UNIVERSIDADE DA BEIRA INTERIOR
Ciências

O impacto da sinalização astrocítica nos efeitos terapêuticos do secretoma de células mesenquimais estaminais no sistema nervoso central

(Versão final após defesa)

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Dissertação para obtenção do Grau de Mestre em
Bioquímica
(2º ciclo de estudos)

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Covilhã, Novembro de 2019

Dedication

I dedicate this dissertation to every teacher I have ever had.

Firstly, to those who taught me how to live, how to be grateful, merciful and kind. My parents.

Secondly, to those who taught me to believe myself, who taught me to be critical and open-minded. To all the teachers at Colégio Singular:
Xico Boca, Fernandinho, Frank, Elton, Raimundo, Fátima, Maria Cecília, Mirian, Adriana, Beatriz.

Lastly, to those who inspired my dreams, my career paths, my sense of ethics.
To those that with their character, have led by example.
To all the professors at Centro Universitário São Camilo:
Sandra Chemin, Fabiana Poltronieri, Rosana Freire, Roseli Espindola, Aline De Piano, Adriana Peloggia, Andrea Fraga, Cinthia Romam, Deise Caramico, Fernanda Patti, Juruce Borovac, Luciana Trindade, Monica Galisa, Rosana Toscano, Vera Frangella e Silvia Martinez.

Acknowledgements

Firstly, I would like to thank my supervisor Prof. Dr. Antonio Salgado, for the opportunity given to integrate his team and to get involved with this project. His guidance, and support were vital for the continuity of this project. His hard work and sense of leadership have been inspirational, and truly something that I will carry for the future.

Secondly, I would like to thank my co-supervisor Prof. Dr. Graça Baltazar whom which without her recommendation, this experience would have never happened, she was the person responsible to introduce me to professor Salgado’s line of research. Additionally, she was the responsible for opening my eyes to the possibility of studying neuroscience, something that up until her classes at UBI I would have never thought about.

Additionally, I would also like to thank everybody that is part of the so called (Tó team) group, they were responsible for the welcoming experience I had during this time at ICVS and their support and friendship were crucial for this work. I can happily say that I made true friends here in Braga.

Also, I would like to thank the support of the girls at the molecular biology services, specially Liliana, for the countless hours at the confocal microscope. Without her help, I would have not been able to do this work.

Special thanks must be given to the people of ICVS as a whole, for the help with random experiments, lending of reagents and helpful tips and discussions. Trying to name everyone would be a bad idea as I probably had help from literally everyone.

I must also thank my now ex-wife Jéssica, her belief in me was ultimately the responsible to bring me where I am now. The partnership we have had possess an immensurable formative role in my life.

Lastly, I would like to thank all my family, for the unconditional support and encouragement.

I would also like to thank the financial support from from Prémios Santa Casa Neurociências - Prize Melo e Castro for Spinal Cord Injury Research (MC-04/17) This work was funded by FEDER, through the Competitiveness Internationalization Operational Programme (POCI), and by National funds, through the Foundation for Science and Technology (FCT), under the scope of the projects POCI-01-0145-FEDER-007038; TUBITAK/0007/2014; PTDC/DTP-FTO/5109/2014; POCI-01-0145-FEDER-029206 and NORTE-01-0145-FEDER-029968. This work has also been developed under the scope of the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER).

Resumo

A capacidade de regenereação do sistema nervoso (SNC) central é limitada, gerando um desafio para a produção de novas terapias. Estudos recentes do nosso laboratório evidenciaram um potencial interessante para a aplicação de células estaminais mesenquimatosas (MSC) como um potencial agente terapêutico para o SNC no qual o seu secretoma (secreção de fatores tróficos e vesiculas) tem sido descrito como o principal mecanismo de ação, capaz de estimular neurogénese e a sobrevivência de células gliais. Neste contexto, astrócitos tem grande importância na homeostase do SNC modulando importantes funcões como neurotransmissão, fluxo sanguíneo e neurogénesis. Portanto, este projeto teve o objetivo de investigar o papel dos astroócitos nos efeitos modulatórios promovidos pelo secretoma de MSC's nomeadamente nos seus níveis de proliferação e nas densidades gliais. Para isso, foi utilizado um modelo animal transgénico que possui o complexo SNARE bloqueado, reduzindo desta forma a transmissão astrocítica. Adicionalmente, a morfologia destes astrócitos foi estudada. Como controlos, foram usados murganhos C57BL/6. Uma semana após a injeção com o secretoma, procedeu-se a avaliações histológicas, os tecidos foram marcados para Ki-67 e GFAP e contramarcados para DAPI. O secretoma apresentou um papel estimulatório sob os níveis de proliferação evidenicados pelo maior número de células positivas para Ki-67 na zona sub-granular do hipocampo nos animais *wild type* e nos SNARE high, sendo a transmissão astrocitica importante para tal processo tendo em vista que, em comparação com animais SNARE low, os níveis de proliferação foram reduzidos, adicionalmente, o secretoma elevou a proliferação de celulas gliais radiais (RGC) apenas nos animais Snare High, mostrando um possível efeito compansatório sob a redução da transmissão glial. Adicionalmente, os animais tratados com secretoma que possuiam expressão do transgene, apresentaram morfologia hipertrófica e mais complexa quando comparados com animais *wild type*. Os resultados encontrados devem ser considerados com parcimónia pela natureza exploratória do presente estudo, vale a pena reasaltar que é a primeira vez que este tipo de abordagns experimentais e análises é desenvolvido neste modelo animal.

Palavras-chave

Secretoma, astrócitos, célula estaminal mesenquimatosa, proliferação, morfologia

Resumo Alargado

A capacidade de regenereação do sistema nervoso (SNC) central é limitada, e os processos fisiológicos que a governam são complexos, criando um desafio na geração de novas terapias. Atualmente diversas classes terapêuticas tem sido estudas e entre elas a terapia celular tem ganhado crescente atenção na comunidade científica.

Estudos recentes do nosso laboratório evidenciaram o potencial a aplicação de células estaminais mesenquimatosas (MSC) como agente terapêutico para o SNC, no qual o seu secretoma (fatores tróficos e vesículas) tem sido descrito como o principal mecanismo de ação, capaz de estimular processos como a neurogénese, diferenciação neuronal, crescimento axonal e a sobrevivência de células gliais, processos essenciais para recuperação funcional do SNC. De fato, dados obtidos em modelos animais, de doenças como a doença de Parkinson ou lesões na medula spinhal mostraram aumentos da capacidade funcional, redução da area de lesão tecidual e redução da resposta inflamatória. Atualmente a assinatura molecular do secretoma é estudadar com objetivo de identificar os componentes responsáveis pelos efeitos benéficos e tem sido dada especial atencão às vesiculas extracelulares como exosomas e microvesiculas, já que estes norteiam a comunicação celular e tem a capacidade de alterar respostas teciduais a lesão em diversos modelos animais.

Neste contexto, astrócitos apresentam elevada importância por estarem envolvidos em diversos processos homeostáticos no SNC, participando de eventos de neurotransmissão, regulação do fluxo sanguíneo e da neurogénese, bem como em respostas a lesões agudas ou doenças de caráter neurodegenerativo. Portanto, este projeto teve o objetivo de avaliar histologicamente, o papel dos astrócitos nos efeitos modulatórios promovidos pelo secretoma de MSC's nomeadamente nos níveis hipocampais de proliferação e sob as densidades astrogliais. Para isso, foi utilizado um modelo animal transgénico que possui o complexo SNARE bloqueado denomeado dnSNARE. Neste modelo, ocorre uma importante redução significativa nos eventos exocitóticos reduzindo desta forma a transmissão astrocítica. Adicionalmente, a morfologia destes astrócitos foi estudada em resposta ao tratamento com o secretoma. Como controlos, foram usados murganhos C57BL/6 com transmissão astrocícita inalterada. Uma semana após a injeção do secretoma, os animais foram sacrificados e procedeu-se à avaliações histológicas, para marcadores de proliferação e para marcadores de astrócitos. Os tecidos foram submetidos a immunohistoquímica para Ki-67 e GFAP e contramarcados para DAPI, e submetidos a microscopia confocal para obtenção de imagens em três dimensões. Foram feitas contagens de células proliferativas na zona sub-granular do hipocampo e no hílus, e para astrócitos na camada celular granular. O secretoma apresentou um papel estimulatório nos níveis de proliferação, evidenicados pelo maior número de células positivas para Ki-67 na zona sub-granular do hipocampo nos animais *wild type* e sob menor dimensão nos SNARE high, evidenciando a importância da transmissão astrocítica neste processo. O secretoma aumentou

a proliferação de celulas gliais radiais (RGC) apenas nos animais SNARE High, mostrando um possível efeito compansatório sob a redução da transmissão glial, o que poderá justificar a maior proliferação na zona sub-granular.
Não foram encontradas deiferenças nas densidades astrogliais entre os diferentes grupos experimentais.
Adicionalmente, os animais tratados com secretoma e que possuiam elevada expressão do transgene, SNARE High apresentaram característica morfologia hipertrófica e mais complexa em comparação com animais WT. Os animais com expressão do transgene baixa Snare Low apresentaram maior complexidade morfológica apenas marcada pela analise de Sholl. Salienta-se que os resultados apresentados devem ser considerados com parcimónia dado a natureza exploratória do presente estudo. Vale a pena resaltar que é a primeira vez que este tipo de abordagens experimentais e análises é desenvolvido neste modelo animal.

Palavras-chave

Secretoma, astrócitos, célula estaminal mesenquimatosa, proliferação, morfologia

Abstract

The central nervous system (CNS) has a limited auto-regeneration capacity, which makes it challenging for the development of new therapies. Previous studies from our lab have demonstrated the applicability of mesenchymal stem cells (MSCs) as a possible therapeutic too for CNS, in which their secretome (e.g. the secretion of trophic factors and vesicles) has been described as the most probable mechanism of their therapeutic action, due to its ability to stimulate/modulate neurogenesis. Glial cells as astrocytes are important players in neural activity and in the modulation of neurotransmission, thereby being crucial elements in neurogenesis. Thus, in the present project, we aimed to evaluate the impact of astrocytes on the effects promoted by the application of MSCs secretome as a therapeutic tool for the modulation and generation of new neurons. For this, MSCs secretome was injected into the dentate gyrus (DG) of the hippocampus of a transgenic animal model (with the SNARE complex blocked) without astrocytic transmission, and therefore with their function partially depleted. Additionally, morphological features of astrocytes were assessed. As controls wild type Black 6 mice, in which the astrocytic function has not been depleted were used. One week after secretome treatment, animals were sacrificed, and brains collected for molecular and histological analysis. Pre-frontal cortex tissue was used to perform a transgene analysis in order to divide the experimental groups in wild type (Wt), Snare high and Snare low (animals presenting different patterns of gene expression). Brains were immuno-stained for GFAP and Ki-67 and assessed under a confocal microscope for proliferations levels at the SGZ of the hippocampus, at the hilus, for counting of radial glial cells at the SGZ and for GFAP+ densities at the DG. Confocal images were also employed for morphological analysis.
Results demonstrated increased levels of proliferation for Wt and Snare high animals at the SGZ when compared to Snare low when treated with secretome, furthermore, secretome increased levels of proliferating radial glial cell in Snare high animals. Morphological assessments revealed increased process hypertrophy and complexity in snare animals treated with secretome. The results could be attributed to trophic factors present in the secretome, previously shown to increase proliferation at the DG and also to alter astrocyte morphology. The impact of transgene expression is harder to explain, nevertheless, impaired exocytosis from astrocytes could have implications for the response to a proliferative stimulus given the established autocrine signaling trough this mechanism.

Keywords

Secretome, astrocytes, mesenchymal stem cell, proliferation, morphology

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Chapter 1 – Introduction

1.1. The central nervous system regenerative capacity

Central nervous system regeneration is a hard feature to achieve in the human diseased brain. Although other species such as the zebra fish and even rodents present considerable regenerative capacity, traumatic lesion to the human brain have devastating effects with severe life-threatening risk and strongly associated co-morbidities (Williams et al., 2014; Otero et al., 2018).

In fact, this inherent incapability for regeneration was challenged by Santiago Ramón y Cajal more than 90 years ago, the results of the experiments were published in his book entitled Regeneration and Degeneration of the Nervous System for the first time in English in 1928. This results presented compelling evidence for the possibility of growing axons either from peripheral or central nervous tissue in response to the exposure to growth-promoting substrates originated from peripheral nerves, challenging his very own decree from only 2 years before "In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated" (DeFelipe, Jones, 1991) (Silver et al., 2015; Williams et al., 2014). Since then our understanding of the cellular and molecular basis of CNS trauma and degeneration have evolved as well as the research avenues that aims to one day provide system-wide regeneration of the CNS tissue.

In aiming for CNS regeneration, a list of barriers must be surpassed in order to subvert the damages caused by an injury or prolonged neurodegeneration, a graphic representation of this barriers as well as the mechanisms that gives support to regeneration is present in (Figure 1) at the end of this section. Firstly, and probably the most important feat to be achieved is a way of promoting neuroprotection of the local cell population but also fostering cell proliferation and differentiation. Secondly, the promotion of sustained and controlled axonal growth coupled with efficient remyelination that targets the enforcement of synaptic connections. Thirdly, angiogenesis needs to exist in order to provide metabolic support to the area in regeneration, and finally, the complete restoration of the local homeostasis is achieved after repair of the blood brain barrier.

In the case of a traumatic injury to the CNS, laceration, contusion and compression are the three pillars of the first injury and the cause for the disruption of local homeostasis, it results in cell death, but also amplifies into a second wave of injury related mechanisms such as ischemia, ionic imbalance mediated cell death, glutamatergic excitotoxicity, inflammation and hemorrhage (Ahuja et al., 2017). This second injury thereby magnify the damage caused by the first insult ultimately leading to cystic cavity and astroglial scar. Therefore, strategies should target the mechanisms of secondary injury in order to prevent the amplification of damage to spread into functional areas (Silva et al., 2013).

Neuroprotection is a key aspect that is currently being given considerable attention and numerous studies using a diverse range of strategies aiming at neuroprotection, did accomplish positive functional outcomes (Santamaria and Guest, 2017).

Currently, some drugs are now being tested in clinical trials with mechanistic goals ranging from (excitotoxicity reduction, hemorrhage and inflammation control, and promotion of neurite sprouting, or angiogenesis). (Ulndreaj et al., 2017).

Additionally, emerging therapies such as stem cell therapy are under intense pre-clinical investigation. In this context mesenchymal stem cells (MSC's) are promising due to their ability to reduce inflammation, and most importantly due to the secretion of neurotrophic factors that have been shown to promote neuroprotection. (Squillaro et al., 2016; Inês et al., 2019)

In regard to axonal damage and regrowth, their molecular characteristics are complex and, currently not fully established, nevertheless, according to the current evidence, it is possible to make some considerations, in this sense, axonal damage varies according to cell type, injury type and severity and also affected area. It is known that upon injury, a myriad of events takes place ultimately leading to axonal degeneration. (Egawa et al., 2017) For instance, the disruption of cell membranes leads to disrupted metabolic coupling among neuron and glial cells generating an environment of neuronal energy and metabolite deficiency, the dysregulation of ionic gradients for instance causes intracellular signaling pathway disturbance and microtube cytoskeleton destabilization, what together culminates in axonal dieback from both ends. (Filous et al., 2017; Silver et al., 2015).

Attempts of intrinsic neuronal regeneration have been reported, and those showcase the existence of regenerative mechanisms contained within neurons that unfortunately are often hampered by molecules of glial origin such as myelin and proteoglycans as both present a strong inhibitory potential against axonal regeneration, demonstrating that axonal injury may not be a static end point, but probably a phenomenon that can be modulated, prevented or even reversed. (Willians et al., 2014; Aboul-Eneinm et al., 2006)

It is now becoming increasingly evident of how important glial cells are for the fate of axonal regeneration and now it is recognized that by creating an extrinsic environment filled with negative cues they hinder the already limited neuronal regenerative potential. Therefore, strategies targeting the modulation of glial cells combined with others aiming to boost the neuronal intrinsic regenerative potential are likely the ones to achieve better outcomes (Willians et al., 2014).

For instance, reactive astrocytes could be targeted to reduce the rates of chondroitin sulfate proteoglycan (CSPGs) production, an extracellular matrix component known to hamper axonal regeneration, additionally, strategies aiming to reestablish astrocyte-neuron metabolic coupling could be feasible to support neurons overcoming metabolic death (Escartin and Rouach, 2013; Siebert and Osterhout, 2011; Bradbury et al., 2002).

Accordingly, dysfunctional oligodendrocytes and myelin derived proteins such as Nogo-A, MAG (mielin associated glycoprotein) and OMgp (oligodendrocyte/ myelin glycoprotein) are a promising target as they have been shown to promote growth cone collapse and neuronal

growth arrest mediated by a CREB inactivation mechanism. For instance, the presence of neurotrophic factors can counteract their actions as they elevate cAMP and P-CREB as well as mTOR, a potent growth promoting protein show to strongly promote axonal sprouting and growth. (Liu et al., 2011; Giger et al., 2010)

Regarding the inflammatory response related to axonal damage, a specific role for microglia and macrophages have been established a long time ago where the injection of pyrogens induced an increased leukocyte presence at the lesion site that was correlated with better neurological recovery as well as reduced intralesional scarring in dogs. (Guth et al., 1994; Guth et al., 2006).

The findings were confirmed decades later in spinal cord injured rats, where LPS treated animals presented higher intraspinal leukocytosis that correlated with a robust axonal growth and functional recovery (Kiger et al., 2009). The understanding of the inflammatory reaction to the spinal cord injury grew since then, and now it is understood that the increased presence of higher numbers of the polarized M2 macrophage the so-called anti-inflammatory macrophage is superior when compared to M1, the inflammatory sub-type. The reason for this might be explained by the fact that M2 cells release activin-A, a molecule that enhances remyelination dependent on oligodendrocyte progenitor cell differentiation (OPCs) (Miron et al., 2013). Although there is a current lack of in-vivo evidence to support the positive role of M2 cells in the promotion of axonal regeneration, infusion of IL-4 into guidance chambers implanted into injured sciatic nerves promoted M2 polarization together with Schwann cell migration culminating in enhanced axonal regeneration (Mokaram et al., 2012).

Furthermore, in a model of spinal cord injury, systemic injection of IL-4 was able to promote increased functional recovery as well as a reduction in the number of cells expressing inflammatory markers such as CD11b/c and iNOS, accordingly, levels of the anti-inflammatory cytokine IL-10 rose considerably in the serum after treatment (Lima et al., 2017).

Taken together, the existing evidence on the possibility of axonal regeneration is multifaceted and a positive outcome will likely arise from an integrated approach that focus at turning the intrinsic genetic neuronal machinery for regeneration on, with the modulation of the external cues in order to promote a more supportive environment for axonal regrowth.

Essentially, after axonal growth, remyelination presents itself as the next challenge to be achieved, and although myelin contains inhibitory proteins for axonal growth, the myelin sheath and myelinating oligodendrocytes are critical for proper axonal function. (Zhang et al., 2013; Maki et al., 2013) In fact, the presence of OPCs (oligodendrocyte progenitor cells) have shown to be important for the generation of mature myelinating oligodendrocytes during injury states. Additionally, OPCs may support axonal regrowth trough the creation of a permissive environment mediated by the B-catenin signaling pathway, and they are now being explored as possible candidates for cell therapy targeting remyelination in different animal models of demyelination. (Kim et al., 2015; Wang et al., 2013; Rodrigues-Frutos et al., 2016)

Another important hallmark to address in order to create an environment that fosters CNS regeneration is the reestablishment of the local microvasculature. Angiogenesis is a

phenomenon that shares conserved molecular constituents to the axonal guidance phenomena. It is a process that starts early in life to ensure adequate delivery of oxygen and nutrients to the developing brain. (Wang et al., 2017)

Upon brain or spinal cord injury, depending on how severe the damage is, disruption of the blood brain barrier (BBB) and blood vessel rupture takes place, and data shows that the reassembling of such structures plays a remarkable importance in reestablishing a homeostatic environment to support regeneration. (Tata et al., 2015)

Recently, a study on rats subjected with spinal cord injury found that increases in angiogenesis correlated with increases in functional recovery as well as sparring of white matter after a treatment with platelet rich plasma (PRP) injection (Chen et al., 2018). The authors attributed the effects to the angiogenic state created by PRP and also revealed the mechanism for such action, pointing for a role of angiopoietin-1 (Ang-1) Tie-2 pathway in mediating the effects encountered, additionally the researchers attested for the presence of an array of angiogenesis promoting molecules such as PDGF and VEGF, contained in PRP.

Additionally, a more mechanistic study found that the delivery of angiogenic microspheres containing VEGF, Ang-1 and FGF-2 was able to promote neural regeneration and motor function gains together with neurogenesis in rats subjected to spinal cord injury. (Yu et al., 2016). The authors discuss the importance of the sustained delivery of such angiogenic molecules to the promotion of a supportive environment for the intrinsic angiogenic response that takes place after SCI, interestingly the presence of such molecules prevented vessel regression 7 days after injury being able to further increase functional vessel density (Yu et al., 2016).

Despite of the clear role of oxygen and nutrient provision and CO2 and metabolite removal provided by new vessel formation, another study presented an interesting result that strengthens the importance of angiogenesis for CNS regeneration, as the sole presence of new vessels, induced axonal sprouting trough prostacyclin production in as experimental model of encephalomyelitis, showing not only the importance of local blood flow but also the existence of local molecular signaling between capillary endothelial cells and neurons at the injury site. (Muramatsu et al., 2012)

Together with the lesion site revascularization, reestablishment of the blood brain barrier (BBB) is essential to achieve regeneration, as the BBB promotes the interface with blood born inflammatory cells and mediators, promoting its functionality is crucial to reduce inflammation and restore local homeostasis (Bejjani et al., 2016; Alves et al., 2014).

For instance, in the event of a neurotrauma the disruption of vasculature integrity leads to the entrance of blood derived molecules into the brain (Chodobski et al., 2011). Fibrin for example, acts on microglia increasing its phagocytic activity, thrombin, ramps up the production of NO (nitric oxide) as well as stimulating its proliferation, and inflammatory cytokines (IL-6 and IL-12) production (Adam et al., 2007; Moller et al., 2000; Ryu et al., 2000).

Similarly, albumin also acts on microglia inducing the production of NO (nitric oxide) and IL-1ß, astrocytes are also responsive to the presence of albumin at the CNS, by expressing receptors to TGF-ß receptor 2 (Ralay Ranaivo and Wainwright, 2010). Apart from these “strict” blood

born molecules, other, parenchymal derived substances exert important roles on the dysfunction of the post-traumatic BBB. Glutamate for instance, is released from various parenchymal cells including infiltrating neutrophils and increases BBB permeability (Palmer et al., 1993). Reactive oxygen species (ROS) have the ability to not only increase BBB permeability, but also allows the invasive capacity of inflammatory cells by inducing the overexpression of cell adhesion molecules such as intracellular adhesion molecule 1 (ICAM1), a similar effect is caused by the presence of TNF-α and IL-1ß, these cytokines, up regulate the expression of E-selectin, and vascular cell adhesion molecule 1 (VCAM1), in addition, these cytokines promote inflammation by increasing the production of chemokines (CXCL1-2 and CCL-2) by endothelial cells and astrocytes leading to increased leukocyte invasion (Bradley et al., 1993; Hess et al., 1994; Stanimirovic et al., 1997) .

Matrix metalloproteinases (MMP) also have an important role in BBB post injury dysfunction, they are produced by many cell types including invading leukocytes and act degrading the basal lamina proteins as well as tight junction therefore disrupting BBB integrity. (Cunningham et al., 2005; Yang et al., 2007).

Taken together, the data mentioned above clearly states the obvious importance of the BBB as a central player in future strategies for CNS regeneration, it also highlights the past flawed view that a single molecule would be able to recapitulate the functionality of the BBB once its disrupted, and take us to the future where a more holistic approach will certainly be the best option for the design of new and efficient therapeutic strategies.

New and exciting alternatives are paving the way for effective therapies aiming at regenerating the damaged CNS, the next chapter will provide an overview of the field of cell therapy for CNS regeneration giving special attention to the use mesenchymal stem cell and its derived products.

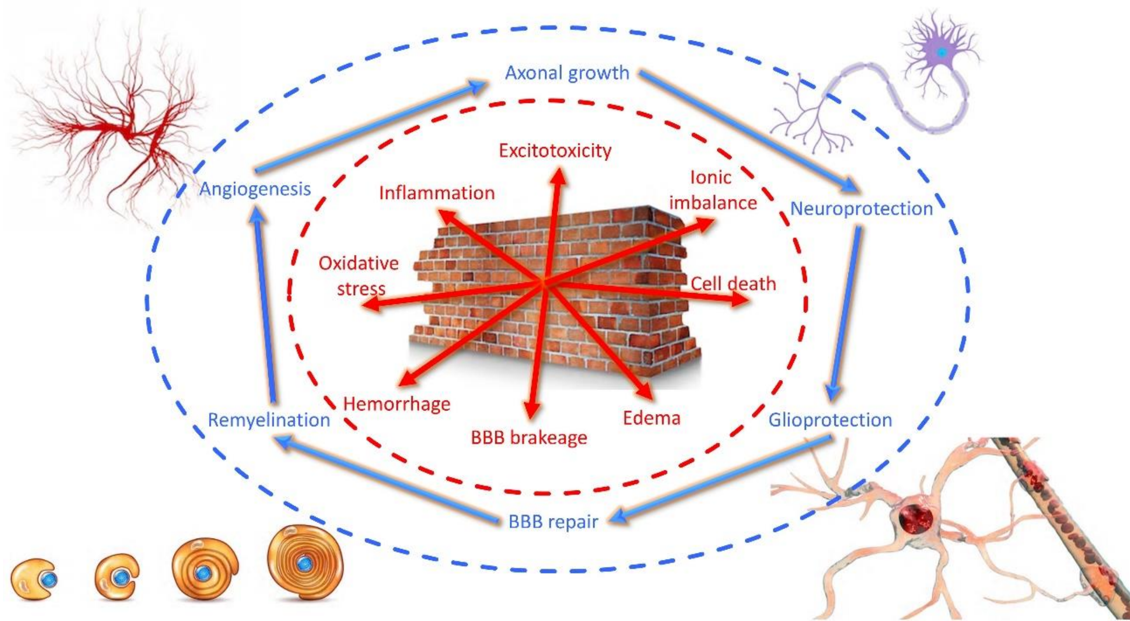


Figure 1. Highlights the physiopathologycal mechanisms that takes place after an injury as well as the physiological phenomena that needs to happen in other to promote regeneration to the CNS. (In red): physiopathological mechanisms and (in blue) the regenerative mechanisms. Abbreviations: BBB, blood brain barrier.

1.2. Mesenchymal stem cells as a regenerative tool

The history of using cells to promote a desired therapeutic outcome is rich and long, for instance, over the last 50 years, over a million hematopoietic stem cell transplants (HSCT) have been performed, putting HSCT as the most well-established cell therapy currently in clinical use. Nevertheless, most cell-based therapies are experimental, with few exceptions reaching clinical trials, in fact, recent analysis have shown that the majority of cell-based therapies are still at early stage of development (clinical trials of phases 1 and 2) focusing on clinical safety and early signs of efficacy respectively (Trounson et al., 2015; Gratwohl et al., 2015).

Cell therapy could be classified according to clinical therapeutic indication, e.g. cardiovascular, neurological or ophthalmological; or if they are isolated from and destined to the same patient (autologous) or if they are transplanted into another individual (allogeneic) transplantation. Additional definition is provided by the EU and discriminates between minimally manipulated cells destined to homologous usage (transplants or transfusions) or those classified and regulated as medicine referred to as (Advanced Therapy Medicinal Products, ATMP's) which are the ones required to demonstrate, quality, safety and efficacy standards in order to obtain marketing authorization before becoming commercially available. (Weissman and Shizuru, 2008)

ATMP's are further divided into gene therapy, somatic cell technology or engineered products and currently, the so far approved ATMP's includes not only somatic cells such as dendritic cells

(Provenge®), cartilage-derived chondrocytes (ChondroCelect® and MACI®) and conreal limbal stem cells (Holoclar®), but also, in vivo gene therapy (Glyberra®).

The rapid progress made in the field and the strong presence of somatic cells leading such innovations, confirms the importance of this area of research.

Although the field of somatic cell technology is not only comprised by stem cells, most of what has been done at the pre-clinical and translational level is related to those cells. In fact, several hundred clinical trials using mesenchymal stem cells (MSC's) or subpopulations of MSC's are currently ongoing around the world and several of them are of phases 2 or 3 (Li et al., 2014).

At figure 2, a graphic scheme summarizes the main tissue sources, advantages regarding therapeutic applicability.

Indeed, in comparison to other cells, MSC's do not present the same ethical constraints as embryonic stem cells nor they have the technical disadvantages of isolation and further expansion when compared to other cells such as neural stem cells (Salgado et al., 2010). Furthermore, MSC's present a general immunosuppressive characteristic upon transplantation becoming a great candidate for allogeneic transplantation as well as being a good candidate for in vitro genetic manipulation aiming for the expression of proteins with regenerative interest (Dai et al., 2005; Meng et al., 2018).

In addition, important aspects put MSC's in an interesting position as a therapeutic tool for multiple conditions, for instance, their widespread availability limits the concerns regarding the amount of starting material as well as the need for invasive techniques of cell harvest. (Teixeira et al., 2017).

Furthermore, as research advances, more tissue sources are identified as candidates, often presenting MSC's with unique therapeutic applications, at the moment, researchers have been able to isolate MSC's from mostly bone marrow, adipose tissue, birth derived tissues (umbilical cord's blood and Warton's jelly, amniotic fluid and placenta), dental pulp, peripheral blood, and skin, as seen in figure 2 (Gronthos et al., 2000; Teixeira et al., 2013; Niezgoda et al., 2017). Also, a smaller number of reports found them present and were able to successfully isolate, characterize, proliferate and pre-clinically study MSC's from synovial fluid, endometrium, muscle and brain. (Jones et al., 2008; Xu Y et al., 2015; Paul et al., 2012)

Another important characteristic is the fact that MSC's present great proliferative potential with minimal senescence across multiple passages. It is worth pointing out that, some studies have identified a breaking point of when the cultured cells start to become considerably senescent, changing its differentiation capacity, reducing its proliferation and also the profile of secreted neuroregulatory proteins (Serra et al., 2018). Therefore, it is currently advisable that for therapeutic purposes, the number of passages as well as the age of the donor are to be given thoughtful consideration (Bonab et al., 2006; Gu et al., 2016). Notwithstanding, MSC's can additionally be genetically engineered to over-express desired therapeutic proteins, in order to improve its regenerative performance trough different approaches ranging from different viral vectors, liposomes, siRNA and CRISPR (Borger et al., 2017; Gerace et al., 2017).

In light of such complexity and in order to standardize and promote its full characterization, it was introduced in 2006 by the International Society for Cell Therapy (ISCT), the minimal criteria for identification of MSC's, the publication defines them as being a plastic adherent, multi-potent population capable of differentiate down the osteogenic, adipogenic and chondrogenic lineages, expressing the membrane antigens CD105, CD90 and CD73, while presenting negative expression of classical hematopoietic markers such as CD44 and CD35. (Dominici et al., 2006)
In fact, many in vivo reports had encouraging uses for MSC's in a multitude of conditions ranging from spinal cord injury (SCI), bone marrow recovery in cancer patients, Parkinson's disease, stroke and graft versus host disease (GVHD), despite of such positive data, the mechanisms of action behind those effects were uncertain (Koc et al., 2000; Li et al., 2001a; Li et al., 2001b; Ikehara et al., 2003).
Mechanistically, in the late nineties trough to the early two thousand, their regenerative potential was attributed to two main aspects, firstly, many studies have showed MSC's to have a great migratory potential towards any given lesion site, with results showing migration and homing capacities to the heart after allograft transplantation, to the brain after nerve injury, to the bone marrow after metabolic storage disorder, and to the kidney following acute tubular damage (Wu et al., 2003; Wynn et al., 2004; Herrera et al., 2007).
The mechanism behind this homing capacity was revealed to be from an interaction between chemokines such as SDF-1 and fraktalkine released from the damaged tissue and chemokine receptors expressed by the MSC's such as CXCR4 and CX3CR1. (Ji et al., 2004)
Together with this homing capacity, the ability to differentiate into new and functional cells, replacing damaged ones to recover tissue homeostasis was the mechanism believed to mediate their actions (Pittenger et al., 1999; Bruder et al., 1998). At around the same time, several reports indicated that MSC's could have an even greater differentiation capacity being able to differentiate beyond the mesodermal lineages, in fact, a genome wide, gene expression analysis, found expression profiles of not only mesenchymal traits but also, endothelial, epithelial and neuronal lineages (Tremain et al., 2001). Although later studies showed MSC's aptitude to present neuronal-like morphology, the experiments revealed this to be an artifact of stressors in the culture conditions that led to cytoskeletal collapse, phenomena that could be mimicked by the use of actin cytoskeleton-disrupting pharmacological agents (Kemp et al., 2011). Additionally, albeit there seems to be an in vivo potential for trans-differentiation of the engrafted MSC's into neuronal or glial progenitors, the rates by which such event happen (1-5% of cells respectively) would hardly explain any of the increased functional results presented by such studies (Salehi et al., 2016) Furthermore, cell-fusion events have also been demonstrated to occur in vivo upon transplantation by different mechanisms to those of trans-differentiation, but in the same way, the frequency by which such phenomenon occurs with fusion rates of 2-11 clones per million cells clearly deem this process unlikely to be the one behind the promotion of any of the functional results (Terada et al., 2002; Woodburry et al., 2000).

Nevertheless, up until that point and without clear notion of the mechanisms behind MSC's actions, a considerable number of evidences was mounting regarding its beneficial impacts for regenerative purposes, especially for CNS applications. (Maltman et al., 2011)

A few years later, Gnechhi and colleagues, in 2005, published a paradigm shifting research, revealing the most probable mechanism of action for the MSC's, opening a field for different ways and technologies to be explored in the way researchers used these cells for therapeutic purposes. His results demonstrated that the therapeutic potential of MSC's was mostly related to the secretion of growth factors to its extracellular environment rather than to their differentiation potential. (Gnechhi et al., 2005).

Indeed, a few years earlier, an array of different growth factors such as brain derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and ciliary neurotrophic factor (CNTF) were identified in the culture media of such cells after exposure to an ischemic brain extract confirming their paracrine capacities (Chen et al., 2002).

Whereas the secretome nowadays, is widely accepted as one of the main drivers behind the therapeutic potential of MSC's, it is worth mentioning that the sole presence of these cells in a damaged tissue present benefits, namely by contact mediated trophic support (Donders et al., 2015; Scuteri et al., 2014).

Another important regenerative mechanism that have only recently began to be explored, is the MSC's capacity to transfer functional organelles such as lysosomes and mitochondria to neighboring cells trough tunneling nanotubes (TNT) and extracellular vesicles (EV's).

The current research on the topic is new and comes mostly from in vitro and in vivo models of respiratory system inflammation or ischemia-reperfusion injury in the contexts of lung and heart disease (Murray and Krasnodembskaya, 2019).

Transfer of mitochondria from MSC's have been reported to happen between endothelial, epithelial, cardiac, renal and immune cells and is followed by physiological enhancement of oxidative phosphorylation and ATP production having important implications in the recipient cell bioenergetics (Hsu et al., 2016). Such process can be mediated by microvesicles, apoptotic bodies and TNT, and it shows to be protective against chronic obstructive pulmonary disease and asthma mouse models (Islam et al., 2012). Furthermore, EV-mediated transfer of mitochondria to macrophages, increases ATP turnover and enhances phagocytic activity in vitro, additionally TNT-mediated mitochondrial transfer promoted macrophage bioenergetics and improved phagocytosis in vitro and in models of *Escherichia coli*-induced lung injury (Phinney et al., 2015).

In another context, TNT-mediated mitochondrial donation from MSC's to cardiomyocytes improves bioenergetics as well as reduces apoptosis, interestingly the reverse transport also occurs in moments of oxidative stress when cardiomyocytes can transfer mitochondria to MSC's triggering anti-apoptotic responses (Koyanagi et al., 2005).

Lysosomal transfer induces the maintenance of both lysosomal pH and pool during physiological stress, reducing the risk of early apoptosis and senescence of endothelial cells in the case of a vasculopathy. Transfer of lysosomes was shown to happen trough TNT endothelial progenitor cells (EPC's) and endothelial cells inducing the rescue of vascular function through mediation of vaso-relaxation properties in models of diabetic-induced vascular senescence. (Yasuda et al., 2011)

Such mechanisms, although underexplored at the moment, represent novel and important features of MSC's in maintaining a responsive communication to its microenvironment and even at this point there is existing data to support the transfer of mitochondria and lysosomes as important mechanisms behind the therapeutic effects of mesenchymal stem cell therapy.

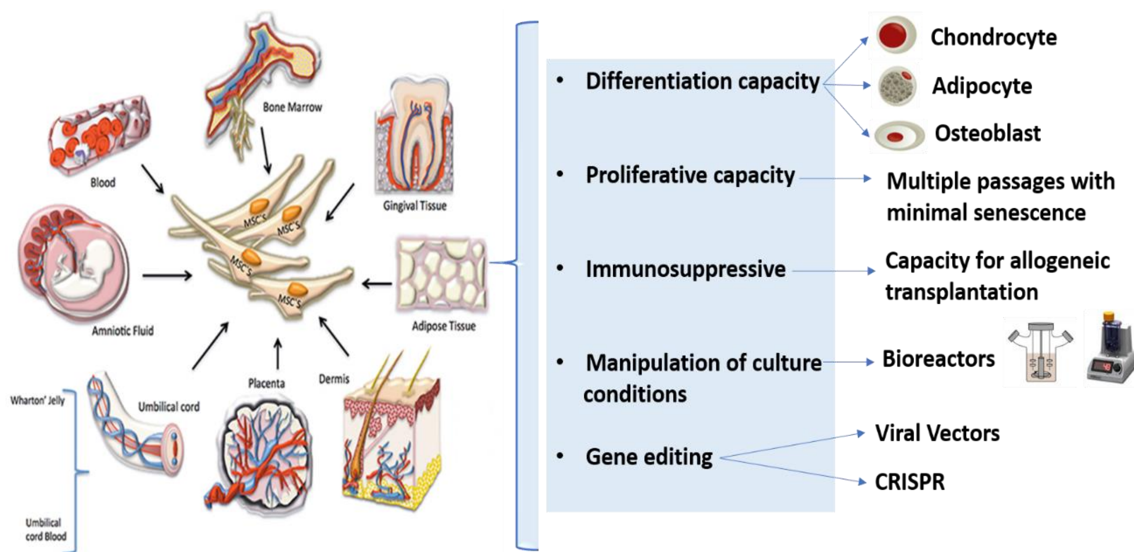


Figure 2. Tissue origin and phenotypical characteristics of MSCs. MSCs are distributed throughout the different tissues of the body, increasing the number of tissue sources for isolation, they present multilineage differentiation potential and possess a high proliferative capacity, additionally, they can be easily cultured in vitro and manipulated using different culture technologies and genetic engineering to increase its regenerative potential. Abbreviation: CRISPR, clustered regularly interspaced short palindromic repeats. Adapted from: Merino-González., et al 2016.

1.3. Secretome of mesenchymal stem cell

Since the beginning of its usage, the term cell secretome have gained more context, reaching areas even outside of stem cell biology or regenerative medicine, for that reason an accurate conceptual definition is hard to be made given the complexity of the biological processes it entails. Nevertheless, secretome could be broadly defined as the myriad of molecules secreted by any given cell, including (proteins, extracellular vesicles, signaling lipids, metabolites and even whole organelles). A graphic depiction of the molecular complexity of the secretome as well as identified CNS processes that it modulates can be found in figure 3.

Further in this section, data from interventional studies applying the secretome for CNS regeneration purposes will be discussed (Praveen et al., 2019; Teixeira et al., 2013).

MSC's secretome has been shown to be extremely responsive to changes in the environment in which the cells are cultured, and as a regenerative product, it offers endless possibilities for modulation of its trophic potential depending on the type of MSC used as well as the culturing protocol of choice. For instance, the usage of hypoxic culture condition has been shown to increase the neuroregulatory profile of the secretome of MSC's, moreover, secretome collected from cells cultured in dynamic bioreactors have a higher profile of trophic factors and is able to induce higher levels of neuronal proliferation and differentiation in vivo when compared to the secretome from MSC's cultured in static conditions. (Teixeira et al., 2015; Teixeira et al., 2016)

Additionally, when primed with pro-inflammatory cytokines, bone marrow MSC's (BMMSC's) secretome presented higher levels of TIMP-1 a key anti-angiogenic protein, furthermore, pharmacological preconditioning has also been shown to induce changes in BMMSC's and ADSC's conditioned media showcasing the broad possibilities for modulation of the trophic profile of the secretome (Maffioli et al., 2017; Ferreira et al., 2018).

Nowadays, such molecular changes in the secretome can be verified and characterized with the aid of multiple technologies, although we should recognize that more robust techniques with smaller detection limits would benefit the field immensely, which by allowing the total molecular characterization of the secretome would enable the full understanding of its mechanistical properties (Teixeira et al., 2017).

At the moment, proteomic-based techniques are the most common for secretome characterization, reflecting the importance of the proteomic research field for general biomedical science, in this sense, techniques such as 2D-eletrophoresis, multiple liquid chromatographic techniques, mass spectrometry, multiplex-type essays and ELISA (enzyme-linked immunosorbent assay) are the ones most used (Teixeira et al., 2017).

Alternatively, and with an aim directed to metabolomics, nuclear magnetic resonance (NMR) spectroscopy can be employed to study the metabolic composition of the secretome (Pereira et al., 2014).

As a matter of fact, several characterization studies based on proteomics have been carried out in the past few years, in which most of them were targeted to clarify differences among the secretome of different MSC's population. In that sense, data from a LC-MS/MS study showed clear differences between the secretory potential of MSC's derived from bone marrow, adipose tissue or umbilical cord tissue, the analysis revealed the expression of 451 different proteins, being 134 of them common among all three cell populations and in spite of such differences all cells presented relevant expression levels of important neuroregulatory proteins, which in the context of the study, answered important mechanistic questions from in vivo interventional studies. For instance, BMMSC's secretome expressed important protective proteins against oxidative stress and apoptosis, whereas ADSC's secretome presented expression profiles important for the prevention of excitotoxicity and inflammation, lastly, the secretome of umbilical cord perivascular cells (HUCPVC's) showed to be appropriate to increase proteostasis and to reduce apoptosis (Pires et al., 2016).

Similar results were obtained from mass spectrometry analysis of the secretome from stem cells of the dental apical papilla (SCAP's) in comparison to BMSC's secretome, only this time, the number of identified proteins were higher (2,046) given the lower detection limit of the applied method, the results reviewed some degree of overlap between the two secretomes, but also highlighted differences among them, for instance SCAP's presented increased secretion of proteins involved in metabolic processes, transcription, chemokines and neurotrophins and decreased secretion of proteins involved with immunomodulation, angiogenesis and developmental processes. (Yu et al., 2016)

Although, extremely relevant, an important caveat of such proteomic data from mass spectrometry is the type of fractionation applied to the sample in order to increase the sensitivity of the measures, for instance, the different biogenic pathways that produce the full composition of the proteome that makes up the secretome is lost, in that sense, previous fractionation of the secretome would be advisable, specially, taking into account the fact that the secretome is composed not only of soluble proteins but also by extracellular vesicles with a rich protein content (Anjo et al., 2016; Yu et al., 2016). Therefore, analyzing the two fractions (soluble protein fraction and vesicular fraction separately would give us insight into the complex mechanisms behind the physiological responses to the secretome.

When applied to in vitro and in vivo models of CNS diseases, the use of MSC secretome is shown to present promising results, for instance, in Parkinson's disease , secretome usage is shown to improve cell survival of TH+ dopaminergic neurons in culture as well as protect them against neurotoxicity caused by MPTP and 6-OHDA toxins, two clinically relevant toxins used in Parkinson's disease models. Furthermore, in vivo application of MSC secretome has shown to rescue the parkinsonian phenotype by reducing local inflammation and oxidative stress, as well as maintaining the TH circuitry of animals injected with 6-OHDA, similar effects were also encountered with the employment of undifferentiated hNPC's (human neural progenitor cells) secretome in the same disease model. Such regenerative outcomes were associated with the presence of secreted trophic factors, BDNF, IL-6, glial cell-line derived neurotrophic factor (GDNF), cystatin C, glia-derived nexin, galectin-1, pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), (FGF-2), endothelial growth factor (EGF), anti-inflammatory, IL-10 and TGF-ß, anti-oxidant proteins, peroxiredoxin-1, superoxide dismutase and protein deglycase (DJ-1) and extracellular matrix enzyme MMP-2 (matrix metalloproteinase 2). (Cova et al., 2010; Park et al., 2012; Kim et al., 2009: Teixeira et al., 2017; Pinheiro et al., 2018). Such pre-clinical data moved research into early clinical trials of safety and efficacy assessment of BMSC or UC derived MSC's. Currently two studies are undergoing patient recruitment and one is currently active. (NCT03550183, NCT02611167, NCT03684122. 2019, Clinical Trials.gov National Library of Medicine)

Regarding spinal cord injury (SCI), several reports have showed that the secretome from MSC's modulates important physiological hallmarks associated with improved disease outcome, for instance, in vitro and in vivo data, supports the use of secretome for axonal sparring, reducing inflammation and apoptosis and maintaining levels of myelination thus promoting axonal

regeneration, neurite outgrowth and glial scar reduction, which in turn mediates functional recovery. Such results demonstrate that the presence of trophic factors in the secretome such as BDNF, VEGF, NGF, IL-6, SDF-1α, HGF and GDNF were responsible to drive those mechanisms (Cantinieaux et al., 2013; (Ciskova et al., 2018; Szekiova et al., 2018; Kingham et al., 2014; Wright et al., 2010). Indeed, such results encouraged researches to move forward, and several clinical trials exploring the role of MSC's transplantation are now undergoing.

Recently, a metanalysis of a systematic review from MSC's transplantation data in humans was published comprising eleven studies and 499 patients, the results reviewed encouraging improvements in sensory and bladder functions although, no differences were seen in motor function or activities of daily living (ADL) when compared to controlled groups. (Xu and Yang, 2019). Such data reveal the potential clinical applications for interventions in sub-acute SCI lesions and encourage the exploration of other treatment strategies such as different routes of administration, use of the secretome with or without cell therapy and also the exploration of combined strategies.

Indeed, more recently, MSC-derived extracellular vesicles, including exosomes and micro-vesicles are being studied for their role in MSC-based cell therapy, such vesicles have been shown to at least partially mediate cell-cell communication, cell signaling and also influence cellular metabolism locally or at distant organs, furthermore, studies have found them able to modulate responses to injury, such as inflammation, and cell survival ultimately influencing disease onset, progression and therapy (Phinney and Pittenger, 2017; Rosca, et al., 2017).

Extracellular vesicles are an heterogenous class of lipid bilayer vesicles containing a diverse cargo. In order to be characterized, EVs could be assigned to their biogenesis pathway within the cell, although, such characterization requires the correct imaging technique to be employed and should capture the exact moment of release by using techniques such as electron or atomic force microscopy (Lotvall et al., 2014). Instead, a more traditional characterization is achieved accordingly to morphological features and specific membrane marker expression patterns. For instance, vesicles coming from endocytic origin are normally termed exosomes, and vesicles coming from the shedding of plasmatic membrane are usually referred to as ectosomes (micro-vesicles or microparticles) (Thery et al., 2018).

Morphologically, exosomes present diameters in the range of 30 to 150nm whereas ectosomes could present diameters of a 100nm to more than one micrometer, Nevertheless, the current MISEV, 2018 guideline suggests a division defined by ranges, where vesicles that are smaller than 100nm or not bigger than 200nm should be referred to as (small EV's) and vesicles that are bigger than 200nm should be called (large/medium EV's). Additionally, they can be categorized by its density (low, middle or high), and also, biochemically based on known membrane markers such as (Annexin A5, CD63, CD9, CD81, TSG-101, HSP-70 and many others). (Thery et al., 2018)

The biogenesis of exosomes follows the typical endocytic pathway, where transmembrane proteins are endocytosed and trafficked to early endosomes, those early endosomes mature into late endosomes in a process where the endosomal membrane invaginates generating intra

luminal vesicles (ILV's), as a result, the multivesicular bodies (MVB's) are formed and are destined to the plasma membrane for exocytosis. The process of intraluminal vesicle formation is governed by sphingomyelinase 2 and is ceramide dependent, whereas the formation of MVB's is governed by the ESCRT machinery, a protein arrangement formed by four different protein complexes (Zorec et al., 2016).

The fusion of the MVB's to the plasma membrane is mediated by different proteins of the Rab family (Ras superfamily of small GTPases) Rab 11, Rab 27 and Rab 35, and its contents are released to extracellular environment (Qin and Xu, 2014).

Although far less complex, the biogenesis of ectosomes also involves both the ESCRT machinery and ceramide, being controlled by the interaction of ARRDC1 (Arestin) and tumor sensitive gene (TSG-101), a process that allows for the assembly and budding of the vesicles. The size of ectossomes can vary broadly, and EV's of over 4µm in diameter have also been reported. Such large sized EV's present a specific membrane shedding marker, ß1-Integrin, and also exhibits in its outer leaflet acid sphingomyelinase and phosphatidylserine, they can contain whole organelles such as lipid droplets and even mitochondria, and although the physiological relevance of its release has not yet been fully revealed the possibility that they can represent apoptotic bodies remains plausible. (Bianco et al., 2009; Bianco et al., 2005)

Additional to the already verified positive effect of the secretome and its existing protein content, early investigations are being made in the context of further characterization of the vesicular component of the secretome aiming for better understanding of the molecular mechanisms governing such findings. In fact, the possibility exists that at least some of the proteins already identified as composing the soluble portion of the secretome are also present inside extracellular vesicles such as exosomes and, in fact, BDNF, FGF, VEGF, MMP's, HGF, IL-10, TGF-ß have been found to be expressed in MSC exosomes (Chisanga et al., 2015) .

Furthermore, many interventional reports now have data from in vitro and in vivo experiments, where the role of MSC's derived exosomes is evidenced, for instance in the promotion of angiogenesis, immunomodulation and myogenesis as well as in the reduction of oxidative stress and apoptosis, having strong implications for, hearth, kidney and autoimmune diseases (Jing et al., 2018). In what concerns CNS pathologies additional benefits were also observed, for instance in stroke, exosomes from MSC's mediated neurite outgrowth and functional recovery by the delivery of mIR-133b in vivo (Xin et al., 2016). Similarly, in TBI they were shown to reduce neuroinflammation by suppressing microglial and astrocyte activation and also by inducing neurogenesis by stimulating neurogenic niches (Yang et al., 2017). Such tendency was also observed in SCI where MSC's exosome injections presented modulatory effects of inflammation and regeneration what led to functional recovery in rats. (de Rivero et al., 2015)

In neurodegenerative diseases such as Alzheimer's, exosomes from ASC's were shown to express functional neprilysin (NEP) capable of degrading Aß aggregates 40 and 42, and in Parkinson's where the presence of catalase in MSC exosomes presented strong neuroprotective function in the 6-OHDA rat model (Katsuda et al., 2013; Chang et al., 2018).

Although extremely encouraging, the results mentioned above gives rise to an important mechanistical question, of where is the beneficial impacts of MSC's secretome therapy coming from? The evidence is clear that either the secretome as a whole or the isolated extracellular vesicles and exosomes possesses a huge potential when applied to different pathological contexts. A solution to this question would emerge from carefully designed studies aimed to test in the same disease context and population, not only the secretome as a whole but also a second arm with the isolated EV's and a third arm with the soluble proteins left from the isolation protocol from the same secretome batch. Coupling such study with a proper molecular characterization of each fraction would likely yield a huge amount of valuable information for the design of future therapies as well as clinical trials and will likely be decisive in the future of cell-free based therapies.

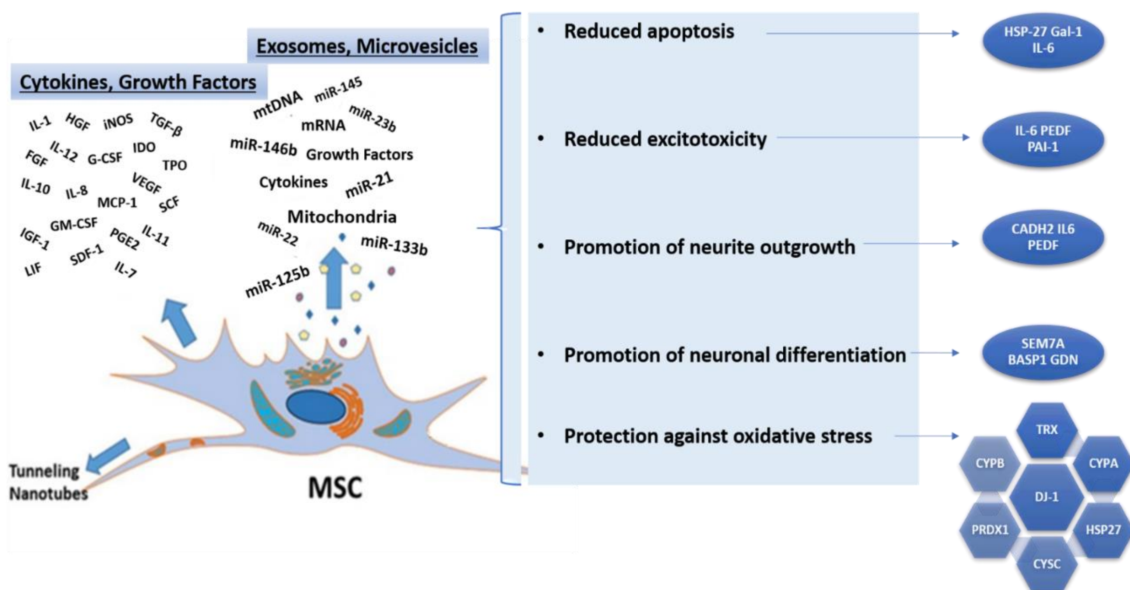


Figure 3. MSCs secretome molecular complexity and function. MSCs trough different secretory pathways secrete an array of molecules that modulate clinically relevant pathological hallmarks of numerous CNS diseases. Abbreviations: HSP-27, heat-shock protein 27; Gal-1, galectin-1; IL-6, interleukin-6; PEDF, platelet endothelial derived factor; PAI-1, plasminogen activator inhibitor 1; CADH2, cadherin-2; SEM7A, Semaphorin 7A; BASP1, Brain acid soluble protein 1; GDN, glial derived nexin; TRX, thioredoxin; CYPA, cyclophilin A; CYPB, cyclophilin B; CYSC, cystatin C; PRDX1, peroxiredoxin-1; DJ-1, protein deglycase-1 Adapted from: Phinney and Pittenger, 2017; Pires et al., 2016

1.4. Astrocyte functions in the CNS an overview

The basis created in the first chapter will help the reader to understand the different molecular aspects necessary to support the different functional roles astrocytes have in the CNS.

This sub-chapter will be divided into the most relevant and well-studied astrocytic functions aiming to construct a final picture that enables easy recognition of key targets for the understanding of pathophysiological implications as well as therapeutic opportunities.

1.4.1. Neurodevelopment

Originally recognized as cells with predominantly structural roles being responsible for the tilling of the brain cytoarquitecture, astrocytes have earned progressive attention and are currently seen as crucial cells for sustaining normal brain physiology.

In neurodevelopment, astrocytes show remarkable importance by coordinating neuronal survival, differentiation and maturation trough contact mediated or the release of its secretome composed of soluble trophic factors, signaling vesicles and extracellular matrix proteins (ECM), what allows for neurite outgrowth and controlled modulation of synapse formation and function (Clarke and Barres, 2013).

These functions are sustained by the continuous communication between neurons and astrocytes what gives the necessary molecular inputs that thanks to the expression of receptors for neurotransmitters are interpreted and translated into astrocytic secretion of molecular cues able to alter neuronal physiological fate.

For instance, in the CNS development of embryonic *drosophila,* astroglia was shown to be responsible to maintain viability of follower neurons while they migrate towards pioneer neurons by cell-cell interaction via axonal guidance (Booth et al., 2000).

Regarding neurite development, astrocytes are involved by secreting lamnin-1, fibronectin and plasminogen activator inhibitor-1, such ECM proteins were responsible for elongation and maturation of neurites after astrocyte muscarinic and also thyroid hormone (T3) stimulation. (Guizzetti et al., 2008; Martinez Rodrigo et al., 2002).

Additionally, astrocytes have been shown to promote synapse formation by the release of thrombospondins (Christopherson et al., 2005), synapse maturation by the secretion of TNF-alfa (Stellwagen et., 2006) and control of synapse function and support of synaptic plasticity by sustaining LTD (long-term-depression) mediated by vesicular ATP release (Pascual et al., 2012).

1.4.2. Neurotransmission

Astrocytes have the ability to infiltrate into the neuropil and wrap its processes around the synapse, by doing that, they can sense the molecular synaptic microenvironment and also respond to it by sending specific molecules with three main targets, modulation of synaptic structural formation, induction of functional changes and in some cases synapse elimination.

The astrocytic involvement in synapse formation have been attested in many synapses such as glutamatergic, GABAergic, glycinergic and cholinergic ones, and multiple molecular signals that govern this process are both contact-mediated or secreted cues (Allen et al., 2017; Chung et al., 2015).

As an example, in the formation and maturation of presynaptic terminals, cholesterol is seen as an essential molecule released by astrocytes into apoE (apolipoprotein E) that is responsible to increase vesicle density and fusion probability leading to increased presynaptic function (van Deijk et al., 2017).

An important step in the establishment of functionally connected synapses is the ability to eliminate dormant or superfluous ones based on its activity. Such process is achieved by the secretion of SPARC (secreted protein acidic and cysteine rich) that antagonizes the positive effects of HEVIN, also, by the secretion of TGF-ß that promotes synaptic c1q expression what leads to c3 receptor-mediated microglial recognition and phagocytosis. In addition, astrocytes also can autonomously eliminate excess synapses via the phagocytic receptors Mertk and Megf10. (Kucukdereli et al., 2011; Schafer et al., 2012)

1.4.3. Brain metabolism

The human brain consumes approximately 20% of the total energy substrate at rest even though it only represents around 2% of the total body mass. This high energetic efficacy is achieved thanks to the considerably high metabolic plasticity of astrocytes (Morita et al., 2019; Falkowska et al., 2015).

Astrocytes are strategically positioned in a way that reveals its central role in CNS metabolism and in fact, these cells sit between the cerebral microvasculature and densely distributed neuronal processes and synapses (Verhatsky et al., 2017). This specific localization is important, because astrocytes are able to cover entire surfaces of capillaries where they express multiple transporters such as GLUT 1, which enables facilitated diffusion of glucose, monocarboxylate transporters (MCT) that facilitates absorption of lactate and also fatty acid translocase (FAT) enabling absorption of fatty acids (Leino et al., 1997; Pierre et al., 2005; Husemann et al., 2001). Such nutritional substrates serve as basic fuel for the different neurochemical reactions required for normal brain homeostasis. Additionally, astrocytes are put in the center of this metabolic control specially because they present a large enzymatic capability as well as being arguably the only stock of glycogen in the brain. Glycogenolysis serves to provide glucose for ATP production, oxidative stress management, synthesis of neurotransmitters and neurohormones, and structural components for brain cells (Belanger et al., 2011). Although the basal blood supply of glucose seems to be enough to sustain neuronal glycolytic requirements throughout normal activities, during periods of high energy demands and in hypoglycemia the relevance of astrocytic derived glucose is increased. For instance, during intense firing patterns, where there is an accumulation of glutamate in the peri synaptic space, astrocytes can absorb the excess glutamate in exchange for lactate and glutamine fueling the metabolic neuronal requirements in the phenomenon known as ANLS (astrocyte-neuronal L-lactate shuttle). (Li et al., 2015; Abe et al., 2006) In addition, evidence from experiments of glucose-deprivation conducted on the optic nerve shows that astrocytes are relevant in maintaining axonal fuel by breaking glycogen down to lactate and transferring it into neurons. (Wender et al., 2000).

Additional metabolic roles for astrocytes involve the supplying of lipids for synaptic membrane and myelin synthesis by neurons and oligodendrocytes respectively.

1.4.4. Blood flow

The high energy demand imposed by functioning neurons to maintain basal brain activity is supported by an extensive vasculature (Mishra et al., 2017). The concept of an interconnected neuro-vascular system has emerged from over a hundred years ago when the term neurovascular coupling (NVC) was first described (MacVicar et al., 2015). Since then, the vascular responses that are followed after surges in neuronal activity have been established as a fundamental hallmark of brain physiology, being currently exploited by many of the non-invasive diagnostic tools such as PET (positron emission tomography), BOLD fMRI (blood oxygenation level dependent functional magnetic resonance imaging) Fnirs (functional near infrared spectroscopy) all exploring such fluctuations of blood flow as a standard measure for neuronal activity (Hendrikx et al., 2019). The earlier mentioned ideal position between neurons and blood vessels likely translates the important role astrocytes have in modulating NVC, for instance, (Paulson and Newman, 1987) demonstrated that K^+ released by astrocytic end-feet in response to neuronal activity can be a viable mechanism to promote local blood flow increase, in fact, this theory was accepted after the study of (Filosa et al., 2006), showed that neuronal activity which is responsible for causing intracellular Ca^{2+} increases in astrocytes, leads to an activation of K^+ channels on astrocytic end-feet that translates in a marked K^+ efflux onto the vasculature resulting in hyperpolarization of VSMC (vascular smooth muscle cells) and consequent arteriole dilation.

Another proposed mechanism for astrocytic modulation of cerebral blood flow is the release of vasoactive agents. Astrocytes can metabolize membrane phospholipids into AA (arachidonic acid) which is the main precursor of vasodilatory molecules such as prostaglandins and EETs (epoxyeicosatrienoic acids). Furthermore, modulation of AA metabolism pathway was shown to reduce functional hyperemia in vivo, and, further in vitro evidence from slice experiments demonstrated that neuronal activity mediated vasodilation was dependent on astrocytic Ca^{2+} increases and concomitant prostaglandin E2 (PGE2) production (Zonta et al., 2003). Additionally, AA metabolite EET's is quickly metabolized into 20-hydroxyeicosatrienoic acid (20-HETE) which provokes vasoconstriction. 20-HETE's role as a vasoconstrictor was further validated, by studies showing considerable reduction in arteriole lumen in hippocampus and retina after 20-HETE's release from activated astrocytes either by Ca^{2+} uncaging or metabotropic glutamate receptor (mGluRs) activation (Mulligan et al., 2004; Metea et al., 2006).

Interestingly, the fact that astrocyte activation provoked mixed results regarding brain vaso-activity in different brain regions, raises the question for a possible dual role for astrocytic control of the brain vasculature.

1.5. Astrocyte signaling (mechanisms and physiological relevance to the CNS)

Astrocytes, currently being referred to as being neural homeostatic cells, have the ability to sense its extracellular environment presenting a key role for information processing in the central nervous system (CNS). This processing has been shown to be mediated by changes in intracellular second messengers such as Ca^{2+} and cyclic adenosine monophosphate (cAMP) and although astrocytes are electrically non-excitable, they possess an extended number of excitable molecules and mechanisms. (Verhatsky and Zorec, 2019; Horvat and Vardjan, 2018 Volterra et al., 2014),

Structurally, astrocytes present plasmalemmal ion-channels and receptors to neurotransmitters, neuromodulators and neurohormones and also cytoplasmatic structures that are able to generate transient elevations in the concentration of such second messengers. (Verkhratsky and Zorec, 2019; Horvat and Vardjan, 2018 Volterra et al., 2014),

1.5.1. AMPc signaling processing in astrocytes

AMPc is a second messenger that thanks to the development of new cAMP reporter technology has had its spatial-temporal dynamics been studied and further understood. Its presence in astrocytes is correlated with the expression of genes with important homeostatic functions such as (extracellular environment control, antioxidant defense, communication and lastly neuronal metabolic and trophic support) (Parpura and Verkhratsky, 2012).

Its actions need to be tightly regulated in space and time in order to maintain a high level of specificity and normal cell function. cAMP concentrations are increased after GPCRs (G-protein coupled receptor) stimulation and for proper selectivity, it signals in physically separated compartments within the cell (compartmentalized signaling), being also the duration of the signaling precisely controlled. (Horvat and Vardjan, 2018)

Astrocytes present a broad expression of GPCRs linked to either Gs or Gi proteins and have been recognized as the main target of the noradrenergic system in the CNS. Illustrated in the figure 1 are the signaling pathways for α-1 and ß adrenaline receptors in rat astrocytes.

Although data with cAMP recordings are still scarce, its elevations in astrocytes have been linked to a reduction in astrocyte swelling (cytotoxic edema) when subjected to a hypotonic environment or a neurotrauma, showing that its activation could have neuroprotective implications. (Vardjan et al., 2016)

1.5.2. Astrocyte Ca^{2+} signaling processing

The current understanding regarding Ca^{2+} signaling in astrocytes came from years of studies employing the calcium imaging technique where the first reports arose from experiences monitoring calcium surges in cultured astrocytes and also in situ after glutamate exposures (Cornell-Bell et al., 1990; Cornell-Bell and Finkbeiner,1991; Finkbeiner, 1992; Wang et al.,

2006). Although such experiments led us to a remarkable advancement in the understanding of how astrocytes respond to neuronal transmission and synaptic activity, they only focused, due to methodological constraints, on the assessment of somatic Ca^{2+} surges, giving us the idea that astrocytes respond to only intense neuronal firing patterns. Further work carried out on astrocytic processes revealed a much more refined response of even synapses of lower levels of activities, suggesting that the Astrocytic Ca^{2+} activity encompasses the whole spectrum of neuronal communication (Volterra et al., 2014).

Presently it is understood that neurons are able to transfer information over to astrocytes mainly by the spillover of synaptic transmitters and factors that are able to bind to high-affinity GPCRs that trigger inositol-1,4,5-triphosphate (IP-3) production and therefore Ca^{2+} release from the endoplasmic reticulum (ER). Once activated this signaling system can generate a wide range of oscillatory Ca^{2+} signals. (Volterra et al., 2014)

Furthermore, other mechanisms of increased intracellular Ca^{2+} concentration exist in astrocytes, namely, the stimulation of Ca^{2+} permanent ionotropic receptors in regions such as the cerebral cortex by NMDA receptor stimulation, the ectopic stimulation of AMPA receptors in the cerebellum and the reversal of Na^{+}/Ca^{2+} exchangers following neurotransmitter uptake and other pumping activities (Berridge et al., 2003; Rizzuto and Pozan, 2006).

The main endpoint triggered by cytoplasmic increases in Ca^{2+} concentration is the release of neuro-chemical mediators named (gliotransmitters) which have been shown to have modulatory activities on other glial, neurons and vascular cells. (Berridge et al., 2003; Rizzuto and Pozan, 2006).

1.5.3. Mechanisms of astrocyte transmission.

Many underlying mechanisms govern astrocyte transmission (gliotransmisson) but in a simplistic way they could be divided into 3 sub-categories namely, diffusion trough membrane pores, passage through membrane transporters or by means of vesicular exocytosis as seen in figure 4. In this section an attempt to summarize the most relevant and well-studied mechanisms of transmitter secretion in astrocytes was made, summarizing the information in figures 5 and 7.

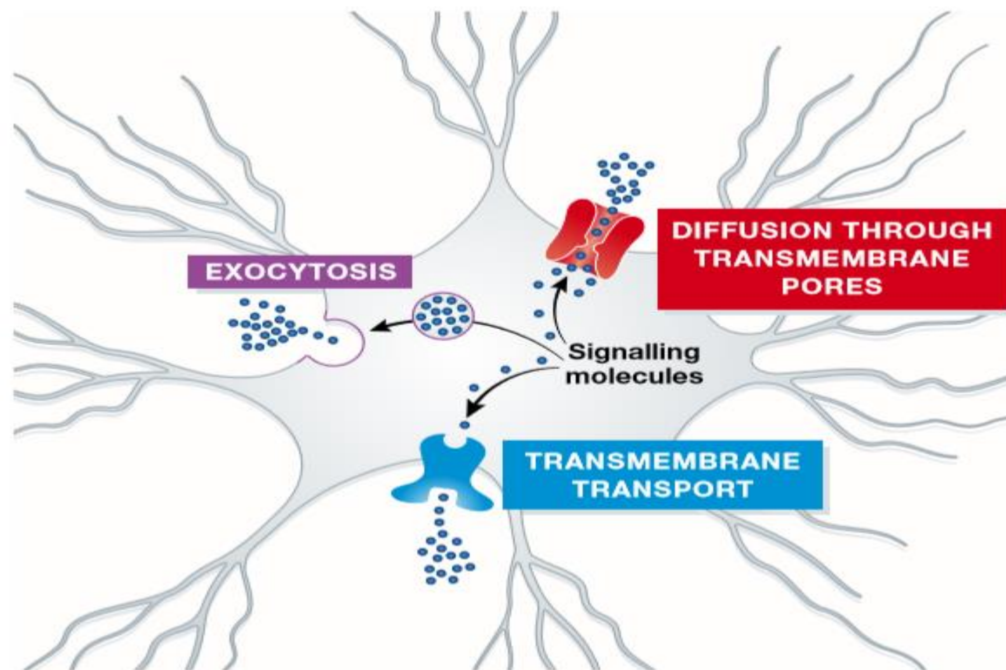


Figure 4. Astrocytes present diverse mechanisms of gliotransmission, from which they maintain CNS homeostasis, responding to neuronal synaptic activity, extracellular ionic concentration, blood brain barrier function and inflammatory insults. Adapted from Verkhratsky et al., 2016

1.5.4. Diffusion of signaling molecules trough membrane pores

Cell swelling is a phenomena that typically occurs after the appearance of an hypo-osmotic condition such as in cases of trauma (stroke/traumatic brain injury/spinal cord injury), and, to compensate for this increase in cell volume, astrocytes open volume-regulated anion channels (VRAC), and trough this channels the passage of inorganic and small organic anions such as aspartate, glutamate and taurine have been reported, especially after the application of highly concentrated KCl, or by culturing the cells in and hypo-osmotic environment (Kimelberg et al., 2004). Several lines of evidence shows a relatively complex control of VRAC anion diffusion, for instance, the levels of cytosolic $Ca^{2+,}$ nitric oxide, hydrogen peroxide, thrombin and activation of kinases all seem to play relevant roles in such secretion pathway, furthermore, ATP mediated astrocytic swelling was shown to induce aspartate, glutamate and taurine release by VRACs being its action attenuated by the use of channel blockers (Rutledge et al., 1998; Kimelberg et al., 1990).

Additionally, there is some evidence in support of GABA release by VRAC after the induction of cell swelling and although the authors confirmed the noninvolvement of purinergic ion-channels and hemichannels pharmacologically, it should take into consideration that the use of anion channel blockers causes direct effects on GABA receptors (Raiteri et al., 2008).

Although the conclusive determination of what transmitters can be diffused trough VRACs has proven complicated due to the lack of specific inhibitors, such secretory pathway certainly deserves the appropriate attention of future studies aiming to dissect the secretory capacity of astrocytes.

Another form of pore formation that is implicated in astrocytic transmitter release is the formation of connexons (GAP-junctions), such structures are formed by the junction of two hexamers of the protein connexin, being the connexin Cx43 apparently the more prevalent in astroglia (Contreras et al., 2003). There is data showing that unpaired connexons may be able to act as functional hemichannels which are able to provide a mechanism of outwardly transmitter diffusion in astrocytes, in fact hypotonicity was able to induce the release of glutamate, aspartate and taurine in hippocampal astrocytes, being their release attenuated by the application of gap-junction blockers. (Contreras et al., 2003) This mechanism was further tested in Cx43 knockout mice which by using astrocyte cultures of such model, the authors were able to show an important reduction in glutamate release right after the exposure to low extracellular divalent cations when compared to wild type controls. (Speidel et al., 2002)

Coincidentally some properties related to hemichannel function correlates well with those of pannexons (non-junctional channels formed by pannexin proteins), having the same ability to communicate the extra and intracellular environments. (Bruzzone et al., 2005) These channels are not activated by fluctuation in extracellular Ca^{2+} but in turn, are activated by increases in cytoplasmatic Ca^{2+}. Although there is a lack of functional evidence for the release of glutamate trough these channels, RNA from pannexins 1, 2 and 3 have been identified in astrocytoma and glioma cells opening the possibility for this pathway to have a considerable role in broader ways of gliotransmission and not just being confined to the well-established release of ATP. Furthermore, pannexin 1 protein expression was detected in cultured astrocytes and in glial-like taste bud cells (Bruzzone et al., 2005).

Another pore forming ion channel, the purinergic P2X receptor may serve as an additional way for signaling amino-acid release in astrocytes (North et al., 2002). P2X receptors are ATP gated non-selective cation channels that shows amplified responses upon low divalent ionic extracellular concentration. There are 7 known types of P2X subunits that can assemble and form a homomeric pore capable of passing molecules of up to 900Da. This receptor has been identified in cultured astrocytes by RtPCR, immunoblotting and immunostaining, and although there is a lack of functional evidence for its actions in vivo, Duan and colleagues in, 2003, have provided evidence for the first time that these channels were able to mediate the release of glutamate and aspartate from astrocytes in vitro after ATP P2X stimulation. Additionally, evidence from hippocampal slices experiments showed an induction of tonic currents in pyramidal neurons caused by NMDA receptor stimulation, the researchers attributed the source of the stimulation to a possible astrocytic glutamate release coming from P2X7 channels as the blockage of this channel using specific antagonists (oATP and Brilliant Blue G) drastically reduced the tonic currents and its overactivation by means of reduced extracellular Ca^{2+}

concentration had the opposite effect, increasing the hippocampal tonic currents, extensively reviewed in (Verkhratsky et al., 2013).
Further in vitro evidence, also adds GABA to the pipeline of gliotransmitter released by P2X7 channels as Wang et al 2002, showed that the application of ATP was able to induce the release of radio-labelled GABA from the RBA-2 astrocyte cell line, action that could be blocked by oATP confirming the release trough P2X7. (Wang et al., 2002)
ATP was also showed to be released by P2X7 receptors, and its currently understood that ATP is responsible for mediating the propagation of intercellular Ca^{2+} waves in astrocytes. (Verkhratsky et al.,2013).
Diffusion of ATP trough anion channels forms an additional option for the release of such transmitter from astrocytes, for instance, astrocytic swelling can open more than one chloride channel. Furthermore ATP-binding cassette (ABC) protein superfamily may act as transporters for ATP, according to Queiroz et al 1997, the ATP molecule would have an additional and also unconventional role in promote the opening of the anion channel instead of only serving for energy donation, nevertheless there is conflicting evidence regarding these findings and the hypothesis remains under intense investigation.
Regarding other nucleotide species such as adenosine and guanosine, the mechanisms of astrocytic release are not as well described and understood. Despite the existence of data showing the release of adenosine trough Ca^{2+} dependent mechanisms from neurons, the most commonly designated mechanism for adenosine release is thought to be trough the equilibrative nucleoside transporters (ENT 1 and 2) that move adenosine trough the plasma membrane according to its concentration gradient. Other mechanisms of release have been identified being the concentrative nucleoside transporter (Na^{+} dependent symporter) able to produce reverse adenosine movements towards the extracellular environment albeit having relatively less importance in mediating adenosine release (Lazarowski et al., 2000; Lazarowski et al., 2003).
In relation to cAMP release from astrocytes, the implied release mechanism has been the passage through the ABC transporter family MRP having implications for neuronal tonic inhibition given that cAMP is degraded by ectophosphodiesterase and ectronucleotidase into adenosine being able to activate A1 receptors (Lantini and Pedata, 2001).
Astrocytes have been showed to be the major source of guanine-based nucleosides, those being present in even higher concentrations when compared to those of adenine-based nucleosides, ischemic and hypoxic events were able to produce sustained increases in adenosine and guanosine levels, and again being guanosine present at much higher levels. (Gu et al., 1996) Albeit the mechanisms of guanosine release were not investigated the authors speculate that guanosine could have been derived from extracellular GTP hydrolysis or released from the cytosol via concentrative N2/cit bidirectional carriers. Hence, these data points to a possible trophic action mediated by the increased concentration of guanosine over time, during and after an ischemic event. (Cicarelli, 1999; Gu et al 1996).

Additional guanosine-based nucleoside release mechanisms are under investigation and in astrocytes the release trough ABC transporter MRP have been pharmacologically tested after stimulation with C-type natriuretic peptides, similar results were achieved after interleukin-b1 stimulation, culminating in the release of cGMP. Despite not been tested mechanistically, the current data allow us to theorize that cGMP rises could be neuroprotective for being a weak competitive inhibitor of kainate receptor with possible physiological implication in cases of glutamate excitotoxicity. (Montoliu et al., 1999).

The release of the nucleotide NAD^+ from astrocytes were shown by Verdeiro et al in 2001, where they showed a steady NAD^+ release after a washing protocol where the culture media was constantly removed. This release was shown to be made viable trough hemichannels which is an interesting fact given that, recently, NAD^+ was shown to have actions on neurons directly by activation of P2Y1 receptors.

1.5.5. Transmembrane transporters

One of the functional hallmarks of astrocytic function is the maintenance of the extracellular environment by means of neurotransmitter reabsorption at the vicinity of synapses with the end goal of terminating the stimulation of the post-synaptic neuron preventing excitotoxicity (Verhratsky and Nedergaard, 2013).

This neurotransmitter homeostasis control is obtained in astrocytes using Na^+ dependent plasma membrane amino acid transporters that drives neurotransmitters into the cell with the aid of Na^+/K^+ gradients. Two predominant types are expressed in astrocytes GLAST-1 (glutamate and aspartate transporter) and GLT-1 (glial glutamate transporter) in rodents, and, the also called excitatory amino acid transporters in humans (EAAT1 and EAAT2) respectively. (Gadea and Lopes Colome, 2001)

In physiological states, the concentration gradients drives excitatory amino acids into the cytosol resulting in the entrance of Na^+ or H^+ together with glutamate or aspartate in exchange of the exit of K^+ into the extracellular milleu, however, throughout a pathological event such as in hypoxia or perturbed ionic states (e.g; in cases of high K^+ extracellular concentrations) the transporter may operate in reverse releasing glutamate or aspartate. (Danbolt, 2001)

In fact, multiple studies have demonstrated by the use of transporter inhibitors that the reverse transport of glutamate and aspartate occurs during periods of ischemia or metabolic impediment. (Verhratsky and Nedergaard, 2013).

Another crucial gliotransmitter, D-serine, has been shown to be internalized in astrocytes by the Na^+ dependent alanine-serine-cysteine transporter (ASCT2), and its transport was found to be coupled to a counter-transport of another neutral amino acid.

D-serine reverse transport although could be induced even in levels of physiological extracellular L-serine concentration showing this to be a possible pathway for D-serine release in astrocytes. (Dun et al., 2007)

Regarding to GABA, and in contrast to the clearance of excitatory glutamate, only approximately 20% of its content is reabsorbed by astrocytes due to the relatively low density of specific transporters for it in the astrocytic plasma membrane. (Schousboe., 2003)
The majority of the GABA that is taken up by astrocytes is metabolized trough the TCA cycle or trough GABA transaminase. This transmitter is taken together with two molecules of Na^+ and one of Cl^-, and studies have shown that the reversal in this transporter operation also occurs, for instance, stimulation with kainate, quisqualate and AMPA all have led to GABA release to the extracellular environment of astrocytes in multiple studies. Astroglia presents GAT1 as the main transporter for GABA and its reverse operation was shown to occur after exposure to glycine in a dose-dependent manner and also independently of Ca^{2+} fluctuation confirming the reverse transport of GABA trough GAT1. (Rishcerson and Wu, 2003)
Another important mechanism of glutamate release from astrocytes is the transportation trough the cysteine-glutamate antiporter, this system is important for the maintenance of basal levels of cysteine for glutathione synthesis and it could serve as a way of releasing glutamate to the extracellular environment. This hypothesis was initially tested by Cavalier and Atwell 2005, in cerebellar slices and it was put forward in vivo by Moran et al, 2005, when the physiological importance of this system was demonstrated after increase in astrocytic cell death by blockage of the transporter, being these results attributed to the reduced synthesis of glutathione.

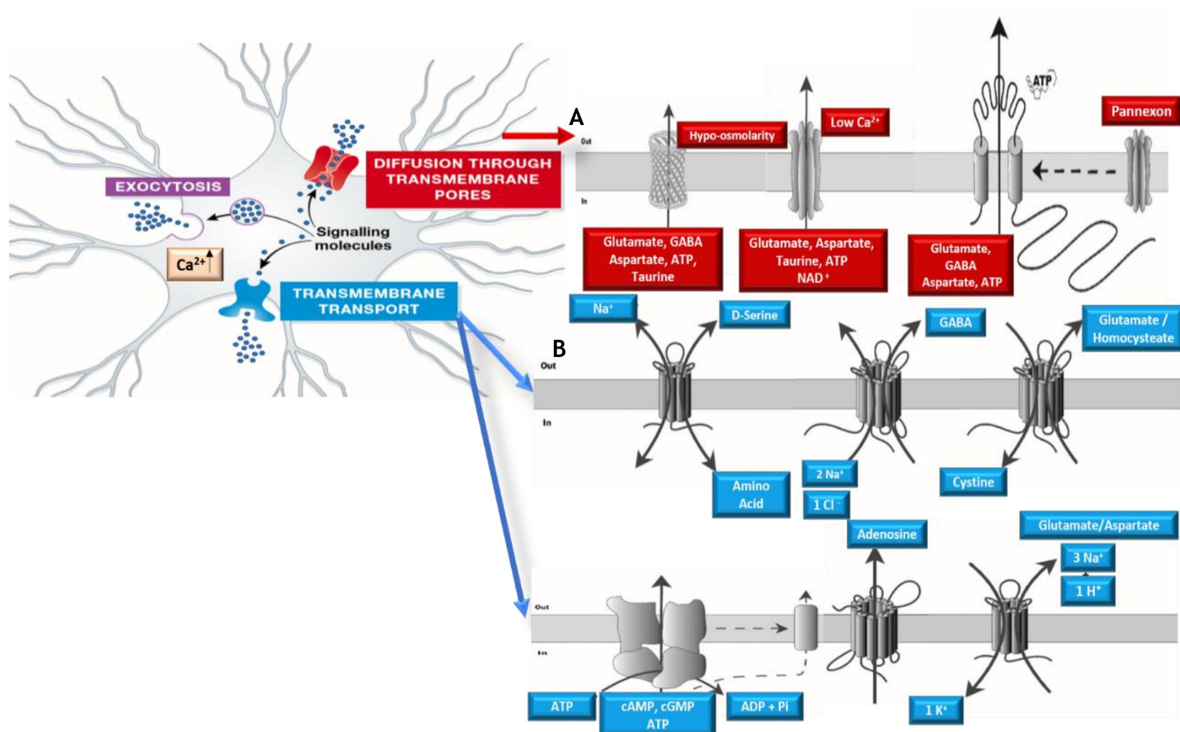


Figure 5. Astrocytes possesses different secretory mechanisms. In this image transmembrane diffusion and transport are shown. A) Transmembrane pores (Red), B) transmembrane transporters (Blue) Adapted from: (Verkhratsky et al., 2016 and Malarkey and Parpura, 2018)

1.5.6. Exocytosis

The ability to secrete a diverse type of membrane bound organelles containing a complex cargo have emerged early in evolution, being this process, a common trait in the majority of eukaryotes (Spang et al., 2015). Exocytosis have been shown to play a crucial role in intercellular communication maintaining normal cell physiology trough the release of important chemical and biological mediators, additionally secondary roles have been proposed such as the important role of targeting receptors and transporters to the plasmatic membrane having important implications in membrane plasticity.

The concept that astrocytes can act as secretory cells is sustained by data that confirms its ability to release vesicles containing neurotransmitters, peptides and growth factors, and although similarly complex to those of neurons, astrocytic exocytosis differs especially considering the dynamics of its kinetics being demonstrably slower, and also in its spatial arrangements and molecular mechanisms (Verkhratsky et al., 2016; Harada et al., 2016).

The underlying molecular mechanism of exocytosis in astrocytes shows to be tied to intracellular Ca^{2+} fluctuations, and in fact, the first experiments conducted by Parpura et al 1994, using HPLC to monitor glutamate release from astrocytes in vitro, showed that an increase in Ca^{2+} is not only sufficient but also necessary to generate glutamate release from astrocytes, such idea was also confirmed by depleting intracellular calcium with thapsigargin (blocker of store specific Ca^{2+} ATPase) or by buffering cytoplasmic calcium with BAPTA both generating considerable reductions in glutamate release (Bezzi et al., 1998; Araque et al., 1999).

Although compelling, the aforementioned studies did not aim to clarify the mode of transmitter release, therefore opening the question if this increases in intracellular calcium were causing the opening of ion channels favoring glutamate release or actually trough the mediation of vesicle exocytosis.

To identify exocytosis as the release mechanism, tetanus and botulinum toxins that are capable of cleaving the VAMP2/3 and SNAP 23/25 proteins selectively, have to be used to block exocytosis, the results shows a blockage of the synaptic effects of gliotransmission, confirming the release of glutamate by exocytosis to be at least a relevant mode of transmitter release. (Jourdain et al., 2007)

Such studies aiming to dissect the molecular machinery of astrocytic exocytosis were also conducted with novel gliotransmitters, for instance D-serine, as since not so long ago we believed the mammalian body to be able to produce only L-isomer amino acids.

D-serine is produced by the enzyme serine racemase using L-serine as a substrate and pyridoxal-5-phosphate as a cofactor, being allosterically activated by the complex ATP/Mg^{2+} (Mothet et al., 2005).

In cultured astrocytes, application of glutamate elevated Ca^{2+} mediated release of D-serine via the activation of AMPA, kainate and glutamate metabotropic receptors. Accordingly, pharmacological agonists of AMPA and Kainate were found to increase intracellular calcium and

subsequent D-serine release, which was reduced by the inhibition of such receptors. Moreover, the cleavage of VAMP2/3 by tetanic neurotoxins and the inhibition of V-ATPase in V-GLUT2 containing vesicles that are colocalized with D-serine supresses agonist-evoked secretion of D-serine, suggesting that D-serine is stored in synaptic-like vesicles and is released from the vesicles in a Ca^{2+} dependent manner. (Martineau et al., 2013)

In conjunction with the previous data, new forms of exocytosis started to be investigated, and the possible release of ATP from astrocytes posed as a new mechanism of exocytosis that did not involve synaptic-like vesicles, but it did involve lysosomes, in fact, astrocytes express a secretory lysosome marker (VAMP7 or TI-VAMP), and vesicles with this marker have been shown to contain ATP and being Ca^{2+} dependently released. Furthermore to test if ATP molecules are stored inside lysosomes, Verderio et al, 2012, labelled lysosomes containing V-NUT (Vesicular nucleotide transporter) with fluorescent ATP, and showed that the inhibition of V-NUT reduced the amount of fluorescently labelled lysosomes, additionally, data from total internal reflection fluorescence microscopy revealed exocytotic events of secretory lysosomes in astrocytes after the application of Ca^{2+} ionophore, ATP or glutamate, conclusively showing that ATP is stored in lysosomes and are released by exocytosis following intracellular Ca^{2+} surges. (Oya et al., 2013).

Albeit the release of the aforementioned transmitters has proven importance in the regulation of synaptic physiology and global neuronal-astrocyte crosstalk, they are not the only transmitters secreted trough astrocytic exocytosis. In fact, a rather complex composition of the different types of vesicles have been described, each of them, having different mechanisms of biogenesis, numerous types of membrane markers and a rich molecular cargo (proteins, peptides, amino acids, metabolites and genetic material). In the future paragraphs, an attempt to exemplify all different types of astrocytic exocytosis will be made, giving special attention to its molecular constituents and secretion machinery.

1.5.7 Molecular machinery of exocytosis

The main effector and evolutionary conserved protein family SNARE is the responsible for most of the processes concerning exocytosis. They can be divided into R and Q-SNARES and the former is associated with the vesicular membrane (also referred to as VAMP), while the later are either integral plasmatic membrane proteins (syntaxin) or just associated with the plasma membrane (SNAP-25/23) (Bohmbach et al., 2017).

Upon increases in cytosolic $Ca2^{+}$ concentrations R and Q-SNARE proteins form a complex by joining one of the Vamp2 and syntaxin SNARE domains each, with two from SNAP 23 to form a quadruple α-helical bundle denominated (SNARE pin) as demonstrated in figure 6, this bundle mediates the docking and fusion of the vesicles with the plasmatic membrane (Harada et al., 2016).

The determinants of exocytosis kinetics are highly heterogenous among different cell types and depends on many different cellular aspects. First, sensitivity of the secretory apparatus to cytosolic Ca^{2+} fluctuations plays a very important role. Second, the progression of the Ca^{2+} fluctuations differ among cell types (Sahlender et al., 2014).

As an example, synaptic exocytosis has an exceedingly fast progression (being often ≤1 ms) as the molecular machinery is in close proximity to the Ca^{2+} source, differently, in astrocytes, exocytosis of synaptic-like vesicles has much slower kinetics which can be attributable to a different expression patterns of SNARE proteins (Verkhratsky et al., 2016).

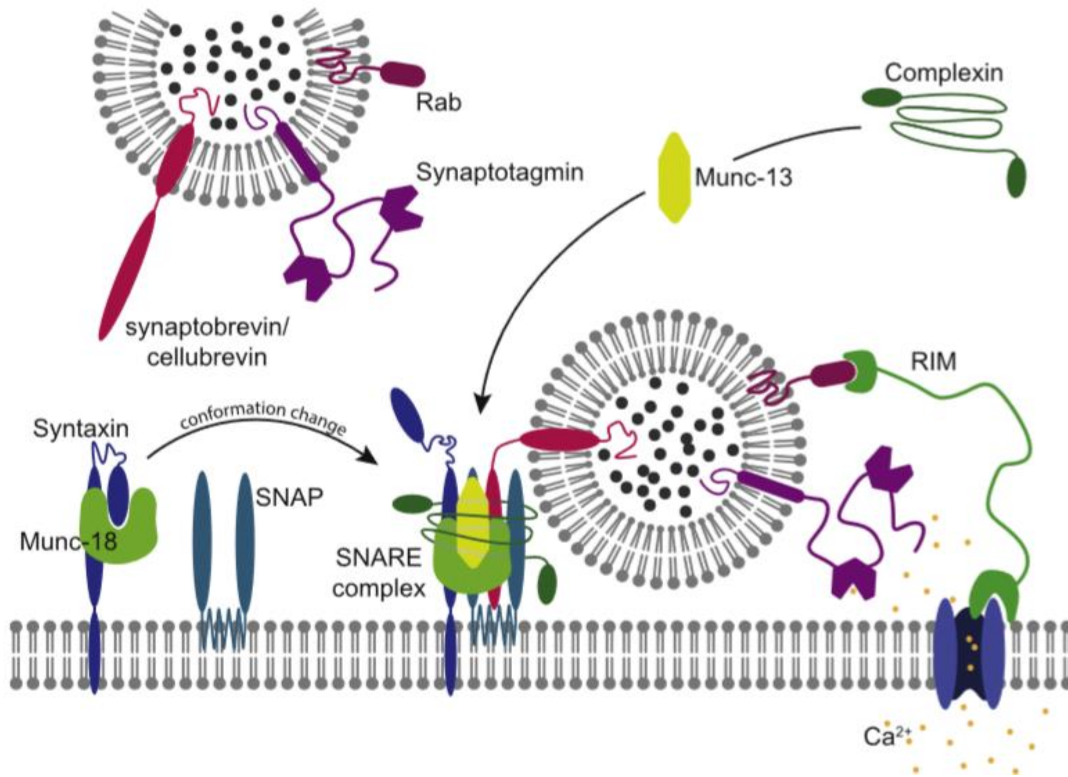


Figure 6. SNARE mediated exocytosis mechanism in astrocytes. Molecular response to intracellular calcium increases. Formation of the SNARE complex and mediation of the synaptobrevin-2/VAMP-2 vesicle docking. Abbreviations: Rab, Ras associated binding protein; Munc 13/18, Mammalian uncoordinated proteins 13 and 18; SNAP, Synaptosomal nerve associated protein; RIM, Regulating synaptic membrane exocytosis protein. Adapted from: Bohmbach et al., 2017.

1.5.8. The diversity of secretory organelles in astrocytes

Different types of secretory organelles are present in astroglia and they are currently divided as extra or intracellular. Intracellular organelles are described as the transport vesicles, lysosomes and other types of secretory vesicles such as (synaptic-like vesicles and dense core vesicles) and extracellular vesicles are represented by exosomes, micro-vesicles, microparticles and apoptotic bodies. These vesicles are characterized as having different morphological aspects such as their size and expression of membrane markers but also differs considerably in their biogenesis, cargoes and release mechanisms (Cocucci et al., 2015).

Typically, the different types of vesicles derive either from the trans-Golgi network or from early or recycling endosomes, although other routes have been identified such as in the case of lysosomes and multivesicular bodies. A depiction of the different secretory organelles is made in figure 6.

1.5.8.1. Synaptic-like microvesicles carry aminoacids

Astrocytic SLMV's range in sizes of 30 to 100nm and appear in pair or groups of 2-15 vesicles, number that is expressively smaller than the neuronal synaptic vesicles that exist in groups of hundreds or thousands. Bigger SLMV's (1-3 µm diameter) have also been identified in hippocampal slices and is believed they could be formed by the merging of smaller vesicles after sustained Ca^{2+} increases or mechanical stimulation, contributing to the pool of extracellular D-serine (Martineau et al., 2013).

An important physiological aspect of secretory vesicles is their ability to concentrate transmitters internally. This is accomplished by the existence of VNT's (vesicular neurotransmitter transporters), six types of VNT's have been identified so far in neurons, including transporters for ATP (VNUT), glutamate (VGLUT1-3), acetylcholine (vAChT), monoamines (VMAT1-2), GABA/glycine (VIAAT, also known as VGAT) and aspartate (sialin/VEAT). Fairly recently, and although its molecular identity has not yet been fully disclosed, data points to the enrichment of SLMV's with D-serine via (VSerT).

Each of the transporter differs from each other in terms of energy coupling, substrate specificity and affinity, forming the fingerprints underlying regulated exocytosis (Blakely et al., 2012).

The expression of VNT's in astrocytes is not as well characterized as it is in neurons, and in fact, conflicting results have emerged considering the expression of VGLUT in cultured astrocytes, for instance, analysis in situ using gene chip microarray, single cell RT-PCR and immunofluorescence produced variable results, in some cases, even objecting the concept of astroglial exocytosis (Li et al., 2013).

Nevertheless, results from immunogold electron microscopy, confocal microscopy and single-cell RT–PCR experiments have demonstrated that sub-populations of astrocytes in the brain not only express VGLUT 1 (Bezzi et al., 2004, Bergersen et al., 2012, Ormel et al., 2012), VGLUT 2 (Bezzi et al., 2004) and VGLUT 3 (Bezzi et al., 2004, Ormel et al., 2012), but also secrete glutamate upon Ca^{2+} rises.

Although the results from in vitro and in situ contrast themselves regarding the co-localization of glutamate and D-serine in the same vesicles, fact is, that astrocytes present a bulk of this vesicles located at peri-synaptic processes as well as in somata, its release is controlled by Ca^{2+} rises and is blocked by astrocytic R-SNARE (VAMP-2 and 3) tetanus toxin mediated cleavage.

1.5.8.2. Dense-core vesicles carry peptides

DCV's are the main compartment of storage and release of neuropeptides and neurohormones for neuroendocrine cells and neurons, such vesicles are also laid with ATP, which likely accumulates via VNUT's, albeit this transporter expression has not yet been reported. (Potokar et al., 2008). Although physiologically important, the overall amount of VAMP-2 labelled DCV respectively to the total number of vesicles is small, representing only 2% of the total vesicle content. In what regards to the ultrastructure of the DCV's in astrocytes they seem similar to those of neuroendocrine and neuronal cells, except for its core that seems much less dense,

similarly to those of neuroendocrine cells, furthermore, morphologically, they are on average considerably bigger than SMLV's (100-600nm) although some ANP (atrial natriuretic peptide) DCV's can be as small as (50nm) in diameter. In cultured astrocytes, DCV's contains the secretory proteins secretogranins 2 and 3 and chromogranins all of them with a clear physiological role not yet established. Additionally, secretogranins have been found to be expressed in DCV's of human brain samples, confirming it's in situ existence. ANP and neuropeptide Y were also identified in vitro astrocytic DCV's, for instance, NPY, a peptide that is widely distributed throughout the mammalian brain, where it acts as a neuroproliferative factor and modulates the growth of vascular tissue, it is released after metabotropic glutamate receptor (mGluR) activation trough exocytosis of DCV's (Barnea et al., 1998, Barnea et al., 2001, Hansel et al., 2001, Geloso et al., 2015).

Accordingly, BDNF (brain derived neurotrophic factor) in the form of its precursor (pro-BDNF) was found to be taken up by astrocytes after strong neuronal stimulation by the p75-neurotrophin receptor, which mediated clathrin attached endocytosis. The internalization of pro-BDNF in single vesicles were confirmed by TIRF (Total internal reflection fluorescence) and confocal microscopy, and its recycling mechanisms were confirmed to happen as astrocytic stimulation with glutamate leads to the secretion of BDNF. It is also important to mention that the release of neurotrophin was blocked by tetanus neurotoxin suggesting an involvement of VAMP-2 mediated exocytosis as the main pathway for BDNF secretion in astrocytes (Lu et al., 2005, Juric et al., 2008, Bergami et al., 2008).

1.5.8.3. Lysosomes and its secretory potential in astrocytes

Lysosomes, an organelle that was first described in 1955 by De Cuve and collaborators, is a prominent intermediate of endo- and exocytotic pathways, operating virtually in all eukaryotic cells including astroglia.

Exocytosis of lysosomes has been shown to be another viable mechanism of transmitter release in astrocytes and in fact secretory lysosomes are the main contributor for the Ca^{2+} dependent secretion of ATP in cultured astrocytes. Presenting diameters in the range of 300 to 500nm they coexist with SLMV's and express specific lysosomal markers such as LAMP 1, Cathepsin D, Rab7 and VAMP7 (Verderio et al, 2012).

The secretion of lysosomes from astrocytes has been shown to rely on the expression of the tetanun resistant VAMP7 as its downregulation inhibits the fusion of ATP-labelled vesicles (Oya et al., 2013).

The kinetics of its release is slow and is triggered by locally restricted Ca^{2+} elevations, differently from the fusion of SLMV's that happens following fast Ca^{2+} spikes. Astrocyte stimulation with agonists or metabolic blockade have been shown to induce regulated exocytosis of such vesicles as confirmed by TIRF microscopy, which was shown to be blocked by the use of Ca^{2+} chelator BAPTA (Andrews and Chakrabarti, 2005).

1.5.8.4. Extracellular vesicles (exosomes, micro-vesicles and ectosomes)

In fact, astrocytes have been shown to release both types of EV's, as seen in figure 6. For instance, ectosome release from astrocytes were shown to happen after the activation of P2X7 purinoceptors by the action of extracellular nucleotides and nucleosides that rapidly activates acid sphingomyelinase causing it to move towards the outer membrane, changing membrane fluidity, what leads to vesicle blebbing and shedding. The mechanism was shown to be at least partially controlled by p38 MAPK cascade activation. As shown by Bianco et al 2009 and Proia et al 2008, in 2-day-old rat cultured cortical astrocytes the diameter of the ectossomes shed varied between 100nm and a 1,000nm.

Ectossomes from astrocytes carry neuroregulatory molecules that can regulate the fate of cells in its microenvironment, for instance, FGF-2, IL-1ß, VEGF, triphosphate diphosphohydrolases and matrix metalloproteinases (Bianco et al., 2009; Proia et al., 2008; Ceruti et al., 2011). Interestingly, astrocytes presented increased release in triphosphate diphosphohydrolases (enzyme that degrades extracellular nucleotides), upon ischemia induction, showing a protective mechanism against increasing levels of ATP released from the damaged BBB cells. (Ceruti et al., 2011).

Regarding astrocytic exosomes, their cargo enrichment has been shown to be at least as complex as any other cell and in fact, recent results postulate the possibility of using them as biomarkers of CNS degenerative disorders such as in Alzheimer disease (AD) and multiple sclerosis (MS) by employing novel methods for recognition of internal proteins, especially in the case of AB42 and GFAP respectively (Goetz et al., 2016).

1.5.8.4.1 The impact of specific protein cargo contained in astrocyte derived EV's.

Although the in vivo evidence for EV´s release from astrocytes is scarce, the evidence for constitutive release of exosomes in vitro is pilling. For instance, different stimuli such as (ischemia, inflammation, oxidative and heat stress) have all been shown to affect EV´s release both quantitatively and qualitatively.

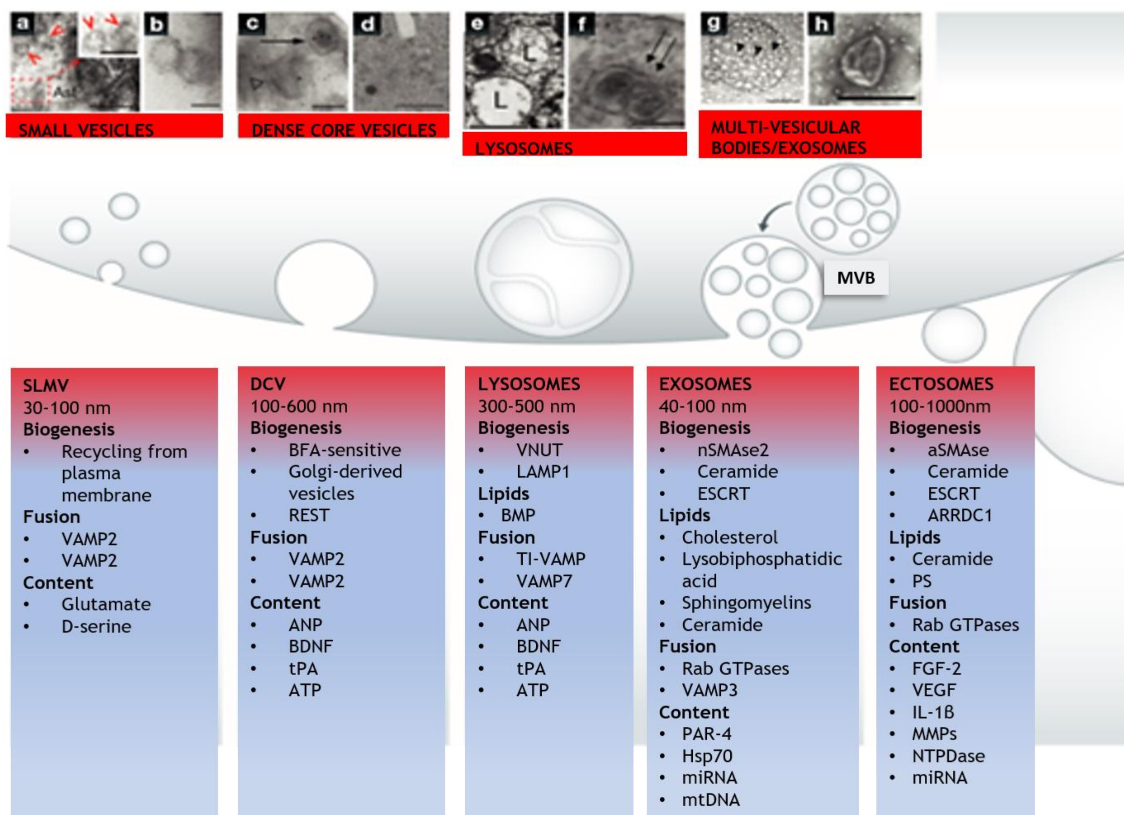


Figure 7. An overview of the different exocytotic organelles in a schematic representation. (a-h) shows ultrastructural electron microscopy images and intracellular localization within the astrocyte. Abbreviations: aSMase, acid sphingomyelinase; BMP, bis(monoacyl glycerol) phosphate; FGF2, Fibroblast growth factor 2; HSP70, 70kDa heat shock protein; miRNA, microRNA; mtRNA mitochondrial RNA; MMPs, matrix metalloproteinases; nSMase2, neutral sphingomyelinase 2; NTPDase, nucleoside triphosphate diphophohydrolases; PS, Phosphatidylserine; REST, RE-1-silencing transcription factor; tPA, tissue plasminogen activator; VEGF, vascular endothelial growth factor.
Adapted from:(Verkhratsky et al., 2016)

The first report for exosomal release from astrocytes came in 2007 from Milligan`s group, where they showed the presence of the classical protein markers Hsp70/Hspc70 in a fraction obtained from a differential-ultracentrifugation protocol. Hspc70 concentrations were increased in the exosomes from conditioned media in response to thermal stress, suggesting a possible role for exosome mediated regulation of stress response by astrocytes. Three years later, results from the same lab showed the release of synapsin-1 in exosomes from astrocytes, and although the authors didn't evidence any functional role of these exosomes on neuronal populations, it has been suggested that synapsin-1 could have a positive effect on neuronal survival and neurite outgrowth. (Taylor et al., 2007, Guescini et al., 2010). Two years later, Wang et al., 2012 demonstrated that the exposure of astrocytes to B-amyloid in-vitro or in-vivo induced exosome mediated release of ceramide and PAR-4 causing increased apoptosis of neighboring astrocytes showcasing the existence of an autocrine mechanism to be further explored.

Additional evidence of different physiological roles for exosome release from astrocytes are under current investigation and early data from such experiments showed they are also able to

secrete EAAT-1 and 2 (Excitatory amino-acid transporters) through small size EV´s, and that these vesicles were able to accumulate aspartate inside them, having a possible regulatory role in the homeostasis of extracellular glutamate apart from the clear role in inter-cellular communication. (Gosselin et al., 2013) Although interesting, the aforementioned studies did not test the possibility of astrocyte-derived exosomal transfer of such proteins over to neurons. Nevetheless, (Dickens et al., 2017) showed that upon an IL-1ß induced brain lesion, exosomes derived from astrocytes were able to promote peripheral leukocytosis.

1.5.8.4.2 The impact of specific miRNA cargo contained in astrocyte derived EV's.

Extracellular vesicles contain a multitude of genetic material making up its internal cargo, together with lipids and proteins. Most of this genetic material, is composed of mRNA and a variety of small non-coding RNA's. Among such RNA species, microRNA's receives the most attention given the fact it can reprogram protein expression in recipient cells (Théry et al., 2018).

MicroRNAs can inhibit transmission by two distinctive mechanisms, being the first one, the targeting of the messenger RNA to direct degradation, and the second one, by interfering with the ribosomal recognition sites to the mRNAs therefore, reducing translation and consequently protein expression (Lafourcade et al., 2016).

The loading of miRNA into astrocytic exosomes have been confirmed in several studies, this process favors the enrichment of some miRNA's over others and it has been shown to be dependent on the KRAS-MEK-ERK pathway. The sorting mechanism involves three specific factors: First, the presence of special miRNA sequences such as uridylated miRNA's and EXOmotifs (special sequences of nucleotides) that function as sorting motifs to incorporate RNA into exosomes. Second, the binding of miRNA's to lipid raft regions in the cytoplasmatic face of the membrane of multi-vesicular bodies, and third, interactions with sumoylated nuclear proteins or AGO2. Although the recognition of such factors has led to tremendous advancement in our understanding of exosomal miRNA loading, the specifics of each microRNA sorting into astrocytes are still lacking, warranting the encouragement of further explorations. (Zhang et al., 2015; Janas et al., 2015; Cha et al., 2015).

To date, the most comprehensive study aiming to dissect astrocyte exosomal composition regarding miRNA's was conducted by (Jovicic and Gitler, 2017) where they were able to quantify the expression of 752 mature rodent miRNAs from astrocyte primary cultures. Interestingly and in accordance with other studies, the miRNA profiles of astrocytic exosomes were different from the profiles of whole astrocytes, supporting the evidence for distinct regulatory mechanisms of miRNA sorting into exosomes. Accordingly, the authors were also able to find a marked enrichment in 5 RNAs (miRNA's 1274a, 1937b, 1937c, 2134 and 2182) that were later confirmed to be tRNA.

Additionally, upon the induction of a model of ALS, (amyotrophic lateral sclerosis) where there is active participation of astrocytes in its etiopathology, the authors failed to detect important changes in miRNA content coming from such astrocytic exosomes therefore, showing that the

toxic effect seen when motor neurons are put in contact with the conditioned media of such astrocytes cannot be attributed to changes in astrocytic exosomal miRNA profile (Jovicic and Gitler, 2017). Furthermore, astrocytes appear to upregulate their exosome secretion upon being cultured together with miR-133b containing exosomes from MSC's and induce a secondary wave of exosome release that correlated with improved functional recovery after stroke in rats. (Xin et al., 2017) All these different mechanisms serve to highlight the elevated complexity regarding astrocyte signaling, and that possible negative interference in any of these events could culminate in the generation of an ethiopathological environment to the CNS. Additionally, the modulation of specific targets or pathways relating to gliotransmission could also represent future approaches regarding therapeutic applications in either disease prevention, management or treatment.

1.6. Astrocytes and pathophysiology of CNS traumatic injury and neurodegeneration - loss of normal function or gain of toxic function

1.6.1. Astrocyte responses to acute focal insults

Reactive astrogliosis is a newly coined term used to define the ability astrocytes have to respond to an insult, being the magnitude of the response dependent on the nature as well as the duration of the insult.

Acute and focal injuries tend to trigger wound repair with tissue replacement where diffuse and chronic diseases provoke gradually escalating tissue changes that ultimately culminate in loss of function (Burda and Sofroniew, 2014). The CNS responses to such insults involve complex cellular interactions among neuronal and non-neuronal CNS cells and also the recruitment of CNS extrinsic cells that gain access to the disease site via the circulation (Dossi et al., 2018).

The research around astrocyte reactivity is progressively gaining momentum, and different phenotypes have now been associated to different disease states or brain areas, for instance, in a focal CNS damage, the response to such insult evolves through three distinctive but also overlapping phases. Broadly, in the first phase there is evident cell death and the instalment of a highly inflammatory environment, in the second, visible cell proliferation occurs and in the third, tissue remodeling takes place (Scuderi et al., 2013).

Astrocytes functionally participate in the initiation and progression of this processes, where in the first moments after the injury (seconds to hours) they remain in situ without migrating towards or away from the lesion site, instead, they osmotically swell and depending on how severe the insult is, they can die, locally or in some cases proliferate (Bardehle et al., 2013; Phatnani et al., 2013).

In a second moment (after the first days), the proliferation of scar-forming astrocyte progenitors takes place leading to the formation of a compact astrocyte scar. Cellular and molecular mechanisms that govern the formation and positioning of the astrocytic scar border remain incompletely understood but are likely to involve a complex interplay of molecular signaling fostering phagocytosis and debris clearance together with signals of cell protection and self-preservation. (Wanner et al., 2013).

Lastly, the third phase of the response to an acute injury begins along the end of the first week and is comprised of tissue remodeling events such as the repair of the BBB and reorganization of the astroglial scar. The role of astrocytes in this phase, remains not totally understood, but it is of known importance, for example, the establishment of a compact interdigitating scar that forms a structural and functional border separating the lesion core containing inflammatory and fibroblast lineage cells from the functional tissue around containing all three neuronal lineage cells presents an intriguing facet of the regenerative process. (Wanner et al., 2013) Data from transgenic loss-of-function studies highlight that the disruption of astrocyte

scar formation, leads to increased lesion size, neuronal death and demyelination, as well as decreases in functional recovery. Furthermore, there is still a huge debate in the field regarding the impact astrocytes have on axonal growth, as in vitro data show them to promote axonal regrowth inhibition based on the synthesis of certain proteoglycans, although in vivo studies present compelling evidence showing axonal regrowth along astrocyte bridges (Herman et al 2008; Wanner et al., 2013).

A better understanding of such molecular mechanisms will surely open up new avenues regarding the possibility of astrocytic scar modulation, targeting an overall reduction in lesion size, for instance, data from experimental studies on ischemic infarcts show that final lesion size can be influenced by subacute metabolic manipulation leading to a possible novel therapeutic strategy (Lo, 2008).

In a different physiopathological context, classically induced inflammatory insults such as in LPS administration where the insult is governed by an intense microglial activation followed by inflammatory cytokine release (IFN-gama, TNF-alfa, c1q and IL-1B) induces a profile of astrocyte activation that is followed by upregulation of a multitude of inflammatory response genes as well as considerable changes in cellular morphology and function, affecting neuronal survival, neurite outgrowth and synaptogenesis negatively, as well as presenting considerable reductions in their phagocytic capacity (Liddelow et al., 2017; Zamanian et al., 2012). In another pathological context, hypoxic ischemic lesions induce a different pattern of astrocytic activation termed A2 astrocytes, these astrocytes show an upregulation of several neurotrophic factor genes as well as being protective against neuronal cell death and capable of promoting axonal growth (Liddelow et al., 2017; Zamanian et al., 2012).

The main pathway thought to be involved in A2 phenotype polarization is the JAK-STAT3 pathway as many studies have implicated its importance in scar-forming astrocyte reactivity after acute brain injury. (Liddelow et al., 2017)

1.6.2. Astrocyte responses to chronic focal insults

Albeit acute focal injury models serve as the probable best way to assess the mechanisms behind astrocyte reactivity, reactive astrogliosis during chronic forms of damage are also observed along-side multicellular responses that resemble those seen in acute insults (Liddelow and Barres, 2017).

For instance, in immune compromised individuals, focal injuries can be infected spontaneously which therefore can evolve chronically and form an abscess. In such cases, reactive astrogliosis is present with formation of an astrocytic scar depending on how lengthy the presence of the insult is. Genetic disruption of astrocytic scar formation in transgenic loss of function studies reveals drastic spread of infection and inflammation throughout adjacent neural tissue presenting devastating effects, demonstrating the importance of astrocyte focal containment of the infection (Drogemuller et al., 2008). A similar relevance is given to astrocytic response in Chikungunya virus infections where they present an important mode of defense governing innate immune response. (Trina Das et al., 2015)

In the case of tumors, both primary and metastatic disease drives reactive astrogliosis in similar ways to what happens in other forms of focal lesion and interestingly non-invasive tumors are contained by encapsulating astrocyte scar in the same way seen in traumatic damage. Accordingly, highly aggressive and invasive tumors are not contained by astroglial scar but on the opposite, induces a different type of reactivity where the derived multicellular response supports the creation of a favorable microenvironment that is supportive of tumor growth, vascularization and metastasis (Silver et al., 2013; Watkins and Sontheimer 2012).

In the context of autoimmune conditions, although they are classically classified as chronically diffuse diseases, individual autoimmune lesions greatly resemble acute focal lesions, for instance, in multiple sclerosis plaques, astrocyte scar separate the lesion core that is devoid of viable neuronal tissue from the surrounding tissue containing all three neural lineages (neurons, oligodendrocytes or astrocytes) pretty much in the same way that occurs in traumatic or ischemic lesions preserving extra lesion area functional capacity (Frohman et al., 2006). Evidences from clinical and experimental lines both point out to a crucial role for scar forming astrocytes in mediating the limited spread of inflammation in CNS autoimmune conditions, but its involvement seems not to be only impactful in disease progression, but it may also be causally related with disease onset (Scuderi et al., 2013; Phatnati et al., 2015; Dossi et al., 2017) For example, loss of function in CNS intrinsic cells such as astrocytes have been related with onset of autoimmune inflammation in some patients that presents auto-antibodies against astrocyte potassium channels Kir4.1 opening up a new rationale for the assessment of the impact the possible gain or loss of function in astrocytes and its relations not only with disease progression but also in disease onset (Srivastava et al., 2012).

1.6.3. Astrocyte responses to chronic diffuse insults

Initially, diffuse insults to CNS tissue tend to be less intense when compared to acute focal lesions such as stroke, rarely causing tissue damage in the beginning of its time course. Conditionally, this damage accumulates overtime and as it progresses, the scattered tissue damage becomes more severe, evoking reactive astroglioses and multicellular responses that finally culminate in the full onset of the disease, presenting BBB breakdown, inflammation and recruitment of leukocytes and although in a less severe scale, they resemble a recollection of smaller acute focal lesions with their on peri-lesion compartment. It is worth mentioning that in many conditions this distributed lesioned area is intermingled with functionally active neurons and viable circuitry and this surely must have implications to synaptic functioning. (Burda and Sofroniew, 2014; Rouach et al., 2017).

The dissection of the existing contribution between astrogliosis and neurodegeneration is challenging and far from advanced. Although experimental and clinical roles for astrogliosis are described in both onset and progression of neurodegeneration, it is hard to pinpoint with certainty its relevance to the disease process as a whole.

Interesting findings relating to astroglial reactive response to neurodegeneration comes from experiments of B-amyloid clearance, the experiments revealed active astrocytic involvement

in the aggregate's clearance both in vitro and in vivo and also, the use of transgenic attenuation of astrogliosis showed an accumulation of B-amyloid in a mouse model of Alzheimer disease as showed in another study (Wyss-Coray et al., 2003).

Another condition where astrocytes respond reactively is Parkinson Disease and in fact the impact of astrocyte is relevant in both disease initiation and progression. Functionally, protoplasmic astrocytes were shown to accumulate alpha-synuclein aggregates early in the disease process and also that alpha-synuclein was transferred from neurons to astrocytes inducing a dose dependent inflammatory response (Song et al., 2009; Lee et al., 2010). Relative to the molecular triggers of reactive astrogliosis, there is the evident role of neurotoxic accumulation of B-amyloid and other protein aggregates, but also, actions for neuronal or synaptic damage have been described to act as triggers specially in ALS (amyotrophic latera sclerosis) or in HD (Huntington disease). Additionally, perturbations to the neurovascular unit leading to disruption of BBB integrity which can foster the recruitment of blood-borne inflammatory cells have also been shown to act as a trigger (Boilee et al., 2006; Maragakis and Rothstein, 2006). Furthermore, this complexity makes it hard to draw a line for where astrocytes become toxic and therefore causally involved in disease onset, or if specific disease aspects turn astrocytes into their reactive form. Understanding these differences will likely present opportunities for the development of future therapeutics aiming at astrogliosis modulation for either disease prevention or treatment.

Chapter 2 – Research objectives

Based on the demonstrated evidence for a complex and yet not fully demonstrated therapeutic mechanism governing the performance of MSC's secretome based therapies applied to CNS regeneration, and considering the importance astrocytes have on CNS physiology, the present work had the following objectives:

- To determine if an impairment in astrocytic VAMP-2-mediated exocytosis presented a modulatory effect on the secretome's performance in inducing proliferation at the level of the hippocampus.
- To assess the impact of MSCs secretome and astrocyte transmission in astrocyte morphological features.

With these purposes we employed a transgenic mouse model of impaired astrocytic signaling named dnSNARE, in which the exocytosis mediated by VAMP-2 molecular machinery is conditionally ablated selectively in astrocyte.

Chapter 3 – Materials and methods

3.1. Bone marrow mesenchymal stem cell culture

Bone marrow MSC's (BMSC's) were resuspended in Alpha-Mem medium (Invitrogen, USA) supplemented with 1 % of antibiotic/ antimycotic (Invitrogen, USA) and 10 % of fetal bovine serum (FBS) (Invitrogen, USA), and plated on culture at a density of 4.0×103 cells/cm2. Subsequently, the culture medium was renewed every 3 days and the culture maintained at 37 °C, 5 % CO2, 95 % air and 90 % relative humidity until confluence.

3.1.1. Secretome collection procedure

The secretome used was collected from BMSC's cultures in passage five (P5) previously plated and kept at a density of 4.0×10^3 cells/cm2 for 3 days in Alpha-Mem medium (Invitrogen, USA) supplemented with 1 % of antibiotic/antimycotic (Invitrogen, USA) and 10 % FBS (Invitrogen, USA). After this, the flasks were washed three times in Neurobasal A medium (Invitrogen, USA) for 5 min and then, washed five times in PBS without Mg^{2+}/Ca^{2+} (Invitrogen, USA). Following this Neurobasal A medium supplemented with kanamycin (1 %) (Invitrogen, USA) was added to the cells. After 24h the secretome was collected, centrifuged at 300*g* for 10 minutes to remove cell debris and then frozen at −80 °C until use.

3.2. Animal model

The mice used for the experiments were a transgenic model named dnSNARE. In this model, the cytosolic portion of synaptobrevin 2 (VAMP-2) protein is overexpressed (amino acid 1 to 96), such procedure causes the blockage of VAMP-2 dependent vesicle fusion, inducing impaired gliotransmission. Such mice are obtained by crossing two lines of transgenic animals. In the first one, GFAP.tTA, the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter drives the expression of the tet-Off tetracycline transactivator (tTA), and the second, which is tetO.SNARE, contains a tet operator (tetO)–regulated SNARE domain for the expression of VAMP-2.

The selectivity of the model is achieved by using the GFAP promoter as the driver for the expression of the "tet-Off" tetra-cycling transactivator. To prevent transgene expression during mice early development doxycicline (Dox) is administered through the animals drinking water supply. Dox usage allows for the conditional suppression of transgene expression, as Dox bind to tTA preventing tet-o promoter activation.

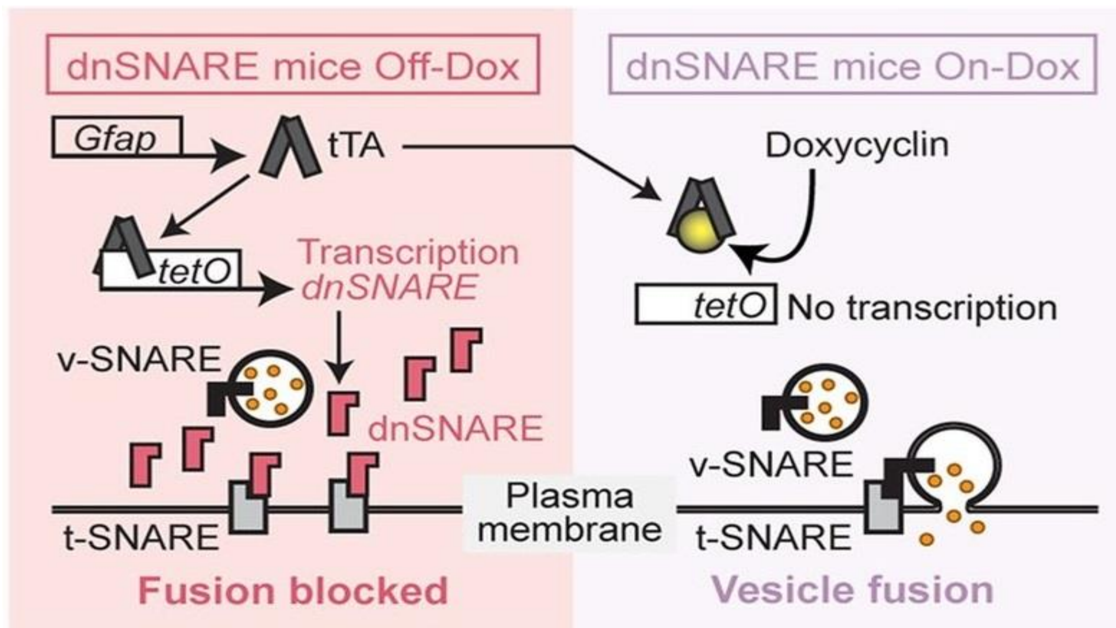


Figure 8. Graphic representation of dnSNARE model: Demonstration of transgene expression when Off-Dox in pink. Mechanism of transcription blockage of transgene expression when On-Dox. Abbreviations: GFAP, glial fibrillary, acidic protein; tTA, tetracycling trans-activator; tetO, tet off; v-SNARE, vesicular snare; t-SNARE, transmembrane SNARE; Dox, doxycycline. Adapted from: Fujita et al., 2014.

3.3. Surgical Procedures

All experiments were conducted using 8 weeks old male dnSNARE mice and its wildtype counterparts C57BL/6 mice (Charles River, Spain). Consent from the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457), was obtained before the experiments. Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council. The animals were housed and maintained in a controlled environment at 22–24 °C and 55 % humidity, on 12 h light/dark cycles and fed with regular rodent's chow and tap water ad libitum. Animals were handled for 1 week prior to the beginning of the injections, in order to reduce the stress induced by the surgical procedures. For the cerebral injections two experimental groups were used (n=17/group in the Secretome group) and n=11 in the Neurobasal group). Young adult mice were anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg). Using a stereotaxic system (Stoelting, USA) and a Hamilton syringe (0.5 µl Hamilton, Switzerland) all injections made in these two groups were bilateral according to previously determined coordinates (Munhoz et al 2006. PNAS) A/P, -2.3 mm, M/L, +-1.3 mm, and D/V, -2.0 mm. The volume injected per DG was 0.250 µl with a rate of injection of 0.1 µl per minute. Two minutes were allowed after each injection in order to avoid any backflow up the needle tract. Neurobasal group was only injected with 0.5 µl of Neurobasal A medium; Secretome group were injected with 0.5 µl of BMSC's secretome. At the end, the animals were sutured and then injected with 100 µl of anti-sedating (Orion Pharma, Finland) in order to recover from surgical procedure.

One week after the injections (week 9), the animals were killed with sodium pentobarbital (Eutasil, 60 mg/kg i.p.; Ceva Saúde Animal Portugal), and transcardially perfused with 4% paraformaldehyde (Merck, Portugal) in 0.1Mphosphate-buffered saline (PBS).

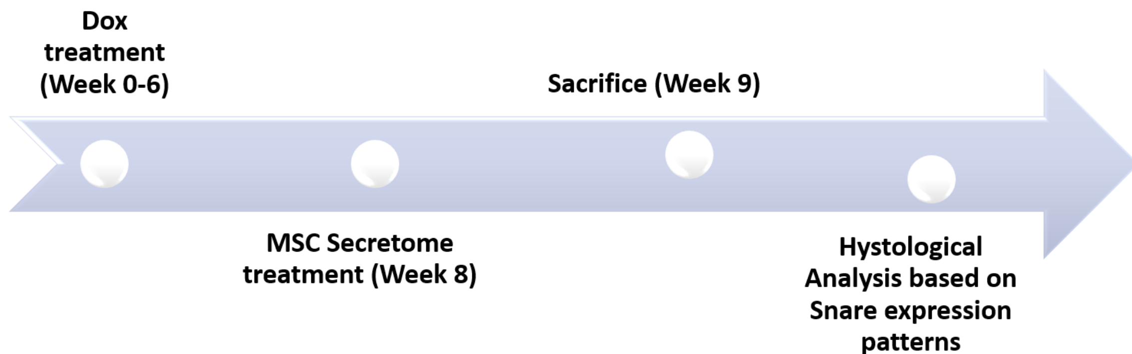


Figure 9. Experimental timeline. Abbreviations: Dox, doxyciclin.

3.4. Transgene expression analysis

3.4.1. RNA isolation protocol

Tissue from pre-frontal cortex isolated upon animal's sacrifice were submitted to total RNA extraction using phenol and guanidine isothyocyanate (TRIzol, Invitrogen). Briefly, tissue was mechanically homogenized with 1mL of TRIzol with the aid of a 10G syringe. To the lysate, isopropanol was added, and the samples were centrifuged for 10 minutes at 12,000xg at 4ºC. The resulting pellet containing RNA was resuspended in 75% ethanol and centrifuged this time for 5 minutes at 7,500xg at 4ºC.

The final RNA pellet was air dried for 5 minutes to allow for total solvent evaporation and was resuspended with RNase free water for quantification.

Quantification was carried out on a NanoDrop (NanoDrop 1000, ThermoFisher), a microvolume sample retention system. NanoDrop functions by combining fiber optic technology with natural surface tension properties to capture and retain minute amounts of sample. The reduction on volume of the sample requirement is essential in order to increase workflow efficiency, allowing for the introduction of quality control steps along the experimental process which in turn promotes greater confidence in the downstream results.

In our case, 2uL of RNA were pipetted onto the fluorospectrometer platform and the upper arm was brought into contact with the sample bridging the gap with the platform. NanoDrop then, calculated RNA concentrations and the purity ratios.

3.4.2. cDNA synthesis

cDNA synthesis was carried out using (Xpert cDNA Mastermix, Grisp), the mastermix is an optimized reaction mix containing a balanced concentration of olido(dT) and random hexamer primers, dNTPs and RNase inhibitors, guaranteeing the preparation of full-length cDNA with minimal degradation.

Briefly, an amount of total RNA totaling 500ng/µL for each sample was used as the template material for the reaction aiming to standardize the starting genetic material concentration in the downstream reactions.

To the varying amounts of RNA samples, 10µL of reaction mix and up to 19µL of RNase free water were added and the total mixtures were submitted to 5 minutes of heat treatment in a thermoblock (Accublock, Lab Net International), at 65ºC to promote removal of possible secondary RNA structures. For cDNA synthesis 1µL of enzyme (Reverse Transcriptase 200U/µL) was added to the samples followed by thermocycler (MyCycler, Biorad) heat up for 15 minutes at 50ºC. Heat inactivation of the enzyme was carried at end of the process by heating the samples at 85ºC for more 5 minutes at the thermocycler.

3.4.3. qRT-PCR for transgene expression

The target of our transgene expression analysis was to enable for the correct division of the different experimental groups according to their degree of synaptobrevin/VAMP2 gene expression. The animals were divided into 3 groups (wild type, SNARE high, SNARE low) and subdivided accordingly to the treatment that was given, being either secretome (SEC) or neurobasal (NBA).

Table 1. Outline with experimental groups

Groups	Interventions	N˚
Wild Type	NBA	3
Wild Type	Secretome	4
Snare High	NBA	5
Snare High	Secretome	5
Snare Low	NBA	3
Snare Low	Secretome	8

The qRT-PCR protocol used was based on the enzyme (Xpert Fast SYBR uni Blue, Grisp), where the mastermix presents all the necessary components for the realization of the procedure apart from primers and template materials. The intercalating dye used in the mix causes negligible inhibition which allows for extremely high sensitivity and specificity. Briefly, a master mix is prepared by adding 10µL of enzyme, 8µL of RNase free water and 0,25µL of the specific forward and reverse primers for the genes of interest and for the housekeeping gene, furthermore

18,5μL of this mixture is added to the PCR plate. For every space in the plate it is added 1,5μL of a 4x dilution in RNase free water of cDNA corresponding to each biological sample.
For every biological sample, two technical samples are assessed, and in every PCR plate, a no-template (NTC) condition for each gene is assessed. Primer efficiency was tested, by developing a five-point concentration gradient where serially reduced amounts of template are added to the mix and completed with RNase free water.
The whole procedure was carried out over ice, covered from light and as fast as possible in order to reduce possible RNase exposure.
The quantification was performed on the thermocycler (7500 Applied Biosystems, Thermofisher) with the following thermal protocol.
After the amplification, a melting curve of the amplified products was built for primer dimer and non-specific amplification assessment.

Table 2. Chart with the qRTpcr cycling protocol for transgene expression analysis.

Nº of Cycles	Temperature	Time	Procedure
1x	95ºC	2 minutes	Enzyme activation
40x	95ºC	5 seconds	Annealing
	60ºC	25 seconds	Extension
Dissociation/Melt Analysis	Up to 95ºC with 0,5ºC increments	Increment every 5 seconds	Quality control

Table 3. Primer sequences used in the transgene expression analysis.

Primer sequence	Sequence 5´3	Tm (UC)	Product size (bp)
Synaptobrevin II-forward	CTGCACCTCCTCCAAACCTTACGTTA	60	297
Synaptobervin II-reverse	GGATTTAAGTGCTGAAGTAAACGATG	60	297
18S-forward	GGACCAGACCGAAAGCATTTG	60	260
18S-reverse	TTGCCAGTCGGCATCGTTTAT	60	260

3.5. Histology and immunohistochemistry

3.5.1 Histological Procedures

Coronal hippocampal sections were obtained by means of a vibratome (Leica VT1000S, Germany). Sections of 40 μm were produced by slicing the whole brains fixed by a solution of 3% agarose gel, with the aid of a stainless-steel blade.

Special attention was given to the angle where the brain was ultimately fixed, which in order to standardize the slicing axis, they were placed rested on the cerebellum after a straight slice had been removed to create a flat surface to match the slicing base of the equipment. Hippocampal slices were used for immunohistochemistry experiments.

After slicing, the sections were kept in 1% Phosphate buffer (PBS) solution supplemented with sodium azide 0,2% in order to preserve the tissue from fungi and bacteria overgrowth.

3.5.2 Immunostaining experiments

Immunohistochemistry was carried out on free-floating hippocampal sections; the goal of this experiment was to answer if the impaired gliotransmission had an effect on the already positive modulation of neuronal and glial proliferation in the (DG) of the hippocampus shown after local application of secretome.

For this, GFAP primary antibody was used to stain astrocytes, anti-Ki-67 was employed to mark proliferative cells (cells that are not at the G0 phase), and as a counter staining, DAPI was used to reveal total cell nuclei.

Regarding the immunostaining protocol, slices are first thoroughly washed in a solution of 1% PBS for 3 minutes under slight agitation. Permeabilization of plasma membrane was achieved by washing the slices with PBS plus 0,5% triton. Nuclear antigen retrieval for Ki-67 transcription factor was done by submerging the slices into 1% citrate buffer in 1,5ml Eppendorf tubes and heating the solution to 80ºC for 20 minutes with the aid of a thermoblock (D1200, LabNet)

After cooling under air hood, the samples were submitted to endogenous blocking by adding 10% fetal calf serum (FCS) for 1,5 hours.

After blocking, overnight incubation with primary antibodies (1/200 anti-GFAP, Dako and 1/100 anti-Ki67, BD Biosciences) was performed in 300µL which is the necessary volume used to easily cover all the slices.

In the following day, the slices are thoroughly washed in 1% PBS for 4 times and secondary antibodies (AlexaFluor 488 and Alexa Fluor 594, ThermoFisher) both at 1/1000 dilution factor are added to the slices for a 2-hour incubation time covered from light.

DAPI counter-staining was performed by adding 150µL of 1/500 solution to the slices for 10 minutes, followed by three more washes.

The slices are then mounted into 60x25 slides (Superfrost, ThermoFisher) with the aid of mounting media (Immumount, ThermoFisher).

Table 4. Antibody Chart. Primary, secondary and counter staining for immunohistochemistry experiments.

Primary antibody	Dilution	Company
Rabbit anti-GFAP	1:200	Dako
Mouse anti-Ki-67	1:100	BD Biosciences
Secondary antibody	**Dilution**	**Company**
Alexafluor 594 (RED) anti-mouse	1:1000	ThermoFisher
Alexafluor 488 (BLUE) anti-rabbit	1:1000	ThermoFisher
Counter Staining	**Dilution**	**Company**
DAPI	1:500	ThermoFisher

3.5.3. Confocal microscopy image acquisition

Photomicrographs for cell counts were taken using (FV1000 Olympus, Laser Scanning Confocal Microscope) with a 40x objective (UPLSAPO NA:0.90), using lasers, 405(Blue), 488(Green) and 559(Red), with a resolution of 640x640px.

In order to standardize the image acquisition and minimize inter-sample variation, photos were taken at a speed of 4μs/pixel, pinhole aperture 110μm, and excitations of 544 for DAPI, 452 for GFAP and 551 for Ki-67.

Images were acquired sequentially, using a time lapse controller for mechanical orientation enabling the construction of a mosaic-like structure of the hippocampus, allowing for cell quantification at all three hippocampal layers (granular cell layer, sub-granular zone, molecular layer and hilus) in a tridimensional manner.

For morphology assessment, the same parameters mentioned above were used, apart from employing a higher resolution of 1084x1084px in order to improve clarity for astrocyte processes segmentation. The images were not submitted to post-processing.

3.5.4 Cell counting

To assess number of proliferative cells Ki-67+ as well as GFAP+ cells at the DG of the hippocampus, the area of the DG was divided into sub-granular zone (SGZ) and granular cell layer (GCL). The SGZ was defined as the three cells layer composing the inner part of the DG, followed by the outer GCL. The hilus was defined as the area between the two arms of the DG as seen in fig.

The area of all parts was determined using the software Image J (NIH Image, Bethesda, USA).

All counting was normalized by area (mm^2) of any given subsection and Ki-67+ cells were counted when nuclei appeared bright red and GFAP+ cells according to normal astrocyte morphology. Radial glial cells were counted at the SGZ whenever a cell expressed the characteristic GFAP+ staining around the nuclei and processes together with a positively stained nucleus for Ki-67.

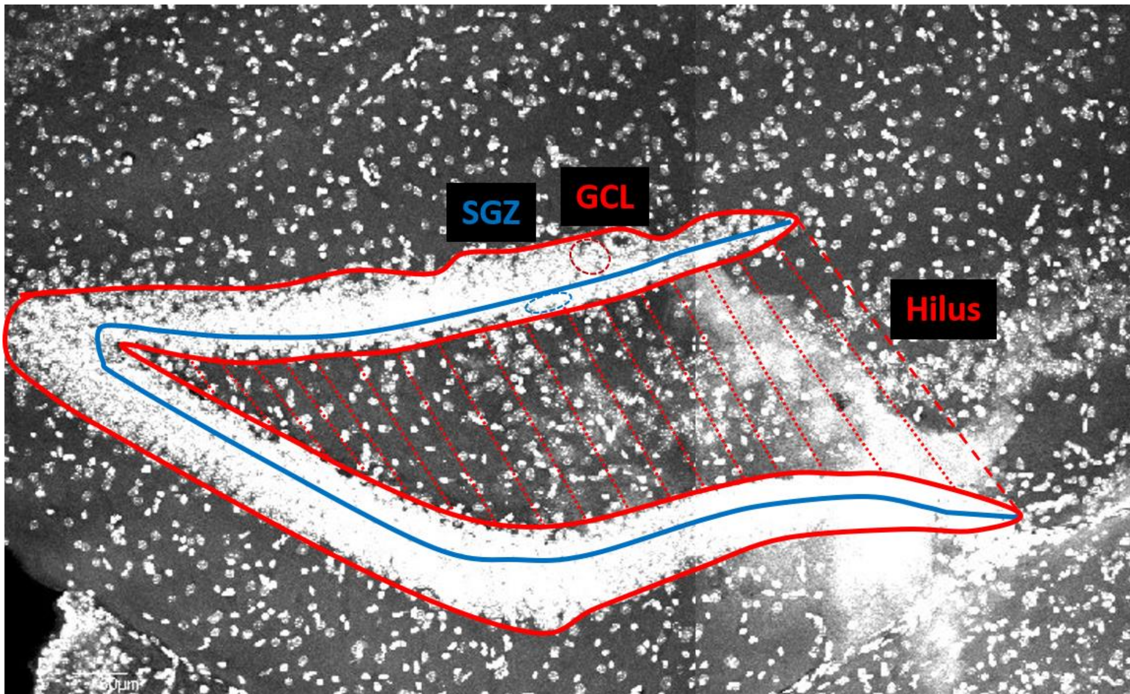


Figure 10. Mosaic photomicrograph of a DAPI channel for better delineation of the dentate gyrus (DG) of the hippocampus. Represented in blue is the sub-granular zone (SGZ) internally, followed by the granular cell layer (GCL) in red externally. Hilus region is the area in between the two arms of the DG.

3.5.5. Morphometric analysis

Astrocytes were readily identified by their characteristic GFAP-positive bushy shape, displaying thicker processes around the DAPI-stained nucleus. Between four and five astrocytes were reconstructed per animal with maximum two cells per slice. Cells with only one DAPI stained nuclei and without truncated processes were considered for reconstruction. The reconstruction of the astrocyte processes was carried using the plugin Simple Neurite Tracer (SNT) from FIJI (NIH, Bethesda, USA). The software, semi-automatically fills the registered paths respecting normal path tortuosity and tridimensional structure suggesting a midline. Due to heterogeneous structure of the astrocytic soma, and its relationship with its processes, the DAPI stained nuclei was consider the initial starting point with every main path being reconstructed from this point. After the cell processes are reconstructed, process length and number are automatically retrieved, and its sums easily calculated by the software. To proceed with the volume analysis, all the paths must be selected, and an adequate threshold must be used as the filling tool starts from the midline and progresses filling the processes outwardly. The threshold must achieve a filling pattern that respects both the thicker and the thinner processes. (0.05 yielded reproducible results across animals).

Regarding the Sholl analysis, all the traced processes were selected and the starting point for the analysis was set at the center of the DAPI stained nuclei. Spherical radius with a 4 µm space was sufficient to provide enough morphological detail. The whole process of morphometric analysis is clarified at (Tavares et al. 2017 Brain Struct Funct)

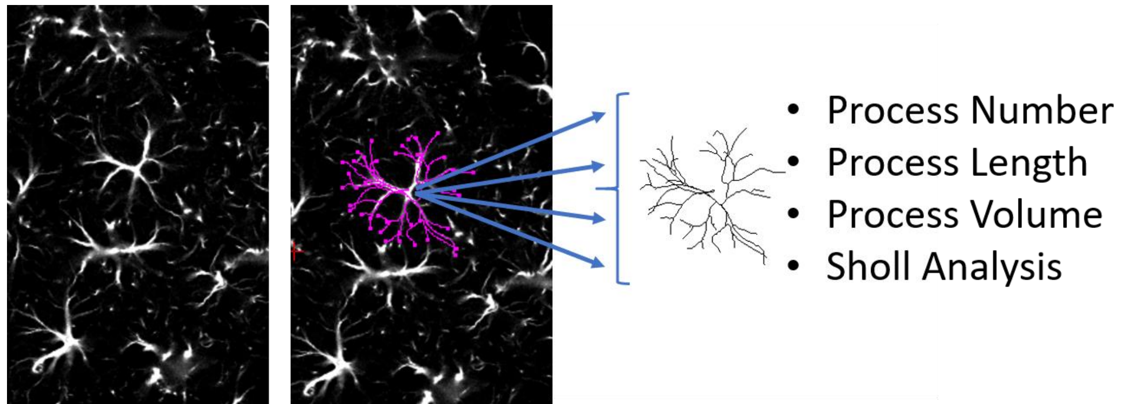


Figure 10. Graphic representation of the astrocyte process segmentation via simple neurite tracer (SNT). In purple is shown the main GFAP+ processes that are included in the multiple analysis.

3.6. Data analysis

Data are shown as the mean ± standard error of the mean (SEM), Statistical analysis was performed using two-way ANOVA, followed by Tukey or Sidak Multiple Comparison Tests. Correlation was performed using Pearson's tests. Values of $P<0.05$ were considered significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ when compared to wild type animals (WT) or control conditions. Data sets were built using excel software (Windows 10, Microsoft, USA). Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) or SPSS version 25 (SPSS 25, IBM, USA).

Chapter 4 – Results

4.1. Transgene expression analysis

In order to establish the validity of the animal model and to inform us of which animals presented impaired levels of astrocytic gliotransmission, qRT-PCR was performed. Alternatively, other authors have reported same results when testing for the expression of reporter genes such as GFP (Sardinha et al.,2017). The graph on figure 11, demonstrates the clear distinction between high and low "expressors". Mean Snare High= 0.016±0.005, n=10; mean Snare Low= 0.001±0.0005, n=13. P=0.0109. The expression levels of Wt animals were undetected as expected.

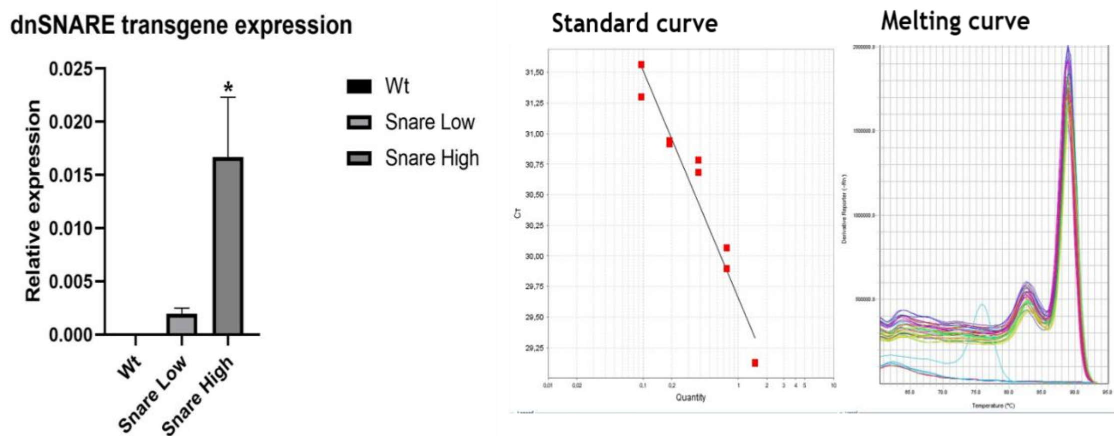


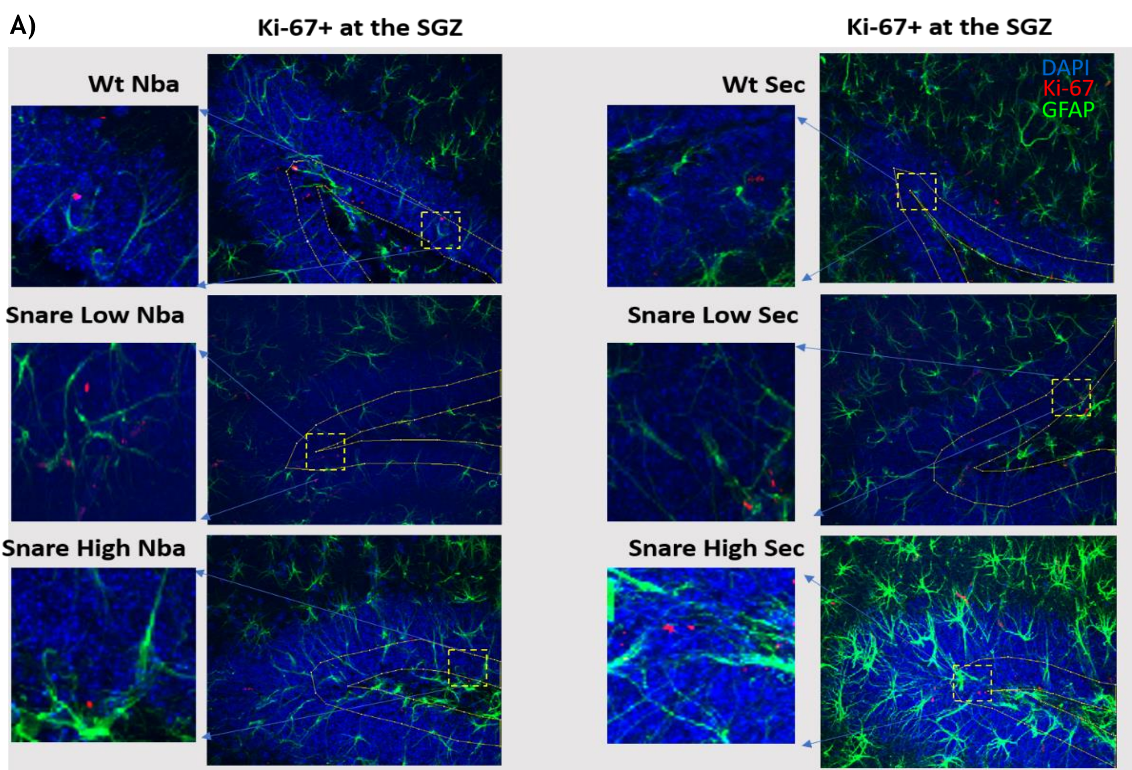
Figure 11. Transgene expression analysis by qRTpcr. Results shows a clear distinction between the so called "low" and "high" expressors. Results shown as mean of relative expression compared to 18s housekeeping gene expression. Data presented as mean±SEM. n=10-13 *P<0.05. Statistical analysis was performed using one-way ANOVA followed by Bartlett's test

4.2. Proliferation levels at the SGZ of the hippocampus

Proliferation is the first event preceding neurogenesis and gliogenesis at the hippocampus, and it is believed to be reduced in disease states (Allen et al., 2016). Additionally, MSC's secretome have been shown to modulate SGZ proliferative state and induce neurogenesis. (Teixeira et al., 2014) The assessment of proliferation was done by counting the number of positive cells at the SGZ of the hippocampus as seen (Figure 12, A-B), cells expressing Ki-67, a highly expressed transcription factor during all active stages of the cell cycle.

Proliferation levels were elevated in Wt and Snare High animals treated with BMSCs secretome when compared to Nba treated animals. There were no differences in proliferation in Snare Low animals.

A)



B)

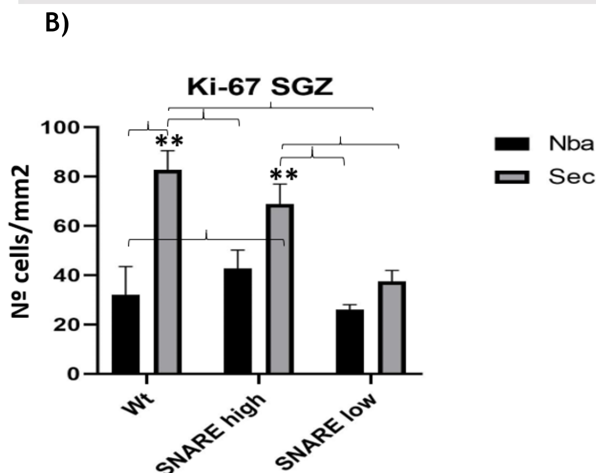
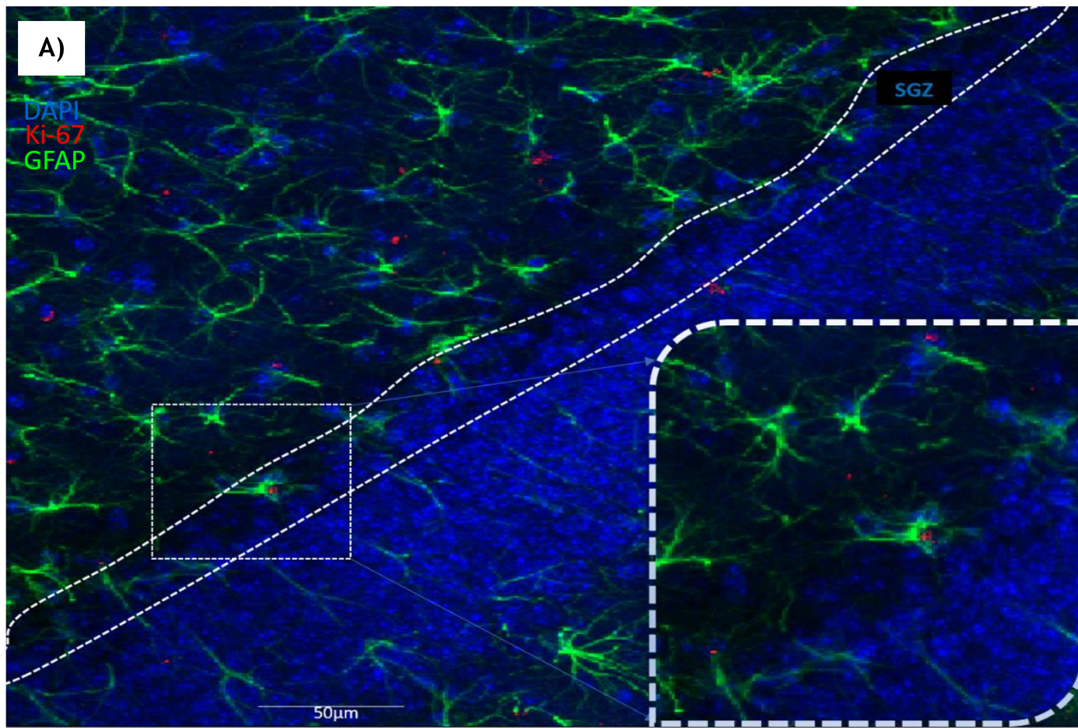


Figure 12. Ki-67 cell counting's. A) Confocal photomicrographs of the DG of the hippocampus showing in blue DAPI stained nuclei, in red Ki-67+ proliferative nuclei and in green GFAP+ astrocytic processes.
B) Graph showing results for Ki-67+ cell counting at SGZ in cells/mm2. Multivariate analysis (Two-way ANOVA) followed by t-test, Wt Sec **P<0.01 when compared to Wt Nba, and Snare High Nba, ***P<0.001 when compared to Snare low Nba and Snare low Sec. Snare high Sec *P<0.05 when compared to Wt Nba and Snare Low Sec, **P<0.01 when compared to Snare low Nba. Data presented as Mean±SEM.

Table 5. Statistical Analysis of Ki-67+ cells at the SGZ-Two-way ANOVA + Tukey test

Marker	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
Ki-67 at SGZ	Wt Sec	82.6±7.8	P Values	0.059	0.031	<0.0001
	Wt Nba	31.9±11.5	F Values	F (2, 22) = 3.209	F (2, 22) = 7.596	F (1, 22) = 23.57
	Snare High Sec	68.8±8.0				
	Snare High Nba	42.6±7.5				
	Snare Low Sec	37.5±4.3				
	Snare Low Nba	25.9±2.1				

Regarding the proliferation of radial-glial cells, cells that are positively stained by GFAP and Ki-67 and can give origin to neuronal or glial progenitors we can see as depicted by (Figure 13 A-B), that in the Snare high Sec group, secretome had a profound impact in increasing its proliferative status.



B)

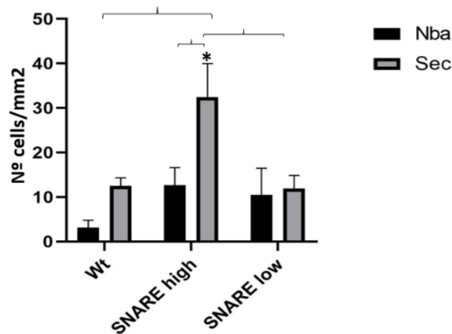


Figure 13. GFAP/Ki-67 double labelled cells at the SGZ. A) Confocal photomicrographs of the DG of the hippocampus highlighting a radial glial cell in the insertion. In blue DAPI stained nuclei, in red Ki-67+ proliferative nuclei and in green GFAP+ astrocytic processes.
B) Graph showing results for GFAP/Ki-67+ double labelling at the SGZ in cells/mm2. Multivariate analysis (Two-way ANOVA) followed by t-test, Snare high Sec *P<0.05 when compared to Snare high Nba and Snare Low Sec, **P<0.01 when compared to Wt Nba. Data presented as Mean±SEM.

Table 6. Statistical Analysis of Ki-67+/GFAP+ cells at the SGZ- Two-way ANOVA + Tukey test

Marker	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
Ki-67+/GFAP+ at SGZ	Wt Sec	12.5±1.7	P Values	0.1574	0.0135	0.0183
	Wt Nba	3.2±1.6	F Values	F (2, 22) = 2.013	F (2, 22) = 5.270	F (1, 22) = 6.494
	Snare High Sec	32.4±7.5				
	Snare High Nba	12.6±3.9				
	Snare Low Sec	11.9±2.9				
	Snare Low Nba	10.5±5.9				

4.3. Proliferation levels at the hilar region of the hippocampus

Regarding the cytogenic capacity of the hilar region, it is known to be a place where glial progenitors migrate and differentiate into astrocytes, interestingly, as seen in (Figure 14 A-B) we found increased although not statistically significant proliferation marked by Ki-67+ expression when gliotransmission was ablated in Snare high animals treated with BMSC secretome.

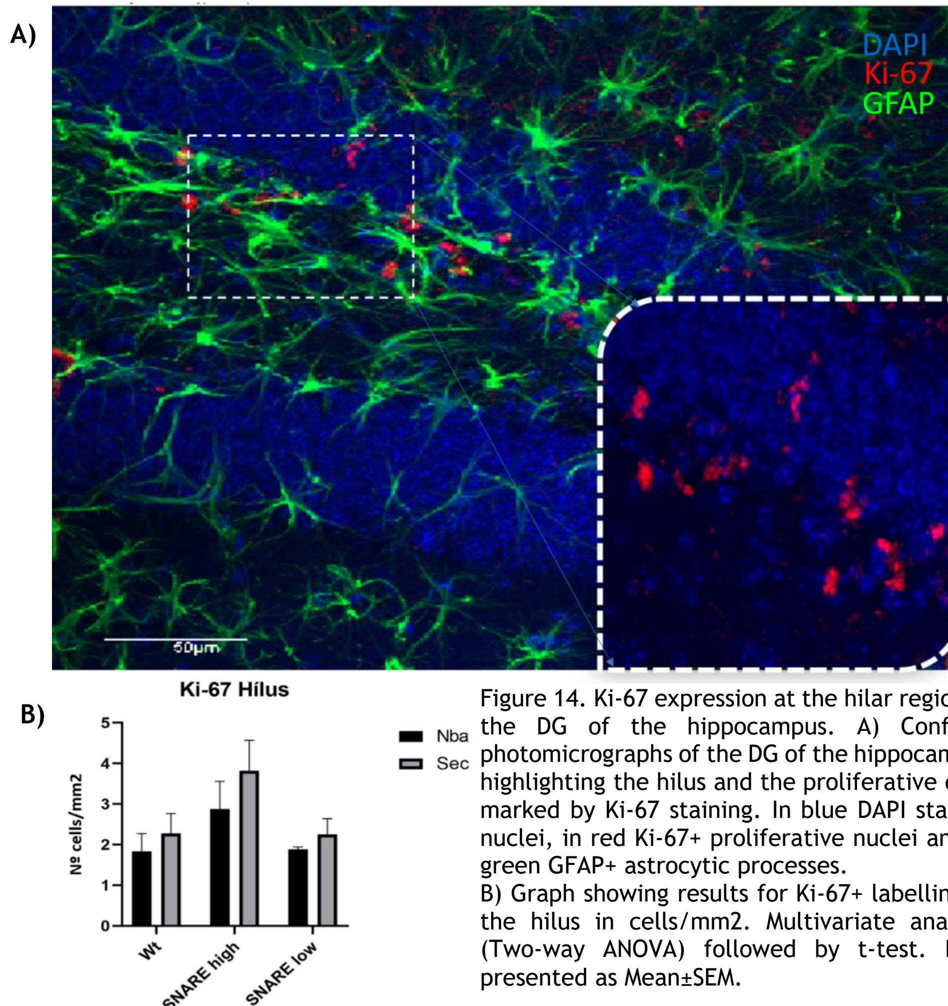


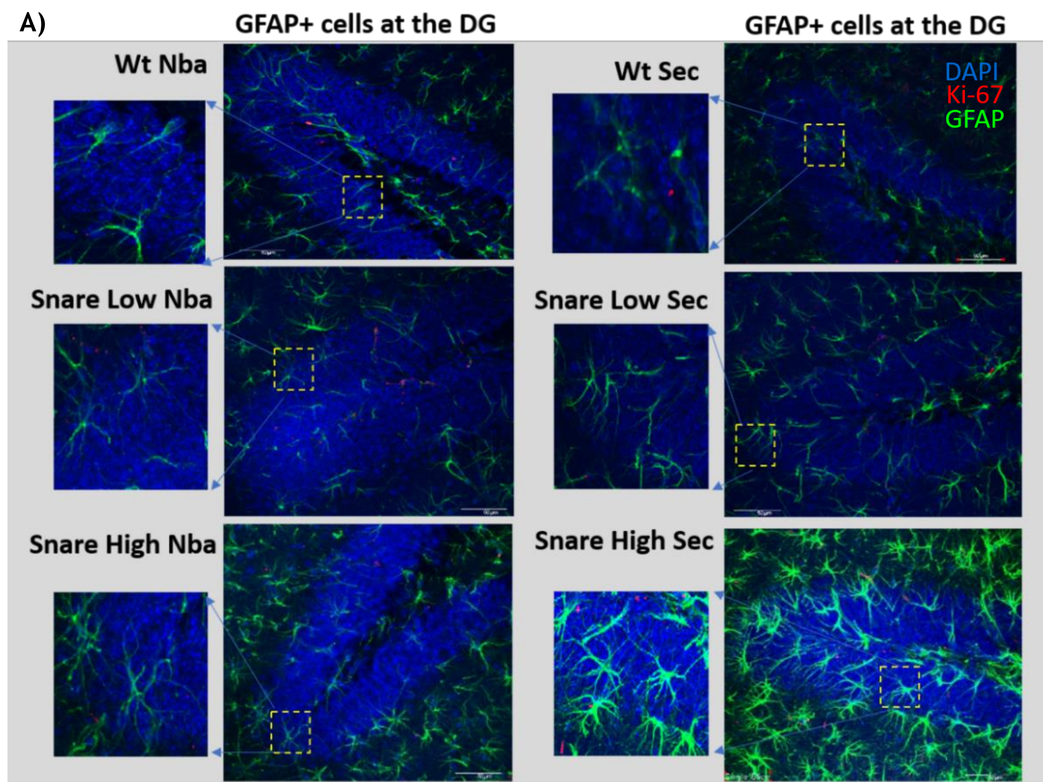
Figure 14. Ki-67 expression at the hilar region of the DG of the hippocampus. A) Confocal photomicrographs of the DG of the hippocampus highlighting the hilus and the proliferative cells marked by Ki-67 staining. In blue DAPI stained nuclei, in red Ki-67+ proliferative nuclei and in green GFAP+ astrocytic processes.
B) Graph showing results for Ki-67+ labelling at the hilus in cells/mm2. Multivariate analysis (Two-way ANOVA) followed by t-test. Data presented as Mean±SEM.

Table 7. Statistical Analysis of Ki-67+ cells at the hilus- Two-way ANOVA + Tukey test

Marker	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
Ki-67+ in the Hilus	Wt Sec	2.2±0.4	P Values	0.8621	0.0529	0.2457
	Wt Nba	1.8±0.4	F Values	F (2, 22) = 0.1494	F (2, 22) = 3.370	F (1, 22) = 1.423
	Snare High Sec	3.8±0.7				
	Snare High Nba	2.8±0.6				
	Snare Low Sec	2.2±0.3				
	Snare Low Nba	1.8±0.05				

4.4. GFAP⁺ cells at the DG of the hippocampus

Although much attention has given to the study of the differentiation potential of radial glial cells, little is known about the generation and differentiation of glial cells at this region. Nevertheless, time-lapse imaging has shown that immature astrocytes migrate radially trough the granular cell layer towards the molecular layer of the DG, where they adopt their final position (Sultan et al., 2015). The (Figure 15 A-B), demonstrates a slight increase in astroglial densities at the DG of the hippocampus in every group treated with the secretome of BMSCs, although the results were non-significant.



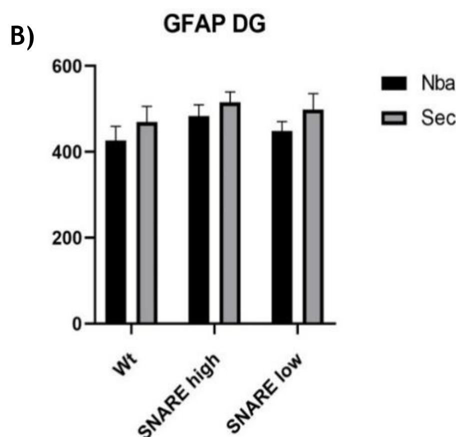


Figure 15. GFAP+ expression at the DG of the hippocampus. A) Confocal photomicrographs of the DG of the hippocampus highlighting GFAP+ expressing cells. In blue DAPI stained nuclei, in red Ki-67+ proliferative nuclei and in green GFAP+ astrocytic processes.
B) Graph showing results for GFAP+ cells counting at DG in mm2. Multivariate analysis (Two-way ANOVA) followed by t-test. Data presented as Mean±SEM.

Table 8. Statistical Analysis of GFAP+ cells at the DG of the hippocampus – Two-way ANOVA + Tukey test

Marker	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
GFAP+ at the DG	Wt Sec	469±37	P Values	0.9664	0.4129	0.1902
	Wt Nba	426±32	F Values	F (2, 22) = 0.03424	F (2, 22) = 0.9211	F (1, 22) = 1.827
	Snare High Sec	515±24				
	Snare High Nba	483±26				
	Snare Low Sec	498±37				
	Snare Low Nba	448±20				

4.5. Astrocytic morphometric analysis

Astrocytes present great morphological complexity, such feature enables much of its homeostatic functions by placing them in close contact with synapses and blood vessels. Dynamic changes in its morphology have been associated with specific phenotypes in response to different physiological and pathological states. Herein we assessed the morphology of astrocytes immuno-stained for GFAP+ a marker that has been shown to correlate with morphological changes, process hypertrophy and overall reactivity. The study was carried, employing an open access tool Simple Neurite Tracer (SNT) that allowed for the verification of astrocytes main processes length, number, volume as well as arbor complexity. In (Figure 16 A, the resulting drawings of the traced astrocytes are demonstrated). In 16, B and C, respectively, it is showed that astrocytes from animals presenting a high gliotransmission inhibition (Snare high Sec) that were treated with secretome presented more and longer processes.

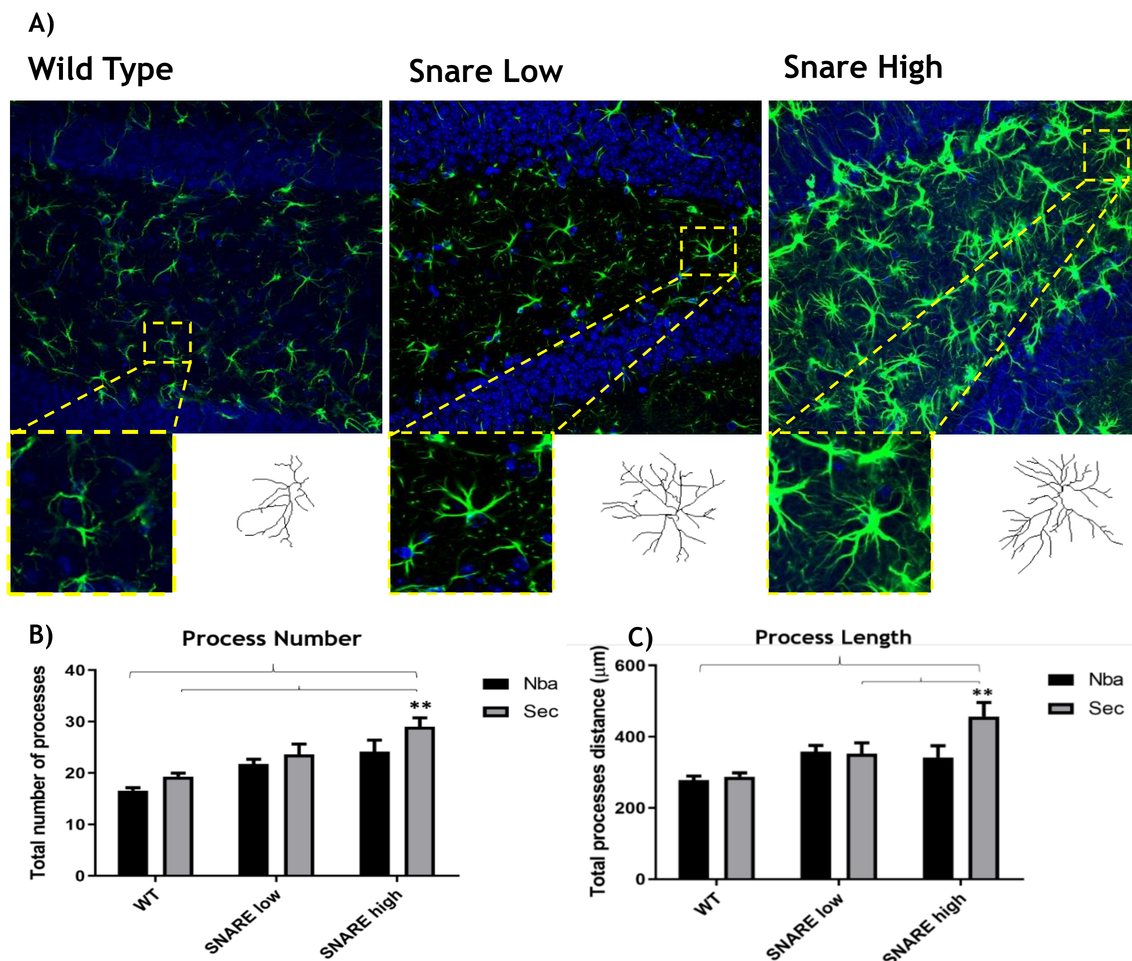


Figure 16. Morphometrical analysis of astrocytes. A) Confocal photomicrographs (max projections) of the DG of the hippocampus highlighting chosen astrocytes for reconstruction with representative SNT traced astrocytes. In blue DAPI stained nuclei, and in green GFAP+ astrocytic processes.
B) Snare high Sec animals present astrocytes with more processes, Snare high Sec *P<0.05 when compared to Wt Sec and **P<0.01 when compared to Wt Nba. C) Snare high Sec animals present astrocytes with longer processes *P<0.05 when compared to Wt Nba and Wt Sec. Multivariate analysis (Two-way ANOVA) followed by t-test. Data presented as Mean±SEM.

Table 9. Statistical analysis of astrocyte process number – Two-way ANOVA + Tukey test

Morphometry	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
Number of Processes	Wt Sec	19.3±0.6	P Values	0.7389	0.0021	0.0797
	Wt Nba	16.5±0.5	F Values	F (2, 22) = 0.3068	F (2, 22) = 8.227	F (1, 22) = 3.376
	Snare High Sec	29.0±1.7				
	Snare High Nba	24.1±2.2				
	Snare Low Sec	23.6±2.0				
	Snare Low Nba	21.7±0.9				

Table 10. Statistical analysis of astrocyte process length – Two-way ANOVA + Tukey test

Morphometry	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
Process Length	Wt Sec	287.8±10.9	P Values	0.1547	0.0117	0.1767
	Wt Nba	278.4±11.1	F Values	F (2, 22) = 2.034	F (2, 22) = 5.488	F (1, 22) = 1.948
	Snare High Sec	456.9±39.0				
	Snare High Nba	341.5±33.0				
	Snare Low Sec	352.0±30.9				
	Snare Low Nba	358.4±17.3				

Regarding astrocyte process volume, despite of a tendency for increased volumes in all experimental conditions when treated with secretome, no statistic differences were found among groups. Nevertheless, positive correlations were seen between astrocyte volume when compared with process number and length as seen in (Figure 17. A-C)

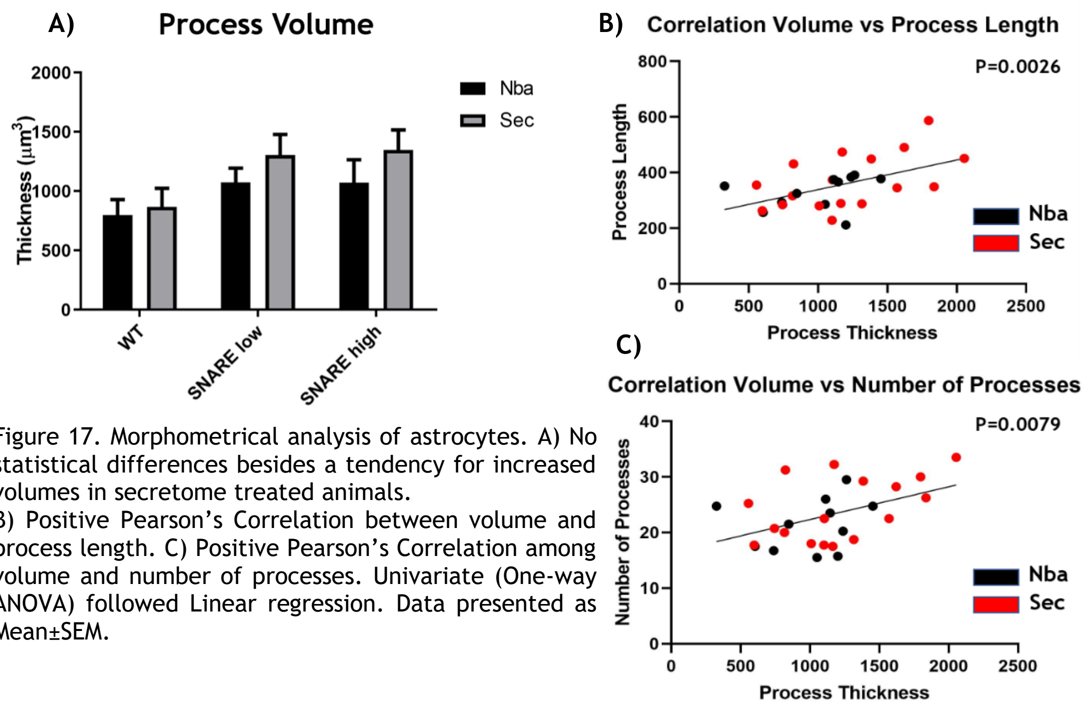


Figure 17. Morphometrical analysis of astrocytes. A) No statistical differences besides a tendency for increased volumes in secretome treated animals.
B) Positive Pearson's Correlation between volume and process length. C) Positive Pearson's Correlation among volume and number of processes. Univariate (One-way ANOVA) followed Linear regression. Data presented as Mean±SEM.

Table 11. Statistical analysis of Pearson's correlation of process volume with process number and length-Linear regression

Morphometry	Volume vs Process Length	Volume vs Process Number
Pearson Correlation	R^2=0.263/P=0.0026	R^2=0.204/P=0.0079
Linear Regression	P=0.0052	P=0.0158

To assess overall arbor complexity, Sholl analysis was employed for 3 different assessments. There was no difference in indirect complexity marked by distance of the last intersection between groups. The classical Sholl analysis showed a shift to the right in multiple distance points, what represents increased complexity in the astrocytes from animals with impaired gliotransmission that were submitted with BMSCs secretome treatment.
Additionally, astrocytes from Snare high animal submitted to secretome treatment presented increased number of intersections.

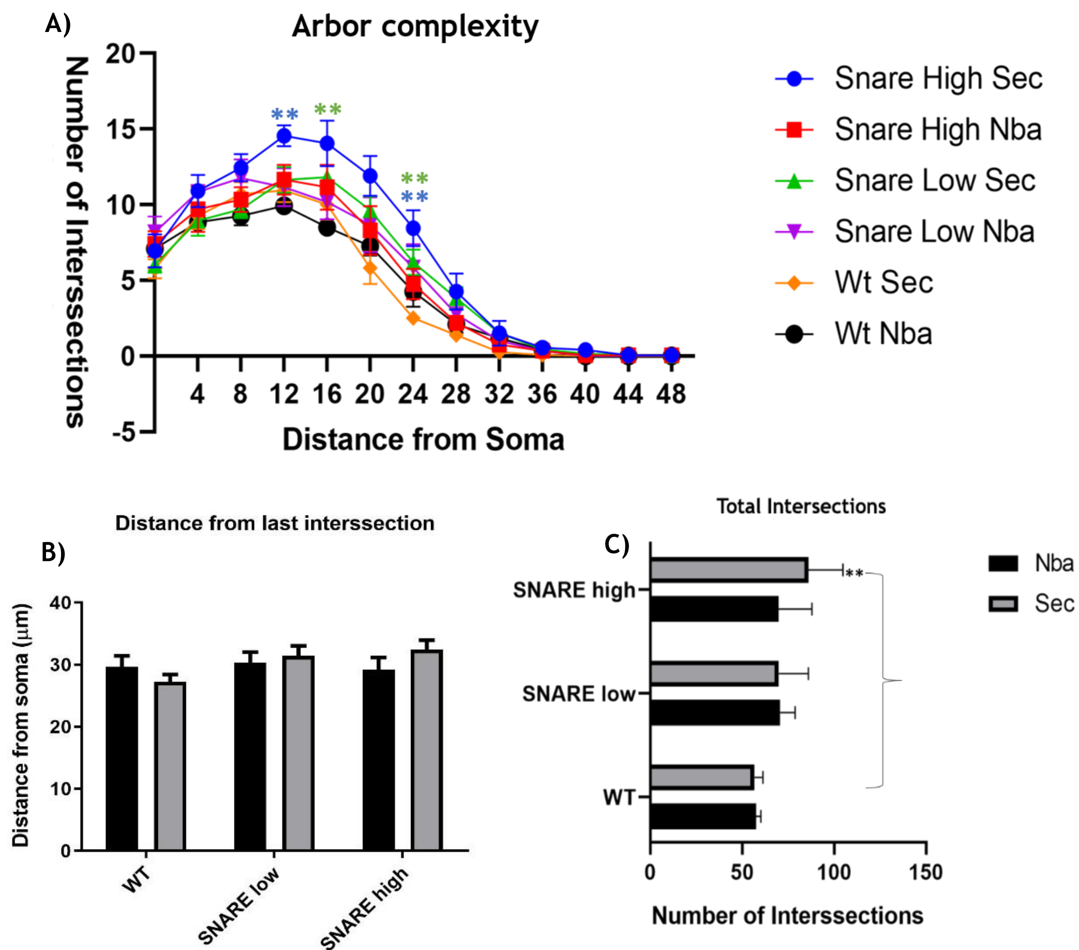


Figure 18. Morphometrical analysis of astrocytes. A) Snare animals treated with secretome presented increased arbor complexity in multiple distance points. Snare high Sec **P<0.01 when compared to Wt Nba and *P<0.05 when compared to Wt Sec. Snare Low Sec *P<0.05 when compared to Wt Nba and Wt Sec

B) Distance of last intersection was the same among experimental groups. C) Snare high Sec animals presented astrocytes with increased number of intersections. Multivariate (Two-way ANOVA) followed Tukey's test. Data presented as Mean±SEM.

Table 11. Statistical analysis of number of intersections - Two-way ANOVA + Tukey test

Morphometry	Experimental Group	Mean±SEM	Significance	Source of Variation		
				Interaction	Genotype	Treatment
Total number of Intersections	Wt Sec	56.6±2.3	P Values	0.4186	0.0203	0.3406
	Wt Nba	54.9±1.2	F Values	F (2, 22) = 0.9062	F (2, 22) = 4.679	F (1, 22) = 0.9489
	Snare High Sec	86.0±8.3				
	Snare High Nba	69.8±8.0				
	Snare Low Sec	69.9±5.6				
	Snare Low Nba	70.6±4.7				

Chapter 5 – Discussion

5.1. Hippocampal proliferative state and astroglial density.

The use of MSC’s secretome for the treatment of CNS diseases has broad therapeutic applications, its capacity to modulate neurogenesis has been implied as a probable mechanism of action in several in vivo studies (Harrel et al., 2019). Since astrocytes represent a major player in the regulation of hippocampal neurogenesis, studying the interactions between astrocytic function and MSC’s secretome application into the DG could help us understand how this highly responsive cell population participate in the complex physiology of the hippocampal neurogenic niche. In fact, early data from our group have demonstrated that the secretome is an active modulator of hippocampal neurogenesis, inducing new-born neuron proliferation and differentiation, furthermore, the secretome is also capable of increasing glial survival in vitro as well as improving astrocytic densities when applied in vivo into the DG (Salgado et al., 2015; Salgado et al., 2010; Ribeiro et al., 2011; Teixeira et al., 2016; Teixeira et al., 2014).
Considering such data, we aimed to study the impacts of impaired astrocytic signaling on the modulatory effects mediated by BMSCs secretome application into the DG. For that, the transgenic dnSNARE mice model was employed which works by overexpressing the cytosolic portion of the SNARE protein synaptobrevin-2 under the control of the GFAP promoter allowing for the selective transgene expression only in GFAP expressing cells. (Pascual et al., 2005)
In that sense, as seen in figure 12 A-C, our experiments revealed that in comparison to the control neurobasal (NBA) injection, BMSC secretome increased levels of proliferation marked by the number of cycling cells (Ki-67+) at the sub-granular zone of the hippocampus in wild type and only in the dnSNARE-high group. Such result could be interpreted in two ways, for instance, the response from the wild-type animals was expected as (Teixeira et al., 2016), also demonstrated the same effect in rats, by showing an increased number of proliferative cells co-expressing the early-neuronal marker double-cortin (DCX) and Ki-67, the results were associated with the increased presence of neuroregulatory molecules such as FGF-2, BDNF, GDNF, IGF-1, VEGF and miR-16. (Teixeira et al., 2014; Teixeira et al., 2016)
Regarding the response of the dnSNARE animals, to the best of our knowledge, this was the first study to explore such outcome on this animal model, nevertheless, it is worth mentioning that astrocytic transmission trough SNARE mechanisms plays an important role in mediating the maturation of adult-born neurons, and the impairment of such function may have induced a proliferative feedback, in order to maintain adequate levels of proliferation. (Sultan et al., 2015)
Importantly, herein we showed data from animals presenting low levels of transgene expression, as shown in figure 11, which is uncommon in studies employing this animal model, in this sense, regarding the proliferation levels, the responses of the low expressing animals appears similar to the observed in wild type animals with the exception of the proliferation

levels in the SGZ, posing an interesting question of whether the effects of the secretome in transgene animals can be isolated or not, specially, given the clear physiological impact of exocytosis blockage. It is worth mentioning that, in studies employing such animal model, the animals expressing lower levels of transgenes are usually disregarded from data analysis (Sardinha et al., 2017)

Interestingly, as showed in figure 13 A-B, the increase in the number of Ki-67+ cells in the dnSNARE-high group could be explained by the increase in the counting of cells co-expressing GFAP and Ki-67+ the so called radial glial cell, effect that was observed only in such experimental group. In fact, it has been demonstrated that radial glial cells can give origin to astrocytes (Cassé et al., 2018). Accordingly, when homeostasis is disrupted in cases of inflammatory responses, the proliferation associated with the establishment of astrogliosis happens due to early commitment of glial precursors to the astrocyte lineage, in fact, during pre-natal brain development, upon the appearance of the first cells exhibiting astrocyte characteristics, neurogenesis decreases in favor of gliogenesis (Costa et al., 2009; Kanski et al., 2014; Nagao et al., 2016).

Furthermore, astrocytes have been shown to negatively regulate neurogenesis by reducing Type-a/Neural stem cell proliferation trough contact mediated NOTCH signaling, a process dependent on intermediate filament protein GFAP and vimentin, what explains why we observed reduced levels of radial glial cell proliferation in the wild type animals. Accordingly, Barkho et al, 2006 identified several proteins such as (IGFBP-6 and decorin) that inhibits the proliferation of neural stem/radial glial cells expressed by astrocytes.

Although such proteins have been identified in the secretome of human MSCs, it is worth mentioning that no study have been conducted with the aim of identifying such proteins in astrocyte exocytotic vesicles nor conditioned media, nevertheless, it is possible that the higher levels of proliferation and radial glial cell counting witnessed in dnSNARE high animals herein could have been due to reduced negative signaling from IGFBP-6 and decorin. (Barkho et al, 2006)

Moreover, astrocytes have also been shown to establish contact with radial glial/neural stem cells by sharing the surface of blood vessels of the molecular layer with processes from neural stem cells, and such interaction has been shown to increase proliferation and neurogenesis. (Kinouchi et al., 2003; Ashton et al., 2012) Thus, demonstrating that astrocytes present a strong influence in controlling the proliferation and differentiation of radial glial/neural stem cells in the hippocampus, and that such influence is dependent on astrocyte's state and protein expression profile as well as molecular cues from its microenvironment. Such molecular cues, could be represented by the trophic factors and extracellular vesicles present in the MSC's secretome as they have demonstrated the potential of changing cellular fate upon application.

Considering the levels of proliferation in the hilar region of the hippocampus, as seen in figure 14 A-B, a robust tendency for higher Ki-67+ expression was observed in the dnSNARE high animals treated with the secretome, albeit, the differences between groups were not statistically significant, this finding is interesting given the actual lack of abundance in

publications reporting hilar proliferative events. Nevertheless, from what has been published, this could have implications for neuropsychiatry disorders as the low levels of proliferation marked by reduced Ki-67 expression in this hippocampal region have been correlated to schizophrenia, (Allen et al., 2016) Furthermore, this result could have been, again, derived from a proliferative response against the dnSNARE transgene expression, specially taking into account that the majority of proliferating progenitors that become astrocytes do it so in the hílus. Such response could be interpreted as a trial to reestablish normal gliotransmission particularly in this animal model, where not every astrocyte presents transgene expression (Han et al., 2011; Sultan et al., 2015; Sardinha et al., 2017).

In what regards the GFAP+ cell density at the DG of the hippocampus, although there were no statistically significant differences between the treatment groups nor between the three phenotypes as seen in figure 15 A-B, an interesting tendency with a strong effect size was seen towards increased densities whenever secretome treatment was present. Indeed, previous results from our lab demonstrates that the secretome when applied to the DG, induces higher GFAP densities when compared to the NBA injected animals (Teixeira et al., 2016). Furthermore, such result makes physiological sense especially given the fact that the secretome contains EGF and FGF-2 which are the main ligands for EGFR and FGFR two highly expressed receptors that define astrocyte progenitors. (Pires et al, 2016; Lasorella et al. 2017).

5.2. Astrocyte morphological responses

The existence of a sustained dialog between neurons and astrocytes is only possible due to the uniquely morphologic features presented by astrocytes in their long astrocytic processes coupled with their highly ramified structure (Sofroniew et al., 2015).
Such complex morphology put astrocytes in the center of neurotransmission events providing strategic points of contacts with synapses and blood vessels (Sofroniew et al., 2015). Indeed, a single astrocyte may enwrap thousands of individual synapses and a myriad of parenchymal blood vessels (Tavares et al., 2017).
At these points, astrocytes have the capacity to sense and respond to different levels of activity trough the release of signaling neuroactive molecules, ultimately altering neuronal activity, microvascular response and behavior processing (Araque et al., 2014).
In fact, considerable morphological distinctions can be seen across species, brain regions and physiological states, and in addition, during certain pathological conditions astrocyte morphology is drastically affected (Sierra et al., 2015). Thus, in the scope of the present research, the study of astrocyte morphology was carried with the purpose of assessing the responses to the secretome injections as well as the influences that the dnSNARE transgene expression had in this cell population.
For that, we employed the use of an open-source tool, Simple Neurite Tracer (SNT), that enables the reconstruction of the astrocytic main processes in a tridimensional manner, the software allows for the analysis of astrocyte morphology from a large number of sample, yet, in a simple, semi-automatic, effective and inexpensive way (Longair et al., 2011).
The animal samples used for such analysis were stained for (GFAP), a broadly used marker for astrocyte main processes which its expression is highly responsive in cases of morphological changes, being one of the main molecular markers employed to asses and study astrocyte reactivity (Oberheim et al., 2012; Sofroniew et al., 2015) .
In this context, we aimed to evaluate astrocyte main processes number, length, volume and overall arbor complexity. As seen in figure 16 A-C, astrocyte process number and length were significantly increased only in the dnSNARE high group treated with secretome, additionally, although no statistical differences among experimental groups were seen for process thickness, a strong and positive correlation between process volume, number and length was present, evidenced by figure 17 A-C. This result reveals the existence of a possible converging stimulatory mechanism among the secretome and the exocytosis blockage in astrocytes that led to overall process hypertrophy.
Such result could be explained partially by the presence of stimulatory proteins present in the secretome of BMSCs such as FGF-2, PEDF, CADH2 and GDN, all molecules previously shown to induce astrocyte process hypertrophy (Pires et al., 2016; Kanemaru et al 2013; Ridet et al., 1997).
Moreover, another potential explanation comes from the fact that, the reduction in exocytotic events seen in dnSNARE high animals inhibits the presence of autocrine ATP signaling events

that normally emanates from astrocytes. Such signaling for instance, has been implicated in the astrocytes ability to control its morphology in response to changes in the microenvironment, therefore, its blockage, could lead to changes in cell morphology (Shen et al., 2017; Schmitt et al., 2012).

Importantly, autocrine ATP/ADP signaling trough 2PY receptors have been implicated in astrocyte release of glutamate, this could have implications for diseases where excitotoxicity plays an etiologic role such as in traumatic brain and spinal cord lesions, epilepsy and neurodegenerative disorders (Nikolic et al., 2018; Allen et al., 2016). Considering arbor complexity, we employed sholl analysis, a method that measures the number and distance of intersections present in each process by creating concentric spheres around the astrocytic soma (Sholl DA. 1953). From such analysis, as demonstrated in figure 18 B, there were no differences regarding the distance of the last intersection which is an indirect measure of arbor complexity. Furthermore, as seen in figure 18 A and C, the total number of intersections was shown to be bigger in the dnSNARE high group treated with secretome, being such result complemented by an increased arbor complexity, as shown by the curve intersection shift to the right. In addition, secretome also increased overall arbor complexity from dnSNARE low animals but failed to do it so in the wild type ones, showing that, indeed the transgene expression was in fact the most important variable mediating such morphological changes.

Taken together, these data shows that the secretome from BMMSCs is a potent mediator of cell proliferation marked by increased Ki-67 staining in the sub-granular zone of the hippocampus of wild type and in dnSNARE high animals, additionally, high transgene expression appears to exert a proliferative pressure in radial glial cells trough mechanisms yet to be determined, and in addition appears to act in a synergistic way together with the secretome to mediate the morphological changes in astrocytes presented herein. This can have implications for the physiopathology as well as treatment of many CNS diseases given the increased relevancy and broad implications that has been given to the role of astrocytes and gliotransmission recently.

Chapter 6 – Conclusions

As a concluding remark, the work that has been presented and included in this dissertation provides important insights of how astrocytic signaling mediated by exocytosis affects the BMSCs secretome performance in modulating the proliferative levels and astrocyte morphology in the mice hippocampus. In fact, we showed astrocytic exocytosis to be crucial for the response to the proliferative stimuli produced by the secretome at the SGZ, by demonstrating reduced levels of proliferation in transgenic mice when compared to wild type. Furthermore, although the lack of gliotransmission did not present an impact on glial densities at the DG, it appeared to generate a proliferative pressure in radial glial cells at the SGZ and at the hilar region that was supported by the secretome treatment. In addition, we demonstrated that whenever there was a high disruption of exocytotic events and presence of secretome the animals presented astrocytes with more complex and hypertrophic morphologies, probably due to a synergistic effect on different signaling pathways caused by the trophic molecules in the secretome and the reduced autocrine signaling due to disruption of exocytotic events.

More studies, aiming to reveal the molecular machinery of biosynthesis, storage and release of gliotransmitters as well as their impact for astrocytic responses to brain pathologies are warranted, especially if we want to one day, harness the opportunities for modulation of this cell population. Similarly, more research should be encouraged in the area of MSC biology, specifically in what concerns the full molecular characterization of the secretome (soluble proteins, extracellular vesicles, metabolites and signaling lipids), in order to provide us with an idea of the transcriptomic impact of applying such a complex cocktail of molecules to achieve a desired therapeutic outcome. Knowing the molecular mediators behind the therapeutic effect of this therapy is of unmeasurable importance in enabling us to translate the so far encouraging effects of pre-clinical studies into the clinics.

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