number or branching of endothelial cell tubes, our results suggest that NSCs have no effect on blood vessel formation in response to depression-associated factors in the circulation. Once again, several other cell types can be involved in the regulation of angiogenesis in depression and endothelial cells can directly respond to circulating factors in the blood. Thus, NSCs' paracrine action does not appear to be involved in the regulation of angiogenesis during depression.

Collectively, the results of this experiment set suggest that NSCs do not modulate blood vessel formation and morphogenesis in the context of disease. Since VEGF is a powerful regulator of angiogenesis, it would be interesting to determine the levels of this growth factor in all tested CMs, to further clarify its potential effects on endothelial cells. Furthermore, considering the strong link between physical exercise, angiogenesis in the brain, and neurogenesis, testing the effects of exercise CM on blood vessel formation in the future could provide valuable insights into the potential role of NSCs in mediating exercise-induced increases in angiogenesis.

4.4 MitCMs and Boosted CM appear to be enriched in neuroprotective metabolites

Determining the composition of the pre-conditioned CMs is an important step to understand its effects and potential mechanisms of action. While often overlooked, metabolites are potent signalling molecules, capable of regulating cell fate and metabolism through specific receptors and transporters (Haas et al., 2016). As of yet, no studies have explored the metabolite composition of the NSC secretome. Therefore, we explored the effects of pre-conditioning on the secretion of metabolites by NSCs.

To characterize metabolite composition, we used gas chromatography-mass spectrometry, the most standardized technique in metabolomics. In fact, it is considered the gold standard for identifying and quantifying small metabolites below 650 Da, particularly small organic acids, alcohols, and sugars (Fiehn, 2016).

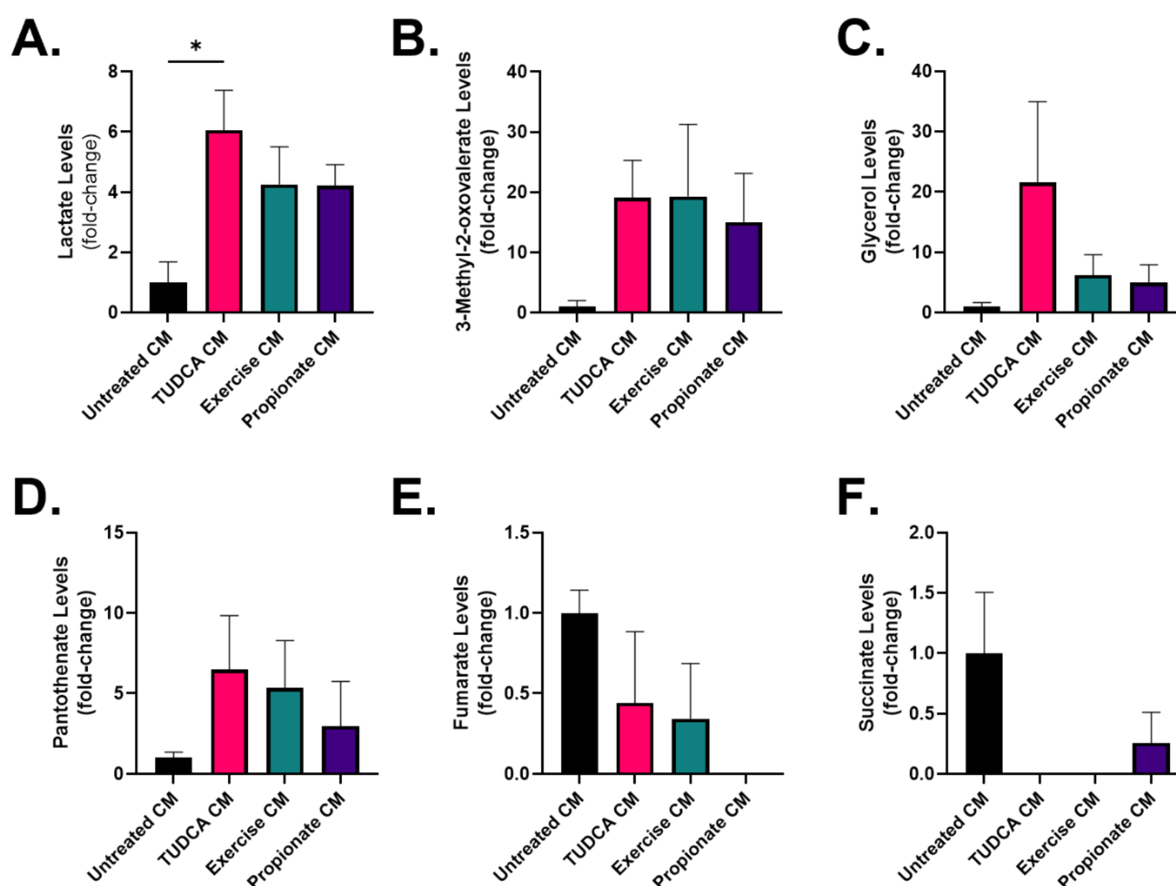

Figure 4.5 – The metabolite composition of mitCMs. (**A**) Lactate, (**B**) 3-methyl-2-oxovalerate, (**C**) glycerol, (**D**) pantothenate, (**E**) fumarate, and (**F**) succinate levels in mitCMs, as determined by GC-MS. Data are represented as fold-change over untreated CM. Data are expressed as mean ± SEM. n = 4. $*p < 0.05$ vs. untreated CM, one-way ANOVA followed by Dunnet's post-test for multiple comparisons. GC-MS, gas chromatography-mass spectrometry.

MitCMs seemed to be enriched in lactate, 3-methyl-2-oxovalerate, glycerol, and pantothenic acid (**Figure 4.5.A-D**), while showing apparent decreases in succinate and fumarate (**Figure 4.5.E-F**).

Specifically, TUDCA CM had significantly increased levels of lactate, compared with untreated CM (**Figure 4.5.A**). Boosted CM also displayed significantly higher levels of lactate (**Figure 4.6.A**), as well as apparent increases in 3-methyl-2-oxovalerate, glycerol, pyruvate, and butyrate (**Figure 4.6.B-E**). In turn, uCMS CM seemed to have increased levels of pyruvate (**Figure 4.7.D**) and decreased levels of propionate (**Figure 4.7.E**).

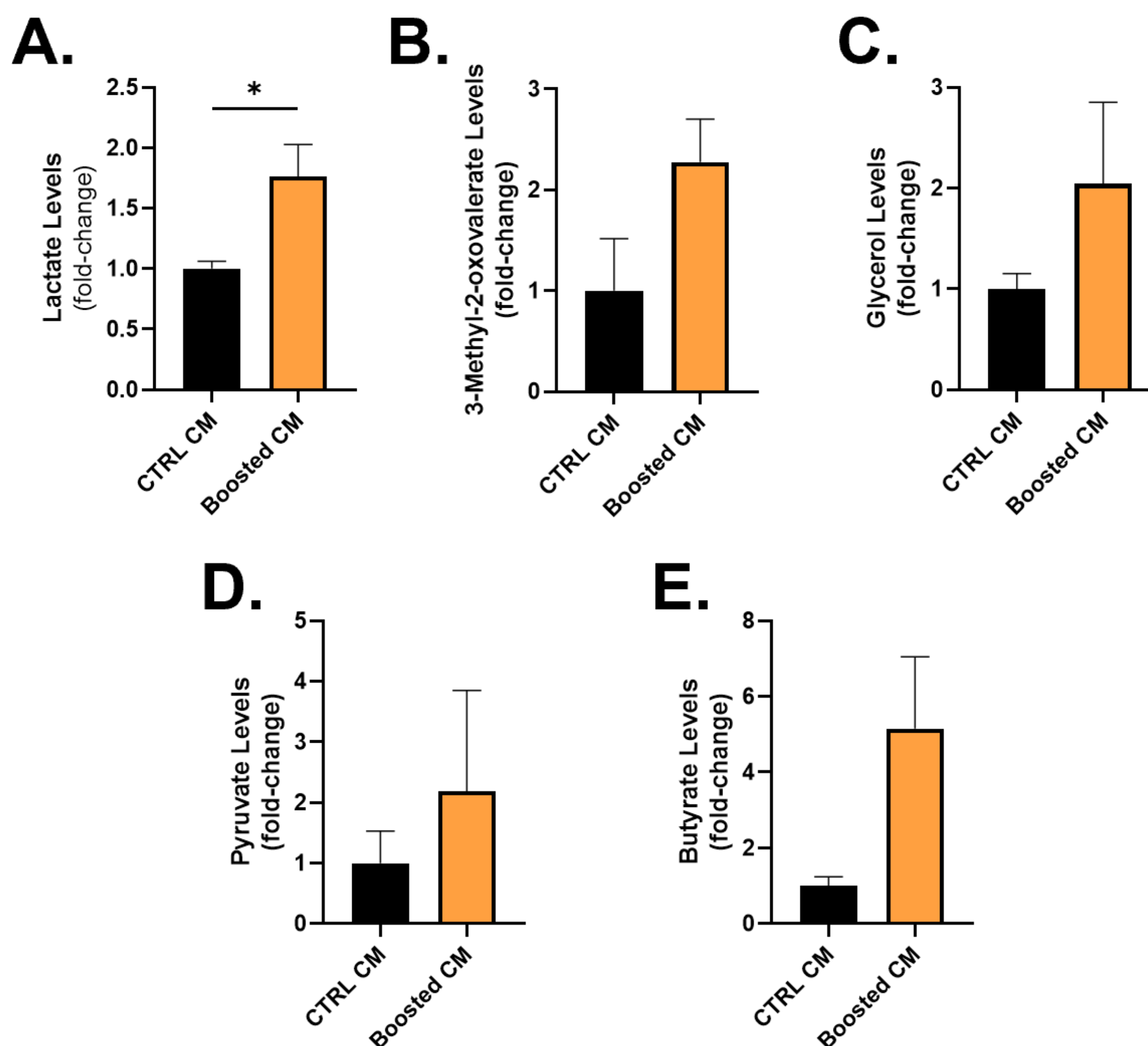

Figure 4.6 – The metabolite composition of boosted CM. (**A**) Lactate, (**B**) 3-methyl-2-oxovalerate, (**C**) Glycerol, (**D**) pyruvate, and (**E**) butyrate levels in boosted CM, as determined by GC-MS. Data are represented as fold-change over CTRL CM. Data are expressed as mean ± SEM. n = 4. *$p < 0.05$ vs. CTRL CM, unpaired and parametric t-test. GC-MS, gas chromatography-mass spectrometry.

Lactate is often considered a by-product of glycolysis, a waste product devoid of biological function shuttled to the extracellular space to prevent intracellular acidification (Rabinowitz & Enerbäck, 2020). However, new evidence shows that it has remarkable signalling properties, regulating processes as diverse as the immune response, angiogenesis, and fibrosis (Magistretti & Allaman, 2018). Akin to other signalling metabolites, lactate can bind to G protein-coupled receptors (GPRs) on the surface of cells—particularly GPR81 and GPR132—inducing a downstream signalling cascade

mediated by second messengers such as cAMP or Ca^{2+}. GPR81 is expressed by several cell types in the brain, particularly neurons, astrocytes, and endothelial cells, highlighting a potential role for lactate signalling in the brain (Magistretti & Allaman, 2018). Lactate also plays a determining role in ensuring adequate energy supply in the brain (Dienel, 2012). The astrocyte-neuron lactate shuttle helps neurons meet energy requirements during intense synaptic activity: synaptically released glutamate induces glucose uptake and lactate production in astrocytes, which is subsequently transferred to neurons to fuel oxidative phosphorylation (Mason, 2017). Since NSCs possess astrocytic features, they may also support neuronal activity through lactate release. In the context of neurogenesis, increased lactate production could play a pivotal role in supporting the high energy demands of new-born neurons. In mice, lactate injections have been shown to increase the survival of new-born neurons in the dentate gyrus through internalization mediated by the monocarboxylate transporter 2 (Lev-Vachnish et al., 2019). In a glioma cell line, exogenous lactate induces metabolic reprogramming, favouring lipid biosynthesis and oxidative phosphorylation (Minami et al., 2021), important pathways during the proliferation and differentiation of NSCs. All in all, the increased levels of lactate in mitCMs and boosted CM may be involved in their ability to promote NSC differentiation, survival, and metabolic reprogramming. Additionally, there might also be a role for lactate in the regulation of microglial activity. These cells are enriched in the monocarboxylate transporter 4 and lactate dehydrogenase B, which catabolizes lactate to pyruvate (Monsorno, Buckinx, & Paolicelli, 2022). As such, microglia may import lactate, oxidizing it to pyruvate to fuel oxidative phosphorylation. In a human microglial cell line, treatment with lactate promoted oxidative phosphorylation, mitochondrial biogenesis, and a shift towards the M2 anti-inflammatory phenotype, which is known to display enhanced phagocytic capacity (Longhitano et al., 2022). In the BV2 microglial cell line, lactate exposure induced proliferation, migration, and phagocytosis (Y. Liu et al., 2020). Thus, lactate may also mediate the phagocytosis-boosting effects of boosted CM. Regarding the vasculature, lactate binds to GPR81 in several blood vessel-associated cell types, increasing the release of VEGF and inducing angiogenesis in the mouse brain (Morland et al., 2017). Strikingly, subcutaneous lactate injections induce a specific increase of angiogenesis in the mouse dentate gyrus, suggesting a potential role for lactate in the promotion of angiogenesis-supported neurogenesis (Morland et al., 2017). A study in rats identified a concomitant increase of angiogenesis and neurogenesis after administration of lactate, further supporting this link (Jing Zhou et al., 2018). Despite the role of lactate in the promotion of angiogenesis, mitCMs and boosted CM failed to induce vascularization in our experiments, suggesting that its presence is counter-balanced by anti-angiogenic factors.

Unexpectedly, mitCMs and boosted CM seemed to be enriched in 3-methyl-2-oxovalerate, a monocarboxylic acid that results from the incomplete breakdown of branched chain amino acids. Typically, it is considered to be a neurotoxin, since its chronic elevation in the brain in maple syrup urine disease causes substantial damage (Blackburn et al., 2017; Mathew et al., 2019). However, transient increases in 3-methyl-2-oxovalerate may be beneficial. A recent study identified increased levels of 3-methyl-2-oxovalerate in the secretome of browning adipocytes (Whitehead et al., 2021). Skeletal myocytes exposed to the secretome or isolated 3-methyl-2-oxovalerate displayed enhanced mitochondrial oxidative metabolism. Furthermore, the study revealed that this metabolite acts through receptors on the

surface of myocytes, activating the cAMP-PKA-p38-MAPK axis. As such, 3-methyl-2-oxovalerate may play a similar role in the secretome of NSCs, promoting a metabolic shift towards oxidative phosphorylation in differentiating neurons and in microglia, thereby supporting neuronal differentiation and microglial phagocytosis. Since 3-methyl-2-oxovalerate's physiological role in the brain has, thus far, been neglected, several experiments would need to be performed to confirm this supposition, particularly to identify receptors for this metabolite in microglia and differentiating neurons. Curiously, 3-methyl-2-oxovalerate has been shown to reduce endothelial cell proliferation in mice, leading to a rarefied vascular network (Andrade et al., 2021). As such, 3-methyl-2-oxovalerate may act as an anti-angiogenic factor, counterbalancing the pro-angiogenic effects of lactate, hence explaining the lack of vascularization in response to pre-conditioned CMs.

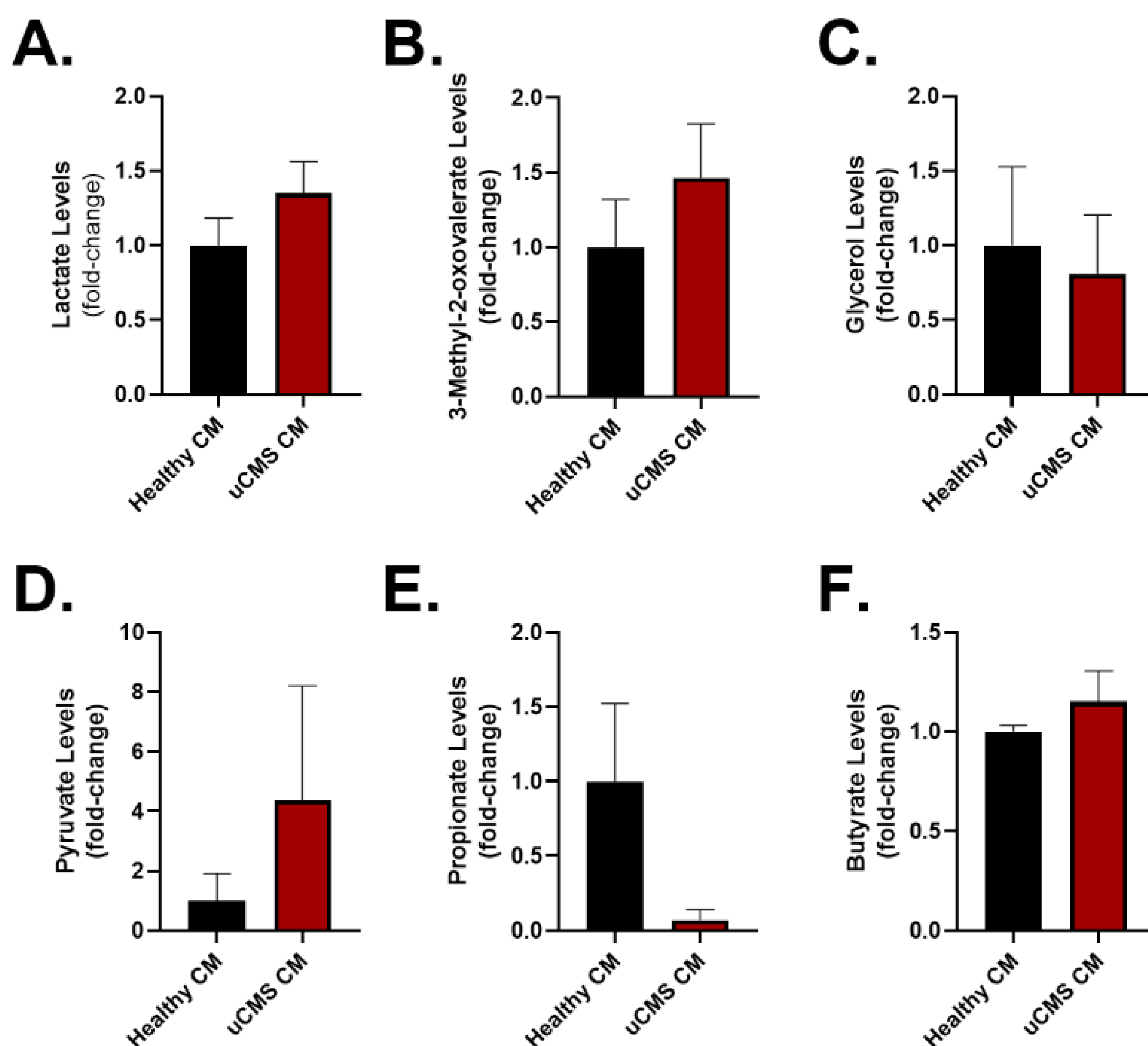

Figure 4.7 – The metabolite composition of uCMS CM. (**A**) Lactate, (**B**) 3-methyl-2-oxovalerate, (**C**) glycerol, (**D**) pyruvate, (**E**) propionate, and (**F**) butyrate levels in uCMS CM, as determined by GC-MS. Data are represented as fold-change over healthy CM. Data are expressed as mean ± SEM. n = 4. GC-MS, gas chromatography-mass spectrometry.

Finally, pyruvate, the end-product of glycolysis, seems to be enriched in boosted CM and uCMS CM. Curiously, extracellular pyruvate can act as a scavenger of ROS. Wang et al observed that treatment of a human neuroblastoma cell line with pyruvate protected them from H_2O_2-induced oxidative stress in a dose-dependent manner (X. Wang et al., 2007). Furthermore, this neuroprotective effect was related to its nonenzymatic decarboxylation, rather than to an improvement of energy metabolism. Both boosted CM and uCMS represent the secretomes of NSCs indirectly exposed to some form of oxidative stress. Boosted cells receive danger signals from NSCs exposed to MPP^+, which inhibits the complex I of the electron transport chain and enhances ROS production (Nicklas et al., 1985); uCMS cells receive the serum of mice with depressive-like behaviour, and a recent meta-analysis found overall oxidative stress to be increased in depression (Black, Bot, Scheffer, Cuijpers, & Penninx, 2015). In these conditions, NSCs may be prompted to release antioxidant molecules, particularly pyruvate, to supress oxidative stress and protect the brain from further damage.

In summary, the results of this experiment have revealed several promising candidates for mediating the neuroprotective effects of pre-conditioned CMs. Particularly, we have identified lactate, 3-methyl-2-oxovalerate, and pyruvate as metabolites capable of promoting neuronal survival and differentiation, with potential implications for the paracrine regulation of neurogenesis by NSCs. Stem cells' secretomes are highly dynamic and extremely sensitive to culturing conditions (Daneshmandi et al., 2020); as such, the large standard deviations associated with these results are unsurprising. To further identify enriched metabolites in pre-conditioned CMs and reach statistical significance, the analysed sample size must be increased.

4.5 Boosted CM is enriched in neuroprotective miRNAs

miRNAs, a class of small, non-coding RNAs, with an average length of 22 nucleotides, have emerged as determining regulators of gene expression (O'Brien, Hayder, Zayed, & Peng, 2018). Generally, they interact with the 3' untranslated region of target mRNAs, inducing their degradation or inhibiting translation, thereby preventing their expression. Importantly, many miRNAs are secreted to the extracellular space enveloped in exosomes, regulating the behavior of surrounding cells (Hu, Drescher, & Chen, 2012). In the context of the neurogenic niches, several miRNAs have been identified as regulators of neurogenesis, microglial activation, and vascularization (Y. Guo et al., 2019; Landskroner-Eiger, Moneke, & Sessa, 2013; Lang & Shi, 2012). Therefore, we decided to investigate the exosomal miRNA composition of pre-conditioned CMs.

In this experiment, we first purified exosomes from pre-conditioned CMs using an exosome isolation kit. Subsequently, we extracted miRNAs from the purified exosomes and identified them using a miRNA array card. Using this approach, we were able to rapidly profile 188 miRNAs in the tested samples. Due to limited resource availability, this experiment was only performed for the disease-related pre-conditioned CMs: boosted CM and uCMS CM.

In boosted CM, we identified 43 miRNAs which appeared to increase relative to CTRL CM, with the largest increases being miR-361-3p, miR-296-5p, miR-18a-3p, miR-342-3p, and miR-106b-3p

(**Figure 4.8.A**). We also observed two apparent decreases: miR-26b-5p and miR-92b-3p (**Figure 4.8.A**). In uCMS CM, we identified 1 miRNA which appeared to increase relative to healthy CM: miR-376a-3p (**Figure 4.8.B**). We also observed 29 apparent decreases, with the largest decreases being miR-21-5p, miR-18a-3p, miR-20b-5p, miR-181a-5p, and miR-652-3p (**Figure 4.8.B**).

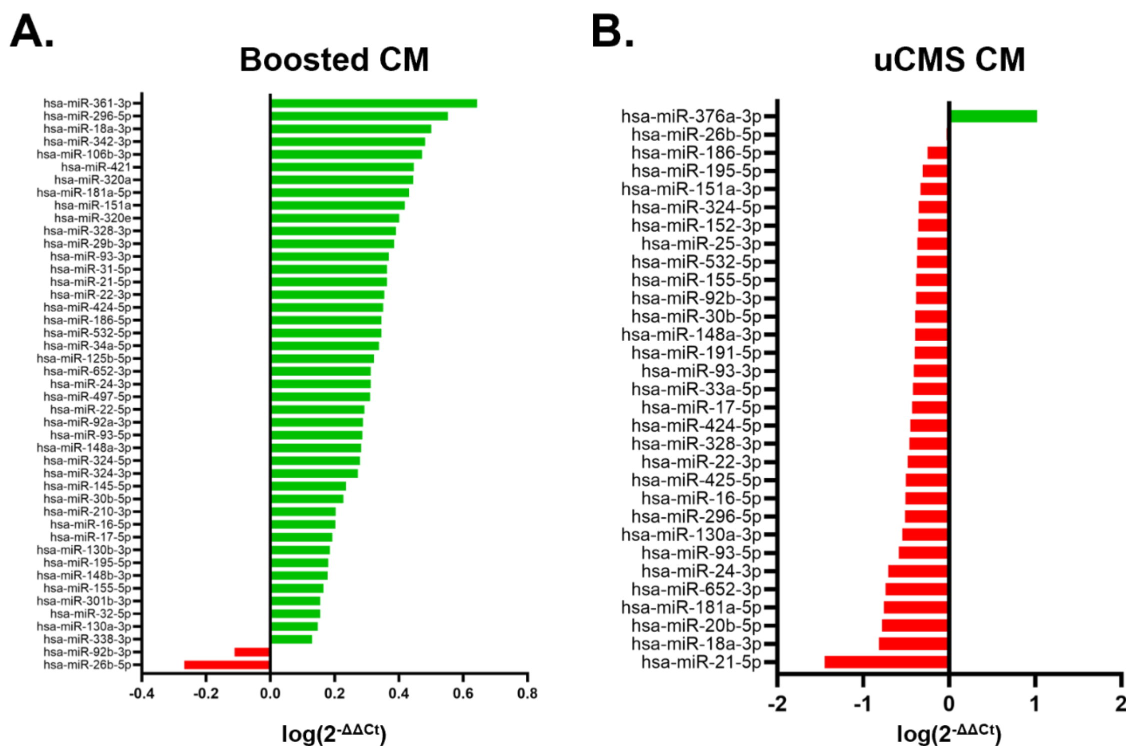

Figure 4.8 – The miRNA profile of disease-related pre-conditioned CMs. Detected miRNAs in (**A**) boosted CM and (**B**) uCMS CM through a miRNA array. Data are expressed as the relative fold expression in relation to CTRL CM and healthy CM, respectively, calculated using the $\log(2^{-\Delta\Delta Ct})$ method. n = 1.

Strikingly, among the identified miRNAs, there was a general increase in expression in boosted CM and a general decrease in uCMS CM. This mirrors the findings related to the metabolite composition of the pre-conditioned CMs, which showed that most metabolites were increased in boosted CM but presented no difference or were decreased in uCMS CM. As a whole, these results suggest that NSCs receiving danger signals from transiently injured NSCs assume a proactive state, increasing the secretion of potentially beneficial metabolites and miRNAs. Contrastingly, depression-associated factors in the serum appear to induce a dormant state in NSCs, inhibiting general secretion of metabolites and miRNAs, which can explain uCMS CM's inability to induce neuronal differentiation and microglial phagocytosis, in comparison with the other pre-conditioned CMs.

Curiously, miRNA-125b was among the identified enriched miRNAs in boosted CM. This increase is particularly relevant, due to miRNA-125b's role in neurogenesis. In fact, a study revealed that this miRNA increases during the neuronal differentiation of two human cell lines and ectopic expression of this miRNA resulted in neurite outgrowth, thereby contributing to neuronal maturation (Le et al., 2009). Furthermore, the authors identified a subset of miRNA-125b target genes involved in the

repression of neuronal gene expression. As such, miRNA-125b may promote neuronal differentiation through the suppression of differentiation-inhibiting pathways. Cui et al confirmed these findings, showing that miRNA-125b enhances neuronal differentiation and migration of rat NSCs, while suppressing proliferation (Cui et al., 2012). They also demonstrated that nestin, the most widely used marker of undifferentiated NSCs and an important promoter of NSC maintenance and self-renewal, is a direct target of miRNA-125b. Hence, miRNA-125b's role in promoting neuronal differentiation is well-established. Boosted CM also appears to be enriched in miRNA-34a, which has similarly been implicated in the regulation of neurogenesis. miRNA-34a overexpression increases rat NSC proliferation *in vitro* and enhances the response to synaptic stimuli (Mollinari et al., 2015). Furthermore, it stimulates NSC proliferation *in vivo* in the adult rat brain resulting in improved behavioral outcomes. Previously in this work, we observed that boosted CM seems to increase the rate of neuronal differentiation. Considering that boosted CM appears to be enriched in miRNA-125b and miRNA-34a, we propose that NSCs exposed to danger signals secrete higher levels of miRNA-125b and miRNA-34a, which may facilitate neuronal differentiation in target cells.

Several microglia-modulating miRNAs also seem to be elevated in boosted CM. miRNA-424, for example, has been demonstrated to decrease microglial activation in a mouse model of stroke (H. Zhao et al., 2013). Additionally, it targets toll-like receptor 4 (TL4), thereby suppressing the inflammatory TL4/MyD88/NF-κB signaling pathway (Z. Li, Wang, & Yu, 2022). Microglia presenting an anti-inflammatory phenotype possess greater phagocytic capacity (S. Guo, Wang, & Yin, 2022); as such, miRNA-424-mediated suppression of inflammation may be involved in boosted CM's enhancement of microglial phagocytosis. miRNA-93 has also been shown to modulate microglial activation *in vitro* and *in vivo*. In a rat model of acute ocular hypertension, miRNA-93 overexpression decreased microglial activation and release of pro-inflammatory cytokines, thereby reducing retinal ganglion cell loss (Y. Wang et al., 2021). As such, increased secretion of miRNA-93 may also be involved in the microglia-modulating effects of the boosted CM.

Lastly, miRNA-21 is one of the most abundantly expressed miRNAs in mammalian cells (Jenike & Halushka, 2021). Here, we detected an apparent increase of this miRNA in boosted CM and an apparent decrease in uCMS CM. A recent *in vitro* study aimed to uncover the role of miRNA-21 in NSCs (W. Zhang, Zhang, Yang, Zhang, & Gao, 2018). Using rat primary NSCs, they revealed that miRNA-21 overexpression increases NSC proliferation through the Wnt/ β-catenin signaling pathway. Furthermore, miRNA-21 enhanced neuronal differentiation and suppressed astrocytic differentiation. These findings are in line with our own results, since the miRNA-21-rich boosted CM seems to induce neuronal differentiation, while the miRNA-21-depleted uCMS CM appears to suppress it. Beyond this novel neurogenesis-boosting role, miRNA-21 has well-established neuroprotective effects. In a rat model of epilepsy, miRNA-21 reduced apoptosis and loss of hippocampal neurons through inhibition of signal transducer and activator of transcription-3 (STAT3) (X. Zhang, Li, Li, Sun, & Zhang, 2020). In a rat model of subarachnoid hemorrhage, miRNA-21-rich extracellular vesicles derived from mesenchymal stem cells promoted neuronal survival and reduced cognitive dysfunction (Gao et al., 2020). Finally, in an *in vitro* model of traumatic brain injury, miRNA-21 was shown to repress Rab11a expression, thereby

preventing excessive neuronal autophagy and protecting injured neurons (D. Li et al., 2019). In summary, miRNA-21 exerts neuroprotective effects through several pathways. Increased secretion of miRNA-21 by NSCs exposed to danger signals from injured NSCs may contribute to the recovery process. Contrastingly, depression-associated factors in the serum appear to suppress miRNA-21 secretion by NSCs, hindering its neuroprotective effects.

In conclusion, the findings of this experiment have highlighted several miRNAs—particularly miRNA-125b, miRNA-34a, miRNA-424, and miRNA-21—which may mediate boosted CM's induction of neuronal differentiation and microglial phagocytosis. These results are preliminary; as such, validating the individual expression of these candidate miRNAs through qPCR in pre-conditioned CMs is necessary to confirm the observed changes. We also identified a general increase in the expression of identified miRNAs in boosted CM and a general decrease in uCMS CM, which suggests that NSCs exposed to danger signals from injured NSCs acquire a proactive state, while NSCs exposed to depression-associated factors acquire a dormant state. Again, this hypothesis would explain uCMS CM's inability to induce neuronal differentiation and microglial phagocytosis relative to the other pre-conditioned CMs.

5

CONCLUSION AND FUTURE PERSPECTIVES

An accumulating body of evidence shows that several cognitive processes, including memory formation, learning, and mood regulation, depend on adult neurogenesis (Oomen et al., 2014). Importantly, adult neurogenesis is modulated by systemic signalling in health and disease through poorly characterized mechanisms (Levenson & Rich, 2007; Winner & Winkler, 2015; Yau et al., 2014). As new studies demonstrate that NSCs can regulate their surroundings through paracrine signalling (Willis et al., 2020), we hypothesized that they can modulate the activity of the various members of the neurogenic niches in response to systemic cues.

NSCs pre-conditioned with metabolic regulators (TUDCA, propionate, and a cocktail of growth factors upregulated during exercise *in vivo*) released a secretome (mitCMs) which appeared to induce neuronal differentiation in differentiating NSCs. These metabolic regulators have all been demonstrated to enhance neurogenesis *in vivo* (Ribeiro et al., 2020; Soares et al., 2018; Yau et al., 2014). Our results suggest that, beyond direct stimulation of neurogenesis, these compounds can also remodel the NSC secretome, signalling to its neighbours to induce neuronal differentiation. Since the metabolic regulators enhance mitochondrial activity and biogenesis in NSCs, these results also highlight a potential role for mitochondria in the regulation of the stem cell secretome. We also tested the effects of these secretomes on microglial phagocytosis since this process contributes to successful adult neurogenesis. Surprisingly, we observed no alterations. This is particularly surprising for the secretome of NSCs pre-conditioned with the exercise-mimicking cocktail of growth factors, since exercise is known to induce both neurogenesis and microglial phagocytosis in the neurogenic niches (Mee-inta et al., 2019; Yau et al., 2014). Thus, our results indicate that increased microglial phagocytosis induced by exercise in the brain is not mediated by the paracrine action of NSCs. Finally, we characterized the metabolite composition of these secretomes and identified increased levels of potentially neuroprotective and neurogenic compounds, particularly lactate and 3-methyl-2-oxovalerate. In summary, our results suggest that stimulating mitochondrial activity in NSCs remodels their secretome, inducing the release of neuroprotective metabolites and enhancing the differentiation of surrounding NSCs. Furthermore, these findings highlight a potential

role for the paracrine regulation of adult neurogenesis by NSCs in response to positive lifestyle changes, such as healthy dieting and exercise.

NSCs pre-conditioned with the secretome of injured NSCs also released a secretome (boosted CM) which seemed to promote neuronal differentiation. We believe this to be a potential compensatory mechanism: in situations of brain injury, there is often a substantial loss of neuronal mass that can compromise adequate brain function (Gorman, 2008); as such, NSCs may release a neurogenic secretome in response to danger signals, in order to restore lost neurons and enhance recovery. We also tested the influence of boosted CM on microglial phagocytosis and observed an apparent increase in cells with high phagocytic capacity. These results further support the hypothesis that NSCs exposed to danger signals release a neuroprotective secretome. After brain injury, there is often an accumulation of cell debris and apoptotic cells that can be highly toxic (Gorman, 2008). In this context, increased microglial phagocytosis is critical to promote the clearance of these remains and prevent the release of cytotoxic and immunogenic intracellular contents (Galloway et al., 2019). As such, our findings indicate that NSCs can regulate microglia through paracrine signalling upon injury, upregulating microglial phagocytosis and thereby contributing to the clearance of cell debris and enhanced recovery. Since adult neurogenesis depends on microglial phagocytosis (Sierra et al., 2010), the paracrine regulation of microglia by NSCs may also indirectly contribute to the generation of new neurons. Since the vasculature supports adult neurogenesis (Bovetti et al., 2007; Chen et al., 2013; Jin et al., 2002), we also tested the effects of boosted CM on blood vessel formation by endothelial cells. Curiously, we observed no changes in vessel formation or morphology. Compensatory increases in angiogenesis have been observed in several situations of brain injury, including stroke and some neurodegenerative disorders (Jefferies et al., 2013; Paul & Elabi, 2022; Ruan et al., 2015). Thus, our results indicate that NSCs do not modulate vascularization through paracrine signalling in response to danger signals. It is therefore possible that the described alterations in angiogenesis in the context of disease may be mediated by other cell types, such as astrocytes or microglia, or may even be a direct response of endothelial cells to injury. To identify potential mediators of the beneficial effects of boosted CM, we characterized its composition in terms of metabolites and miRNAs. Similarly to mitCMs, we observed increased levels of lactate and 3-methyl-2-oxovalerate, suggesting that these secretomes can enhance neuronal differentiation through shared mechanisms (Lev-Vachnish et al., 2019; Whitehead et al., 2021). We also identified apparent increases in miRNAs involved in neuroprotection, neuronal differentiation, and microglial activation, particularly miRNA-21, miRNA-125b, and miRNA-424 (Le et al., 2009; Z. Li et al., 2022; W. Zhang et al., 2018). Succinctly, these findings suggest that danger signals enhance the neuroprotective effects of the NSC secretome, promoting the release of beneficial metabolites and miRNAs which may mediate its ability to stimulate neuronal differentiation and microglial phagocytosis.

Finally, we tested the influence of pre-conditioning with serum from mice with depressive-like behaviour, to characterize changes on the paracrine regulation of the niche by NSCs in the context of depression. The resulting secretome (uCMS CM) seemed to decrease neuronal differentiation. Deficient hippocampal neurogenesis is a recognized feature in both animal models of depression and in patients with major depressive disorder (Yun et al., 2016). Furthermore, some scientists believe that decreased

adult neurogenesis in the hippocampus can cause some of the symptoms associated with depression (Jacobs et al., 2000). As such, these findings indicate that the paracrine action of NSCs may be involved in the pathogenesis of depression, since NSCs appear to release a neurogenesis-supressing secretome in response to depression-associated factors in the circulation. Interestingly, uCMS CM also seemed to decrease the number of highly phagocytic microglia. Several studies have reported abnormal rates of phagocytosis in animal models of depression, with detrimental consequences to healthy neurons and synapses (Bassett et al., 2021; Han et al., 2022). In this regard, NSCs may attempt to revert abnormal microglial behaviour through paracrine signalling in response to depression-associated factors in the circulation, thereby preventing unnecessary damage to healthy tissues. Similarly to boosted CM, no changes in blood vessel formation or morphogenesis were observed in response to uCMS, further consolidating that the NSC secretome does not appear to modulate vascularization in the context of disease. Regarding the metabolite composition of the uCMS CM, we detected an apparent increase in pyruvate; in the extracellular environment, this metabolite can act as a scavenger of ROS (Desagher, Glowinski, & Prémont, 1997). Since depression has been linked to increased oxidative stress (Black et al., 2015), secreted pyruvate by NSCs may play an important neuroprotective role, contributing to the antioxidant defence system. In terms of miRNAs, we observed a general decrease, contrasting with the general increase observed in the boosted CM. As such, depression-associated factors seem to supress the secretion of miRNAs by NSCs. Altogether, these findings suggest that depression-associated factors induce a dormant state in NSCs, downregulating secretion and hindering its capacity to promote neuronal differentiation and microglial phagocytosis.

Most findings in this work are preliminary results since they represent trends rather than statistically significant alterations. Therefore, the next logical step will be to increase the sample size across all experiments, to accentuate the observed differences and clarify the effects of the pre-conditioned CMs. Considering the relevance of neurotrophic factors in the regulation of adult neurogenesis, it would also be interesting to characterize the protein composition of the secretomes. Furthermore, validation of the candidate miRNAs identified in the array through qPCR needs to be performed to confirm the observed changes. An exciting new study demonstrated that NSCs can transfer mitochondria to neighbouring cells through exosomes (Peruzzotti-Jametti et al., 2021). Since mitochondrial oxidative phosphorylation drives neuronal differentiation, mitochondrial transfer from NSCs to differentiating neurons could also be an important mechanism through which NSCs support neurogenesis. To test this hypothesis, it would be interesting to label the mitochondria of donor cells with MitoTracker, followed by collection of the secretome and incubation with target cells. If MitoTracker-fluorescence is detected in target cells, we can use this experiment to compare the level of mitochondrial transfer according to different NSC pre-conditionings.

In summary, the results presented in this thesis reveal that NSCs can regulate the members of the neurogenic niche in response to systemic cues, uncovering a previously unknown role for these cells in the paracrine regulation of adult neurogenesis. They also show that, in response to metabolic regulators, but also to danger signals from injured cells, NSCs release a neuroregenerative secretome, which appears to increase neuronal differentiation and microglial phagocytosis. In contrast, NSCs exposed to

the serum of depressed mice seem to acquire a dormant state, and the resulting secretome failed to induce neuronal differentiation and microglial phagocytosis. Therefore, it is possible that the paracrine action of NSCs may also be involved in the pathogenesis of depression. These findings have undoubtedly contributed to our understanding of the regulation of NSC paracrine activity and its effect on neurogenesis. Considering the emerging role of dysfunctional adult neurogenesis in neurodegenerative and neuropsychiatric disorders, a deeper understanding of its regulation is key to develop new, ground-breaking therapies. We also identified several metabolites and miRNAs in neuroprotective secretomes—particularly lactate, 3-methyl-2-oxovalerate, pyruvate, miRNA-21, miRNA-125b, and miRNA-424—which may mediate its positive effects. Identifying beneficial factors in neuroprotective secretomes is an important first step in the development of cell-free therapeutic strategies, which hold great promise for regenerative medicine since they subvert many of the functional and logistical disadvantages associated with cell-based therapies.

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