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Astrocyte-derived GDNF is a potent inhibitor of microglial activation

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Table of contents

Resumo

A doença de Parkinson é caracterizada pela perda selectiva de neurónios dopaminérgicos na *substantia nigra pars compacta*. A origem desta doença não está completamente esclarecida, no entanto têm sido propostas diversas hipóteses em relação aos possíveis factores envolvidos na degeneraração dos neurónios nesta zona. Entre elas, encontra-se a neuroinflamação, que é cada vez mais reconhecida como o principal factor na patogénese da doença de Parkinson, e inúmeras evidências sugerem que as células microgliais são a fonte predominante de inflamação que contribuir para a neurodegeneração dopaminérgica. Os astrócitos desempenham funções vitais na manutenção da função normal do cérebro e diversos estudos sugerem que estes podem actuar como reguladores fisiológicos prevenindo as respostas microgliais inflamatórias excessivas. No entanto, pouco se sabe sobre a forma como os astrócitos modulam a activação microglial. Dada a relevância das interacções astrócitos-microglia na regulação da neuroinflamação, é importante identificar mediadores envolvidos neste processo, os quais podem actuar como agentes anti-inflamatórios naturais no cérebro. Neste trabalho, o principal objectivo foi o estudo dos efeitos de mediadores solúveis libertados pelos astrócitos na activação microglial induzida pelo agente inflamatório Zymosan A, assim como identificar a natureza desses mediadores. Para a determinação do efeito destas moléculas na actividade microglial, culturas primárias de microglia do mesencéfalo ventral foram previamente expostas, a meio condicionado pelos astrócitos (ou meio de cultura – controlo), e posteriormente tratadas com 5 µg/mL de Zymosan A. Estudos anteriormente indicaram que esta concentração de Zymosan provoca um aumento acentuado da actividade fagocítica microglial, bem como da produção de espécies reactivas de oxigénio, sem no entanto induzir a morte destas células. No presente estudo, verificou-se que a pré-incubação das células microgliais com o meio condicionado pelos astrócitos foi capaz de prevenir o aumento da actividade microglial induzida pelo Zymosan A, mantendo a actividade fagocítica bem como os níveis de espécies reactivas de oxigénio em níveis de controlo. Para avaliar a natureza dos mediadores solúveis libertados pelos astrócitos que preveniam a activação

microglial induzida pelo Zymosan A, foram usados anticorpos de forma a bloquear a acção de alguns factores neurotróficos conhecidos pelas suas propriedades neuroprotectoras na *substantia nigra*. Assim, o meio condicionado pelos astrócitos foi tratado com anti-GDNF, anti-CDNF e anti-BDNF, separadamente, e adicionado às culturas de microglia posteriormente expostas a Zymosan A. O factor neurotrófico derivado de células da glia (GDNF) parece ser um mediador solúvel capaz de prevenir completamente a activação microglial induzida pelo Zymosan A, sendo que os restantes mediadores parecem não exercer qualquer efeito na prevenção da activação microglial. Para confirmar este facto, silenciou-se especificamente o GDNF em culturas de astrócitos, recolhendo-se posteriormente o meio condicionado por estas culturas a aplicando-os a culturas de microglia. Este meio condicionado pelos astrócitos silenciados para o GDNF, não foi capaz de prevenir a activação microglial induzida pelo Zymosan A. Por último, para esclarecer se este efeito era directo e se não haveria outras moléculas a auxiliar o efeito exercido pelo GDNF nas células microgliais, quantificaram-se os níveis de GDNF no meio condicionado pelos astrócitos, e com base nesta quantificação, três concentrações de GDNF, 100 pg/mL, 200 pg/mL e 400 pg/mL, diluído em meio de cultura, foram testadas para avaliar os seu efeitos de prevenção da actividade microglial. Observou-se que todas as concentrações de GDNF suprimem a activação microglial induzida pelo Zymosan A. No entanto, não se verificou um efeito dose – resposta como era de esperar. Os resultados obtidos neste trabalho demonstram que o GDNF é um factor neurotrófico derivado de astrócitos, com capacidade de modular as respostas inflamatórias microgliais. Este efeito do GDNF poderá contribuir para o desenvolvimento de uma possível terapia de prevenção contra a neuroinflamação, e indirectamente reduzir o desenvolvimento da doença de Parkinson.

Palavras-chave: Microglia, Astrócitos, Neuroinflamação, Doença de Parkinson.

Abstract

Parkinson's disease is characterized by the selective loss of dopaminergic neurons in the *substantia nigra pars compacts*. The aetiology of this disease is not completely clarified; however several hypotheses have been advanced regarding the loss of dopaminergic neurons. Among them, neuroinflammation has been increasingly recognized as a major factor in the pathogenesis of Parkinson's disease, and increasing evidence suggests that microglial cells are a predominant source of inflammation contributing for the dopaminergic neurodegeneration. Astrocytes play vital roles in the maintenance of the normal brain function and diverse studies suggest that they could act as physiological regulators preventing excessive inflammatory microglial responses. However, little is known regarding how astrocytes may modulate the microglial activation. Due to the relevance of astrocytes-microglia interactions in the regulation of brain inflammation, it is important to identify the mediators involved in this process, which could act as natural anti-inflammatory agents in the brain. In this way, the major goal of the present work was to evaluate of the effect of soluble mediators release by astrocytes on microglial activation induced by the inflammatory agent Zymosan A, as well as to identify the nature of these mediators. For the determination of the effect of these molecules in the microglial activity, ventral midbrain microglial primary cultures were previously exposed to astrocytes conditioned media (or culture medium – control), and then treated with 5 µg/mL ofZymosan. Studies previously made indicated that this concentration of Zymosan provokes an accented increase of the microglia phagocytic activity and increased ROS generation, showing no citotoxici effect to the cells. However, the pre-incubation of the microglial cells with astrocytes conditioned media was capable to prevent the characteristic increase of the phagocytic activity and ROS production induced by Zymosan A, which levels remained at control levels. To evaluate the nature of the soluble mediators release by astrocytes able to prevent the microglial activation, specific antibodies recognizing some neurotrophics factors known by its neuroprotectors properties of *substantia nigra*. were used to block their action on the astrocytes conditioned media. Those antibodies were: anti-GDNF, anti-CDNF and anti-

BDNF added separately to the astrocytes conditioned medium. Using these condicioned media, we observed that the glial cells line-derived neurotrophic factor (GDNF) seems to be a soluble mediator capable to completely prevent the microglial activation induced by Zymosan A, whereas, the remaining mediators do not exerted an effect on microglial activation. To confirm this fact, specific knockdown of GDNF was achived in astrocytes cell cultures and the resultant condicined medium from this cultures, when applied to microglia cultures before Zymosan A, were not capable to prevent its activation. Finally, to clarify if this effect was an isolated of GDNF or if other molecules were laso involved the levels of GDNF on the astrocytes conditioned media were quntidied by ELISA assay. Based on the obtained values, three concentrations of GDNF (100 pg/mL, 200 pg/mL and 400 pg/mL), diluted in culture medium, were tested to verify its capability to prevent microglia activation induced by Zymosan A. The results have shown that the three concentrations of GDNF were capable to suppress microglial activation induced by Zymosan A. However, the achieved protection was not in a dose dependent manner, as initially expected.

Taken together, the results obtained in this work demonstrate that GDNF a neurotrophic factor expressed by astrócitos, has the capacity to modulate the microglial inflammatory response. In this way, GDNF could be use to develop a potencial therapy to prevent neuroinflammation, and in this way contributing to the reduction of the development of Parkinson's Disease pathogenesis.

Keywords: Microglial, Astrocytes, Neuroinflammation, Parkinson's disease.

List of Abbreviations

6-OHDA	6-Hydroxydopamine
ACM	Astrocytes Conditioned Media
AD	Alzheimer's Disease
AP	Parkinson's Disease
Aβ	Amyloid-β
BDNF	Brain-derived Neurotrophic Factor
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CDNF	Cerebral Dopamine Neurotrophic Factor
CNS	Central Nervous System
CR3	Complement Receptor 3
DCFDA	2'-7'-dichlorodihydrofluorescein diacetato
GDNF	Glial-derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFR	GDNF family receptor
HIV	Human Immunodeficiency Virus
HO-1	Hemeoxygenase-1
IFN	Interferon
IL	Interleukin
IL-1ra	IL-1receptor antagonist
LB	Lewy Body
LPS	Lipopolysaccharide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
MS	Multiple Sclerosis
NADPH	Nicotinamida Adenine Dinucleotide Phosphate
NGF	Nerve Growth Factor
NO	Nitric Oxide
NTF	Neurotrophic Factor
PBS	Phosphate Buffer Saline

PFA	Paraformaldehyde
RNAi	RNA interference
ROS	Reactive Oxygen Species
SN	Substantia Nigra
SNpc	*Substantia nigra pars compact*
SOD	Superoxide Dismutase
TGF	Transforming Growth Factor
TH	Tyrosine Hydroxylase
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
ZyA	Zymosan A

Chapter I

INTRODUCTION

1. Neuroinflammation and neurodegenerative diseases

Inflammation is the first response of our body's immune system to pathogens or irritation. Inflammation is a two-edged sword. In acute conditions, it protects tissue against invading agents and promotes healing. On the other hand, when chronically sustained, it can cause serious damage to host's own tissue. While the central nervous system (CNS) has been known as an immune privileged organ, increasing evidence demonstrate that inflammation is actively involved in pathogenesis of a number of neurodegenerative diseases including multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), and Human immunodeficiency virus (HIV)-associated dementia (McGeer, Itagaki et al. 1988; Raine 1994; Banati, Daniel et al. 1998).

The hallmark of neuroinflammation is the activation of microglia. Activation of microglia is believed to contribute to neurodegenerative processes through the release of proinflammatory and/or cytotoxic factors, including interleukin (IL)-1, tumor necrosis factor-α (TNF-α), nitric oxide (NO), and reactive oxygen intermediates (Kim, Mohney et al. 2000), that amplify the inflammatory response by activating and recruiting other cells to the site of brain lesion. In addition, microglia can release potent neurotoxins, which may cause neuronal damage. Sustained overactivation of microglia has been observed in multiple neurodegenerative diseases (Kim and Joh 2006).

The most characteristic feature of microglia is their rapid activation in response to pathological changes in the CNS. They respond not only to changes in the brain parenchymal integrity but also to very small alterations in their microenvironment, such as imbalances in ion homeostasis that precede pathological changes (Gehrmann, Banati et al. 1993). Although they have a critical role in host defense by removing invading microorganisms and neoplastic cells, or by secreting neurotrophic factors (NTF), microglia may aggravate the effects of inflammation and cause neuronal degeneration (Kim and Joh 2006).

1.1. Parkinson's disease

PD is the second most common neurodegenerative disorder after AD. PD prevalence is age-associated, with approximately 1% of the population over 65 – 70 years of age, increasing to 4 – 5% at 85 years-old (Lee, Tran et al. 2009). In 90 – 95 % cases, PD occurs in an idiopathic manner, whilst in the remaining 5 – 10 % of cases, a genetic mutation is present (Toulouse and Sullivan 2008). PD primarily affect areas of the brain involved in motor control, and initially manifests clinically as a resting tremor, slowed and reduced amplitude of movement, bradykinesia, absence of normal unconscious movements, postural instability and muscle rigidity (Wolters 2008; Tansey and Goldberg 2009). During the progression of the disease, non-motor areas of the brain become affected (Fahn 2003; Dickson, Fujishiro et al. 2009).

PD is characterized by the degeneration of dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) in the midbrain, and loss of their ascending projections to the striatum (caudate – putamen). This decrease in dopaminergic tone leads to the loss of control of voluntary movements. By the time a patient has been diagnosed with PD, approximately 80% of striatal dopamine has been lost and the disease is quite advanced. Although the loss of dopaminergic neurons within the SNpc is the primary pathological feature of PD, widespread neuronal loss also occurs in the locus coeruleus, the dorsal motor nucleus of the vagus and glossopharyngeal nerves, the nucleus basalis of Meynert, and in later stages, neuronal loss occurs also in the neocortex (Braak, Del Tredici et al. 2003). However, the loss of dopaminergic neurons in the SNpc is most acute and is responsible for the majority of the clinical manifestations of the disease. It is of note that the lateral SNpc shows more vulnerability than the medial part (Fearnley and Lees 1991), possibly due to differential messenger RNA (mRNA) profiles in cell death-related genes, mitochondrial complex I genes, glutathione genes and pro-inflammatory cytokine genes, amongst others (Duke, Moran et al. 2007).

Post-mortem PD brain, is characterized by the presence of abundant round eosinophilic insoluble cytoplasmic inclusions called Lewy bodies (LBs). These protein aggregates accumulate in neurons and lead to neurotoxicity and loss of

dopaminergic neurons (figure 1) (Koo, Lee et al. 2008; Mori, Tanji et al. 2008). LBs in PD patients have been shown to contain α-synuclein and ubiquitinated proteins (Lees, Hardy et al. 2009) as well as several other proteins. PD is also characterized by the presence of an accumulation of activated microglia within the SNpc (McGeer, Itagaki et al. 1988). However, the exact reasons for the neurodegeneration and specific cellular manifestations of sporadic PD are unknown.

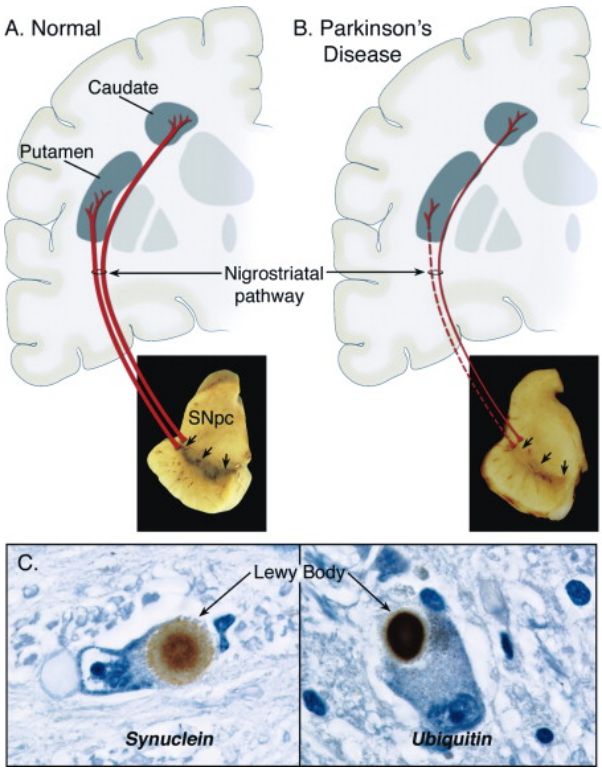

Figure 1: Neuropathology of PD. Schematic representation of the normal (A) and diseased (B) nigrostriatal pathway. Photograph shows the depigmentation of the SNpc due to dopaminergic neurons degeneration. Immunohistochemistry of LBs in SNpc dopaminergic neurons (C) (Dauer and Przedborski 2003).

1.2. The etiology of PD

For the vast majority of PD cases, the etiology remains unknown even though both genetic and environmental factors are likely to be implicated (Vance, Ali et al. 2010). The environmental hypothesis was strongly suggested about 20 years ago after the report of a parkinsonian syndrome in young adults that were intoxicated by a neurotoxin called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

(MPTP) which selectively destroys nigrostriatal dopaminergic neurons (Broussolle and Thobois 2002). Exposure of mice to environmental chemicals such as MPTP and rotenone in mouse produces the symptoms akin to PD and therefore these neurotoxins are commonly used in experimental studies of PD. Another environmental toxin, paraquat (a commonly used herbicide) has also been implicated in the onset of PD (Abdulwahid Arif and Ahmad Khan 2010). The neurotoxicity of these chemicals is accompanied by the blockade of electron flow from NADH dehydrogenase to coenzyme Q. The agents with the ability to improve mitochondrial respiration and ATP production have been shown to exert beneficial effects in PD patients as well as in the animal models of PD (Abdulwahid Arif and Ahmad Khan 2010).

Comparatively, the genetic hypothesis of Parkinson's disease has gained considerable interest during the last decade (Broussolle and Thobois 2002). In rare genetic forms of PD derive in mutations in the genes encoding α-synuclein, leucine-rich repeat kinase 2, Parkin, PTEN-induced putative kinase 1 and DJ-1 (Tsuji 2010). Genetic studies have identified mutations in α-synuclein and ubiquitin C-terminal hydroxylase L1 as rare causes of autosomal dominant PD and mutations in parkin as a cause of autosomal recessive PD. Functional characterization of the identified disease genes implicates the ubiquitin-mediated protein degradation pathway in the hereditary forms of PD and also in the more common sporadic forms of PD (Kruger, Eberhardt et al. 2002).

To date, the most effective treatment for PD remains the administration of a precursor of dopamine, L-dopa, which, by replenishing the brain in dopamine, alleviates almost all PD symptoms. The chronic administration of L-dopa, however, often causes motor and psychiatric side effects which may be as debilitating as PD itself. Moreover, as of yet there is no evidence that L-dopa therapy can impede the neurodegenerative process in PD (Teismann, Tieu et al. 2003).

2. Microglia

The term “microglia” refers to cells that reside within the parenchyma of the nervous system, that share many if not all the properties of macrophages in other tissues, but that in their non-activated or resting state (figure 2A) have a characteristic “ramified” morphology not seen in resident macrophages of other organ system (Rock, Gekker et al. 2004). Microglia are derived from myeloid cells in the periphery and are resident macrophages-like cells in the CNS. Constitute about 12% of all glial cells (Zhang, Hu et al. 2010) and distribute throughout the normal brain whit their ramified processes being close together but not touching each other (McGeer and McGeer 2008). Microglia has been described as distinct cellular population with specific morphologic characteristic. These cells have short size and a soma that does not exceed 5 – 10 µm (Kurpius, Wilson et al. 2006). Microglial cells are characterized by the presence of numerous filipodia and pseudopodia surrounding the cell surface, and well-developed dense bodies and vacuoles in the cytoplasm, suggestive of their active phagocytotic activity (Kim and de Vellis 2005). In the mature brain, microglia typically exist in a resting state and express of cell surface antigens, such as CD14, major histocompatibility complex molecules, chemokine receptors, and several other markers (Zhang, Hu et al. 2010).

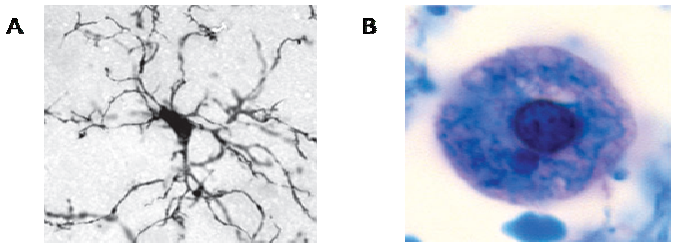

Figure 2: Comparison of microglia in resting state (A) and active state (B) (Ransohoff and Perry 2009).

As a response to alterations in the environment, particularly neuronal damage or presence pathogenic agents, microglial cells exhibit marked morphological changes, proliferate, become phagocytic, and upregulate the expression of a large number of molecules such as cytokines, adhesion molecules, membrane receptors, and transcription factors. Microglial cells are readily active andundergo a dramatic transformation from their resting state into an amoeboid

(round, oval) morphology (figure 2B) (Zhang, Hu et al. 2010). This process, called microglial activation, is a physiological response aimed at protecting the affected neural tissue (Saura, Tusell et al. 2003). However, due to their capacity to produce highly neurotoxic species, chronically activated microglial cells may participate in the pathogenesis of neurodegenerative disorders such as PD or AD (Saura, Tusell et al. 2003). Accumulated evidence suggests that microglial cells are associated not only with brain pathology but also with the normal physiology in the brain (Nakajima and Kohsaka 1993).

2.1. Microglial Activation

Microglia is exquisitely sensitive to disturbance of their microenvironment. The early and rapid response of microglia is entirely consistent whit the role of tissue macrophages as the first line of defense against infection or injury (Ransohoff and Perry 2009). The substances freed from injured cells of the CNS set in motion the microglial activation, and induce the changes in gene expression and reorganization of cellular phenotype (Kreutzberg 1996; Streit 2002). The cells of microglia detect the changes in its environment through the expression of a great number of cellular surface receptors and nuclear receptors that play a critical role in the initiation and/or modulation of its immunitary responses. These receptors recognize factors such as the complement receptor 3 (CR3), immunoglobulin's, molecules of cellular adhesion, steroids, bacterial products, *misfolded* proteins and cytokines (Hanisch 2002; Moller 2002; van Rossum and Hanisch 2004).

One of the most commonly used methods of activating microglia both *in vitro* and *in vivo* is the application of the endotoxin lipopolysaccharide (LPS). LPS is bacterial endotoxin derived from Gram – negative bacteria and was the first agent to be described in the literature as activator of microglial cells (Hetier, Ayala et al. 1988; Kim, Mohney et al. 2000). Microglia respondes actively to the LPS with the consequent release of a great variety of cytokines, NO and proteases. These effects are mediated by the toll-like receptor (TLR) 4 that has been related with the detection of mycobacterials infections (Takeda, Kaisho et al. 2003). LPS-induced activation of microglia results in the production of

cytokines and chemokines such as IL-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-10, IL-12, IL-18, transforming growth factor-β (TGF-β) and TNF-α by microglia. These cytokines, in turn, potentiate microglial activation by binding to receptoras expressed by microglia (Kim and de Vellis 2005). It was demonstrated that injection a single dose of LPS into the nigrostriatal pathway induces a strong macrophage/microglial reaction that leads to degeneration of dopaminergic neurons in the SN (Herrera, Castano et al. 2000).

One other inflammatory agent that induces selective microglia activation is Zymosan A (ZyA), a substance derived from the cellular wall the *Saccharomyces cerevisiae* (Fitzpatrick, Haynes et al. 1964). ZyA is capable of stimulating microglial cells through the CD11b of CR3 (also termed Mac1). CR3 is a member of the β2 family of integrins expressed in plasma membranes of mammalian phagocytes and natural killer cells. It is a heterodimeric type I transmembrane glycoprotein, consisting of a CD11b α chain noncovalently associated with the CD18 β subunit (Le Cabec, Cols et al. 2000). The ZyA, when injected in animals, provokes inflammation by inducing of the release of an ample gamma of inflammatory mediators. These include active components of complement system (Pillemer and Ecker 1941), prostaglandins and leukotrienes (Humes, Sadowski et al. 1982), platelet-activating factor (Roubin, Mencia-Huerta et al. 1982), oxidative metabolism (Nauseef, Root et al. 1983), and lysosomal acid hydrolases (Bonney, Wightman et al. 1978). Since ZyA is not degradable, phagocytosis results in a prolonged inflammatory response (Volman, Hendriks et al. 2005).

Mis-folded or aberrant proteins such as amyloid-β (Aβ) are capable of activating microglia via scavenger receptors, which are up-regulated in the brain of AD patients, or by initiating the phagocytosis of the pathological protein by microglia (Rogers, Strohmeyer et al. 2002). It has also been shown that microglial phagocytosis occurs in response to aggregated α-synuclein, the major component of LBs in PD (Zhang, Wang et al. 2005).

The serum factors thrombin and immunoglobulins initiate microglial activation through protease-activated receptor 1 and Fc receptors, respectively (Stangel and Compston 2001; Suo, Wu et al. 2002). ATP released from damaged

neurons, has also been demonstrated to activate microglia (Davalos, Grutzendler et al. 2005) by binding to purinergic receptors, expressed by microglia.

2.2. Consequences of microglial activation

Activated microglia has been shown to play key roles in both the developing and adult CNS. Upon activation in the adult CNS, microglia act primarily as scavenger and in brain tissue remodeling to restore and protect brain structures and functions (Nimmerjahn, Kirchhoff et al. 2005).

Local microglia extend out their processes to surround the area of insult (Davalos, Grutzendler et al. 2005) and as a result damaged cells are engulfed by the microglia via phagocytosis, removing any potentially damaging material from the area and protecting the neighbouring cells. Microglial cells share with other cells of the myeloid lineage the ability to secrete a multitude of immunomodulatory molecules such as cytokines, chemokines, neurotrophins and reactive oxygen and nitrogen species that mediate the communication with surrounding cells (Garden and Moller 2006). Cytokines are low molecular-weight proteins that are usually classified as either pro- or anti-inflammatory and are thought to signal both by paracrine and autocrine ways. While pro-inflammatory cytokines have the ability to elicit a sustained immune response, anti-inflammatory cytokines act to down-regulate an immune response by binding to appropriate receptors expressed on microglia and initiating an autocrine signalling process. Cytokines have numerous effects on CNS function including growth promotion, inhibition and proliferation of astrocytes and oligodendrocytes (Hanisch 2002), modulation of neurotransmitter release (Zalcman, Green-Johnson et al. 1994), and behavioral alterations such as memory impairment (Yirmiya, Winocur et al. 2002). Chemokines act primarily as chemoattractants to draw additional microglia to the site of injury, while NTF such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) released from microglia have been proposed to participate in the survival and regeneration of neurons (Nagata, Takei et al. 1993; Batchelor, Porritt et al. 2002) and to prolong the existence of

microglia and to regulate their function (Elkabes, DiCicco-Bloom et al. 1996). Activated microglia can also produce and release both reactive oxygen and nitrogen species due to catalysis by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Babior 1999) and these highly reactive free radicals can kill surrounding pathogens. It has also been reported however, that microglial-derived free radicals can cause neuronal cell death and so they have been implicated in the pathogenesis of neurodegenerative conditions (Chao, Hu et al. 1992).

3. Astrocytes

Astrocytes, also known as astroglia, are the most abundant cells in the CNS constituting 20 – 50% of the cerebral volume (Zhang, Hu et al. 2010). They are characterized by its prolongations and great structural complexity. These cells are also characterized by a dense matrix of ramifications, some of which assists in the formation of the blood brain barrier by contacting and involving local vascular walls (Kim, Kim et al. 2006; Koehler, Gebremedhin et al. 2006). Astrocytes also contribute to brain homeostasis in several ways, including buffering of extracellular K^+, regulating neurotransmitter release, releasing growth factors, and regulating the brain immune response (Gee and Keller 2005). Astrocytes also play a role in capturing through active uptake and neurotransmitters released during synaptic transmission (Nedergaard, Ransom et al. 2003). Some evidence suggest that these cells still execute functions such as neuronal differentiation, regulation of the axonal orientation, formation of synapses, cerebral plasticity and communication (Mosley, Benner et al. 2006).

As already it was mentioned, astrocytes are dynamic cells that keep homeostasis in the undamaged SNC. These cells express numerous receptors that allow them to be responsive to neurotransmitters and growth factors, small molecules and toxins. In fact, when astrocytes perceive disturbances of brain homeostasis, its metabolic activity increases, as well as the production of trophics factors, which attributs them a bigger capacity to protect other cells in

the brain from energy depletion, and from na overload of toxic free radicals (Mosley, Benner et al. 2006).

The characteristics that are more frequently associated with the astrocytic response to insults involve cellular hypertrophy, astrocyte proliferation, process extension and interdigitation, and increased production of the intermediate filaments glial fibrillary acidic protein (GFAP), vimentin and nestin. The "reactive" astrocytes may also exacerbate tissue damage as they release pro-inflammatory cytokines such as TNF-α, arachidonic acid metabolites, NO and reactive oxygen species (ROS) that can adversely affect cell survival (Liberto, Albrecht et al. 2004). Depending on the disease context, astrogliosis can be viewed as a beneficial event for promotion of neuronal survival by the production of growth factors and neurotrophins that support neuronal growth, or detrimental for neuronal functions by the formation of glial scars (Dong and Benveniste 2001). The function of reactive astrocytes is not well understood, and both harmful and beneficial activities have been attributed to these cells (Sofroniew 2005).

3.1. Factors release by astrocytes

Astrocytes are responsible for the production of cytokines and chemokines, however this expression is scarce in the normal CNS. Aberrant expression of cytokines and chemokines occurs in CNS diseases such as AD, MS, PD and brain injury/trauma (Dong and Benveniste 2001). Both *in vitro* and *in vivo* studies have documented the ability of astrocytes to produced interleukins, interferons, colony-stimulating factors and chemokines (table 1) (Dong and Benveniste 2001).

Table 1: Cytokines/ckemokines production by astrocytes (Dong and Benveniste 2001).

Cytokines	Chemokines
IL-1α and β, IL-5, IL-6, IL-10	RANTES
IFN-α and β	IL-8
TNF-α	MCP-1

TGF-β	IP-10
G-CSF, GM-CSF, M-CSF	

Cytokines and chemokines have been implicated in the initiation, propagation and regulation of immune and inflammatory responses by astrocytes (Benveniste 1998).

Astrocytes also secrete various soluble factors known as neurotrophic factors. These molecules have pleotropic effect on neurons; being involved in the survival, maturation, differentiation and development of neuronal cells. Accumulating evidences suggests that neurotrophic factors play a role in neurodegeneration as well (Siegel and Chauhan 2000). Neurotrophic factors have been categorized into various families such as NGF superfamily, GDNF family (distantly related to the TGF-β-superfamily), neurokine superfamily and non-neural growth factor superfamily (table 2).

Table 2: Neurotrophic factors and this family (Siegel and Chauhan 2000).

NGF-superfamily	Nerve growth factor
	Brain-derived neurotrophic factor
	Neurotrophin (NT)-3, NT-4/5 and NT-6
TGF-β superfamily	Glial cell line-derived neurotrophic factor
	Neurturin
	Persephin
	Artemin
Neurokine superfamily	Ciliary neurotrophic factor
	Leukemia inhibitory factor
	Interleukine-6
	Cardiotrophin-1
	Oncostatin-M
Non-neuronal growth factors	Fibroblast growth factor (FGF) -1 and FGF-2
	Epidermal growth factor
	Insulin-like growth factor
	Bone morphogenetic protein

3.1.1. The GDNF

GDNF was originally identified and purified from media conditioned by the B 49 glioma cell line and was characterized by its ability to promote the survival and differentiation of dopaminergic neurons during development (Lin, Zhang et al. 1994). GDNF is a distantly related member of the TGF-β superfamily that is glycosylated, the disulfide-bond homodimer, has a molecular weight 33 – 45 kDa, while the monomer has a molecular weight of 16 kDa after deglycosylation (Lin, Doherty et al. 1993; Lin, Zhang et al. 1994). The actions of GDNF are mediated by the activation of the GDNF family receptor (GFR) alpha, a multi-component receptor complex comprising the transmembrane RET tyrosine kinase. Four members of this family have been identified (GFRα1 – 4) with GDNF binding preferentially to GFRα1. It is believed that the GDNF dimer forms a complex with GFRα and that this complex induces the dimerization of RET (figure 2) (Sariola and Saarma 2003; Enomoto 2005). GDNF-activated RET could also induce different biological responses such as morphological transformation, proliferation, cell migration, neurite elongation and neurite branching. In cells lacking the RET receptor, alternative mechanisms which involve neuronal cells adhesion molecules were suggested for GDNF-mediated activities (Sariola and Saarma 2003; Donatello, Fiorino et al. 2007).

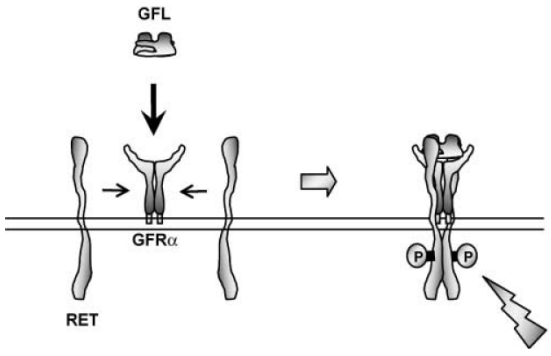

Figure 2: Glial cell line-derived neurotrophic factor (GDNF) family ligand (GFL)-induced activation of the RET receptor tyrosine kinase. A GFL dimer binds to the GDNF family receptor (GFR) α to form a (GFL) $(GFR\alpha)_2$ complex, which then induces dimerization and successive phosphorylation of the RET tyrosine kinase (Jing, Wen et al. 1996).

The GDNF is present in many different regions of the developing nervous system, and this localization depends on the local activity and the stadium of

development of the individual. Detectable levels of GDNF were observed in the thalamus, hippocampus, cerebellum, cortex, spinal marrow and SN, among others local of the nervous system (Choi-Lundberg and Bohn 1995; Del Fiacco, Quartu et al. 2002).

Studies in primary cultures of midbrain ventral showed that GDNF increases the number tyrosine hydroxylase (TH) positive cells and its morphologic complexity and functional and neuronal maturation (Schaller, Andres et al. 2005). In adult rats, GDNF mRNA levels are significantly higher in the striatum than in the SN suggesting a neuronal maintenance role for GDNF in the adult nervous system, mainly as a target-derived NTF for dopaminergic neurons (Choi-Lundberg and Bohn 1995; Oo, Ries et al. 2005). Several studies strongly suggest that GDNF is an essential player in the development of the dopaminergic nigrostriatal system, and that even slight disturbances in the system maturation due to GDNF deficits might compromise dopaminergic survival in the adulthood (Saavedra, Baltazar et al. 2007). GDNF was been considered the more powerful neurotrophic factor in protecting dopaminergic neurons from the degeneration induced by the toxins MPTP or 6-hydroxydopamine (6-OHDA) in animal models of the PD (Rosenblad, Martinez-Serrano et al. 1998).

Although the neurotrophic effect of the GDNF is well documented, its effect on microglial cells remains is less clear. Nevertheless, there are some results indicating that GDNF is able to regulate microglia activity. In the study development in primary rat microglia, Chang and its collaborators showed that GDNF increased the NO production, the activity of superoxide dismutase (SOD) and the phagocytotic capability but had no effect on the secretion of the pro-inflammatory cytokines TNF-α and IL-1β (Chang, Fang et al. 2006).

4. Microglia-astrocytes interactions

The presence of oxidative stress and inflammatory activity is one of the significant pathological features of PD (Kim and Joh 2006). The presence of inflammation is generally indicated by the accumulation of activated microglia in damaged areas of the brain. Particularly high numbers of activated microglia have been found in post-mortem brains of PD patients, predominantly in the SNpc in the vicinity of the degenerating dopaminergic neurons (Long-Smith, Sullivan et al. 2009). Kim and collaborates revealed that the SN had the highest brain density in microglia, thus making post-mortem mesencephalic neurons more sensitive to neurodegeneration-mediated inflammation (Kim, Mohney et al. 2000). The pivotal role of activated microglia-mediated dopaminergic neuronal degeneration has been demonstrated in the rotenone model of PD (Gao, Hong et al. 2002). Wu *et al.,* (2002) and in studies showing that inhibition of microglial activation prevents dopaminergic neuronal loss in MPTP-treated mice (Wu, Jackson-Lewis et al. 2002). Activated microglia has also been implicated in the pathogenesis and progress of PD.

Astrocytes, the most abundant cells in the brain, may have a role in controlling microglial over-activation (Yang, Min et al. 2007). Astrocytes and microglia are the brain representatives of the general immune system, and can, under pathological conditions, act as immune competent cells. Upon activation, the reactive glial cells gain a number of potentially neurotoxic potencies, e.g., via release of inflammation-promoting mediators and oxidative radicals. As long as these factors remain under strict control, reactive glial cells can be seen as having an undoubtedly beneficial role in defense and repair. However, an escalating pathological glial activation which involves both microglial and astrocytes may contribute to secondary neuronal damage (Markiewicz and Lukomska 2006).

The interactions that occur between astrocytes and microglia after a CNS damages are complex and wide unexplored. However, it is known that cytokines and growth factors are important mediators of the glial communication. The discovery of the molecular mechanisms responsible for the microglia-astrocytes interactions has been difficult to attain through the use of of

in vivo models. Therefore, the majority of the experimental data has been generated for studies *in vitro*. It was already demonstrated that microglial morphology is influenced by astrocytes that play an important role in regulating differentiation and microglial down-activation (Streit, Walter et al. 1999). Some authors had described the role of astrocytes in the process microglia ramification, although the process of astrocyte signaling is still controversial (Suzumura, Sawada et al. 1990; Eder, Klee et al. 1997; Eder, Schilling et al. 1999; Liu, Brosnan et al. 1994; Tanaka and Maeda 1996; Tanaka, Toku et al. 1999). Data from literature show that of rat microglial cells modify its morphology from amoeboid to ramified, when cultivated on a top of an astrocytes monolayer (Liu, Brosnan et al. 1994; Tanaka and Maeda 1996). However, they did not detect any alteration in microglial morphology when cells were exposed to astrocytes conditioned media (ACM). Recently, it was reported by Min and its collaborators that soluble factors freed by astrocytes are capable to suppress interferon (IFN)-γ-induced microglial inflammatory responses through the expression of hemeoxygenase-1 (HO-1). These results suggest that astrocytes could cooperate with microglia to prevent excessive inflammatory responses in the brain (Min, Yang et al. 2006).

5. Aims of this thesis

The microglial activation is mainly intended for the neuronal protection. However, in several neuropathologies, associated with chronic inflammation, the inflammatory products derived from the activated microglia can also contribute for the neuronal loss. Since astrocytes have been suggested as main regulators of microglial activation, a deeper knowledge of the possible factors freed for these cells that prevent the change of microglia from an anti- to a pro-inflammatory state, can make possible new approaches in the study of the astrocytes-microglia interactions and in the control of the neuroinflammation in pathological situations.

The aim of this study was to characterize the effect of soluble mediators released by astrocytes in controlling microglia activation induced by the pro-inflammatory agent Zymosan A and to identify the molecules responsible for this mechanism.

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Chapter II

ASTROCYTE-DERIVED GDNF IS A POTENT INHIBITOR OF MICROGLIAL ACTIVATION

Keywords: microglia, astrocytes, neuroinflammation, Parkinson's disease.

1. Abstract

Neuroinflammation is recognized as a major factor in Parkinson's disease pathogenesis and increasing evidence suggest that microglia is the main source of inflammation contributing to dopaminergic degeneration. Several indications suggest that astrocytes could act as physiological regulators preventing excessive microglia responses. However, little is known regarding how astrocytes modulate microglia activation. In the present study we shown that astrocytes secrete factors capable of modulating microglial activation by regulating the phagocytic activity and microglial levels of reactive oxygen species induced by Zymosan A. Both parameters were highly diminished in cells incubated, for 24 h, with astrocytes conditioned media (ACM). It is described that glial cell line-derived neurotrophic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF) and brain-derived neurotrophic factor (BDNF) may play a neuroprotective role in the nigrostriatal system. It will be that these neurotrophic factors are among the astrocyte-secreted molecules involved in microglial protection against its activation. Protection provided by ACM against Zymosan A induced microglia activation was abolished when the GDNF present in the ACM was abrogated using an antibody. This effect was not observed when ACM was neutralized with anti-CDNF, anti-BDNF or with a GDNF antibody previously inactivated by heat. In addition media conditioned by astrocytes silenciated for GDNF was not able to prevent microglial activation, whereas supplementation of non-conditioned media with GDNF was able to prevent the activation of microglia evoked by Zymosan A. Taken together the results strongly suggest that astrocyte-derived GDNF has a major contribution to the control of midbrain microglia activation. This effect can have a major contribution to inhibit neuroinflammation, and thus protect from neurodegeneration.

2. Introduction

The presence of inflammation is generally indicated by the accumulation of activated microglia in damaged areas of the brain. Particularly high numbers of activated microglia have been found in the brains of post-mortem PD patients, predominantly in the *substantia nigra pars compact* (SNpc) in the vicinity of the degenerating dopaminergic neurons (Long-Smith, Sullivan et al. 2009). Kim and collaborators revealed that the high density of microglia in the SN, makes mesencephalic neurons more vulnerable to inflammation-induced neurodegeneration (Kim, Mohney et al. 2000). The presence of oxidative stress and inflammatory activity is one of the significant pathological features of PD (Kim and Joh 2006). Astrocytes, the most abundant cells in the brain, may act in preventing microglial over-activation (Yang, Min et al. 2007). However, the interactions that occur between astrocytes and microglia after neuronal damages are complex and wide unexplored. It is known, that cytokines and growth factors are important mediators of the glial communication. The understanding of the molecular mechanisms responsible for the microglia-astrocytes-interactions has been difficult to achieve. However, it was demonstrated that astrocytes play an important role in the regulation of microglia morphology, differentiation, and state of activation (Streit, Walter et.al. 1999). Moreover, it was recently reported by Min and collaborators that soluble factors freed by astrocytes are capable of suppressing interferon (IFN)-γ-induced microglial inflammatory responses through the expression of hemeoxygenase-1 (HO-1). These results suggest that astrocytes could cooperate with microglia to prevent excessive inflammatory responses in the brain (Min, Yang et al. 2006). The microglial activation is mainly directioned for the neuronal protection, making part of regenerative process. However, in several neuropathologies, associated with chronic inflammation, the products derived from the activated microglia can strongly contribute for the neuronal loss. Thus, having in account that astrocytes are main regulators of microglial activation, one great knowledge of the possible anti-inflammatory molecules released by these cells to prevent the microglia in a pro-inflammatory state, can

possible new approaches in the study of the astrocytes-microglia-interactions and in the control of the neuroinflammation in pathological situations.

Thus, the aim of this study was to evaluate the role of soluble mediators released by astrocytes on the control of microglia activation status and to identify the mediators involved in this effect.

3. Materials and Methods

3.1Microglial cell cultures: Animals were handled in accordance with the national ethical requirements for animal research, and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Postnatal ventral midbrain glia cultures were prepared based on previously reported protocols (McCarthy and de Vellis 1980; Saura, Tusell et al. 2003; Culbert, Skaper et al. 2006). Briefly, the ventral midbrain of postnatal day 4 Wistar rat pups was dissected, carefully stripped of the meninges, and put in iced phosphate buffer saline (PBS: NaCl 140 mM, KCl 2,7 mM, KH_2PO_4 1,5 mM and Na_2HPO_4 8,1 mM, pH 7,4). The tissue was then mechanically dissociated with a 5 ml pipette, followed by 5-10 sequential passes through 20, 22 and 25 gauge needles. Finally, the cells were passed through a 70 µm mesh, pelleted by centrifugation (3K18C Bioblock Scientific; Sigma Laboratory Centrifuges), and suspended in high glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies) with 10% Fetal Bovine Serum (FBS, Biochrom AG), and 100 units/ml penicillin plus 100 µg/ml streptomycin (Sigma), and plated into 175 cm^2 poly-D-lysine (Sigma)-coated culture flasks (BD Falcon) at a density of 2 x 10^6 cells/cm^2. The cultures were kept at 37 ºC in a 5% CO_2, 95% air atmosphere. The medium was changed every 4 days. On day 12, culture plates were shacked at 160 rpm (AGITORB200, Aralab) during 4 hours to detach microglial cells to the supernatant, leaving astrocytes in the adherent monolayer. The supernatant was collected and centrifuged for 10 min at 1200 rpm (3K18C Bioblock Scientific; Sigma Laboratory Centrifuges). The pellet was then ressuspended and microglial cells plated into 1,1cm^2 or 0,9 cm^2 poly-D-lysine-coated

coverslips and kept at 37 ºC in a 5% CO_2, 95% air atmosphere for 13 day. Culture medium was replaced every 4 days.

***3.2 Preparation of ACM*:** Astrocytes were grown to confluence. On reaching confluence culture medium was changed and after 24h the ACM was collected and store at -80ºC until use. The astrocytes were trypsinyzed and plated into 3,5 cm^2 culture well coated whit poly-D-lysine at a lower density (47,000 cells/cm^2).

3.3 ELISA technique: ELISA for GDNF was performed according to manufacturer's directions using the GDNF E_{max}® ImmunoAssay System (Promega). Briefly, 96 well plates were coated overnight with anti-GDNF mAb at 4ºC. Plates were washed, blocked with block & sample 1X buffer provided in the kit, for one hour at room temperature and incubated with 100 µL samples, in triplicate, for six hours at room temperature with shaking. After washing, plates were incubated with anti-Human GDNF pAb overnight at 4ºC, followed by a two hours incubation with anti-Chicken IgY, HRP conjugate. Substrate (TMB One Solution) was added for a final 15 minutes incubation and after added 1N hydrochloric acid for stop reaction. Absorbance measurements were taken at 450 nm for protein concentration determination, and 540 for background measurements using a SpectraMax M2 microplate reader (Leitor de ELISA). GDNF concentrations (pg/ml) were determined against a standard curve using manufacturer's instructions.

3.4 Transfection of siRNA in astrocytes: A sequence of sense and anti-sense oligonucleotides corresponding to the rat GDNF cDNA 5'-ACUGACUUGGGUUUGGGCUACGAAA-3' was used for GDNF silencing. When astrocytes achieved 30 – 40% of confluence the medium was replaced by DMEM without penicillin and streptomycin and 12 hours later the cells in each well were incubated with 50 nM RNAi sequence plus 0,006 mg of

Lipofectamin^{eTM} 2000 (Invitrogen) in opti-MEM (Gibco). Six hours after the medium was changed and cells were maintained in culture for further 36 hours. ACM was collected and store at -80ºC until use. The double stranded siRNA and BLOCKi^{TTM} Fluorescence Oligo were synthesized by Invitrogen (Carisbad, CA, USA).

3.5 Western blot analysis: To confirm that GDNF was silenced by the sequence used, GDNF protein levels were determined by Western blot analysis. Cells were lysed on ice using in a lysis buffer containing 25 mM Tris, 2.5 mM EDTA, 2.5 mM EGTA, 0.2 % Triton X-100, 1 mM DTT, 1 mM PMSF and 25.57 µM Leupeptin. The concentration of protein was determined using the Bradforf method and Bovine Serum Albumin (BSA) as standard (Biorad Protein Assay). Samples were desnaturated by adding sample buffer (100 mM Tris, 100 mM Glycine, 4 % SDS, 8 M Urea and 0,01 % Bromophenol blue) and heating at 100ºC, for 5 minutes. Proteins were separated by SDS-PAGE and transferred to a polyvinyldiene diflouride (PVDF) membrane (Amersham Life Sciences). The membrane was then blocked with 5 % non fat milk (Regilait, France) in TBS-T (0,1 % Tween 20 in a 20 mM Tris and 137 mM NaCl solution), for 1 hour, at room temperature. Incubation with anti-GDNF (1:1000; Santa Cruz Biotechnology) diluted in TBS-T was done overnight at 4ºC. After being rinsed tree times with TBS-T, the membrane was incubated for 1 hour, at room temperature, with an anti-rabbit antibody (1:20 000; Amersham Biosciences) diluted in TBS-T. The membrane was then incubated with the ECF substrate (ECF western Blotting Reagent Packs, Amersham) for 5 minutes. Protein bands were detected using the Molecular Imager FX system (Biorad) and quantified by densitometry analysis using the Quantity One software (Bio-Rad).

3.6 Total RNA extraction, cDNA synthesis and RT-PCR analysis: Total RNA from astrocytes was extracted in TRI reagent (Sigma) according to the manufacturer's instructions. To assess the quantity and integrity of total RNA,

its optical density was determined by spectrophotometry (Pharmacia Biotech, Ultrospec 3000, Denmark), and RNA extracts were analyzed by agarose gel electrophoresis. Total RNA (1 µg) was denatured for 5min at 65°C in a reaction containing 10 mM deoxynucleotide triphosphates (Amersham, Uppsala, Sweden) and 0.3 µM of random primers (Invitrogen, Karlsruhe LMA, Germany). Reverse transcription was carried out at 37°C for 60min in a 20 µl reaction containing reverse transcriptase buffer 5X, 0.1 M DTT and 200 U of M-MLV RT (Invitrogen). The reaction was stopped at 75°C for 15min. PCR reactions were carried out using 1 µl of cDNA synthesized from astrocytes in a 25 µl reaction containing 1X Taq DNA polymerase buffer (20mM Tris-HCl and 50mM KCl), 0.2 mM deoxynucleotide triphosphates (Amersham), 2 mM of $MgCl_2$ (Promega, Madinson, USA), 5 µM of each of forward and reverse primers (Table 1), and 2 units of Taq DNA polymerase (Promega). After an initial step at 95°C to desnaturation the cDNA, the cycling conditions used are described in the table 1. PCR fragments were analyzed in 1% agarose gel containing ethidium bromide. Each primer set specifically recognized only the gene of interest, as indicated by amplification single band of the expected size.

Table 1- Details of specific primers used for amplification of GDNF, CDNF and BDNF genes in astrocytes cDNA by conventional PCR.

Oligo name	Sequence (5'- 3')	Amplicon size (bp)	Cycle conditions
GDNF – fw	ATTCCTGGCGTTACCTTG	368	95ºC at 30' 61ºC at 30' 40 cycles 72ºC at 30'
GDNF – rv	CTGTATTCCGTCTCCTTGG		
CDNF – fw	GATTCTACAACTCCTTGCTAAC	63	95ºC at 30' 45ºC at 30' 35 cycles 72ºC at 30'
CDNF – rv	GCTCTTCCTCTATGGTATCG		
BDNF – fw	CGTGATCGAGGAGCTGTTGG	343	95ºC at 30' 58ºC at 30' 40 cycles 72ºC at 30'
BDNF – rv	CTGCTTCAGTTGGCCTTTCG		

3.7 Immunocytochemistry: After exposure to Zymosan A for 24 hours, microglial cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 1% Triton X-100 in PBS for 5 min. Blocking was performed by incubation with 20% FBS in PBS containing 0.1% Tween-20 for 60 minutes at room temperature. The cells were then incubated for 60 minutes at room temperature with the following primary antibodies, according to the aim of the experiment: rabbit anti-GDNF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CDNF (1:400; Sigma Aldrich), and rabbit anti-BDNF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, cells were incubated for 60 minutes with the secondary antibodies conjugated to anti-rabbit Alexa Fluor® 488 (1:1000; Molecular Probes). Cultures were observed on a Zeiss inverted microscope (Axiobserver Z1, Zeiss) using a 63x lens.

3.8 Stimulation of microglia: On culture day 13, microglial cells were exposed to DMEM (control) or ACM. After at 24 hours incubation Zymosan A at concentration of 5 µg/mL (Sigma) was added to the culture medium.

3.9 Phagocytosis assay: To evaluate the effect of ACM on the functional activity of primary microglia cultures, we studied the phagocytic activity of cells. Control cultures and cultures exposed to Zymosan A were incubated for 15 min with 0.01% fluorescent 1 µm polystyrene microspheres (Molecular Probes) and then washed with DMEM to remove non-engulfed microspheres. Finally, cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes. For quantification of phagocytic cells, more than 30 different fields per slide were analyzed on a Zeiss inverted microscope (Axiobserver Z1, Zeiss) under a 63x magnification.

3.10 Determination of cellular ROS levels: ROS levels were measured using the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). The DCFDA assay is based on the principle that DCFDA is oxidized by ROS and converted to fluorescent 2′,7′-dichlorofluorescein. Cells exposed for 5 hours

to Zymosan A were incubated with DCFDA (50 µM) in culture medium, for 1 hour, at 37 °C. The fluorescence emitted was read in a spectrofluorometer (FluoroMax®-4; Horiba) at 485/535 nm DCFDA.

3.11 Data analysis and statistics: Data are expressed as percentages of values obtained in control conditions or as percentages of the total number of cells, and are presented as mean ± S.E.M. of at least three independent experiments, performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by the Dunnett's test or Bonferroni's Multiple Comparison Test. Values of $P < 0.05$ were considered significant. All statistical procedures were performed using GraphPad Prism 5 Demo (GraphPad Sotware Inc., San Diego, CA).

4. Results

4.1 Effect of ACM on microglial activation induced by Zymosan A

In order to assess the effect of the ACM in the phagocytic activity of microglial cells induced by Zymosan A, microglial cells of rat ventral midbrain, were incubated with ACM or DMEM (control), and later treated with Zymosan A 5 µg/mL. After a 24 hours exposure to Zymosan A, the phagocytic activity (figure 1B) and ROS levels (figure 1C) were evaluated. Strong punctuated fluorescent cells (figure 1A) were considered phagocytic cells. Zymosan A treatment increases the number of phagocytic cells by 121% ± 26,753 and ROS levels by 67% ± 14,617 when compared with the control. Microglial cells pre-incubated with ACM for 24 hours before the Zymosan A treatment did not exhibit any increase in the ROS levels or the number of phagocytic cells. These results suggest that soluble factors release by astrocytes could regulate the microglial activation state.

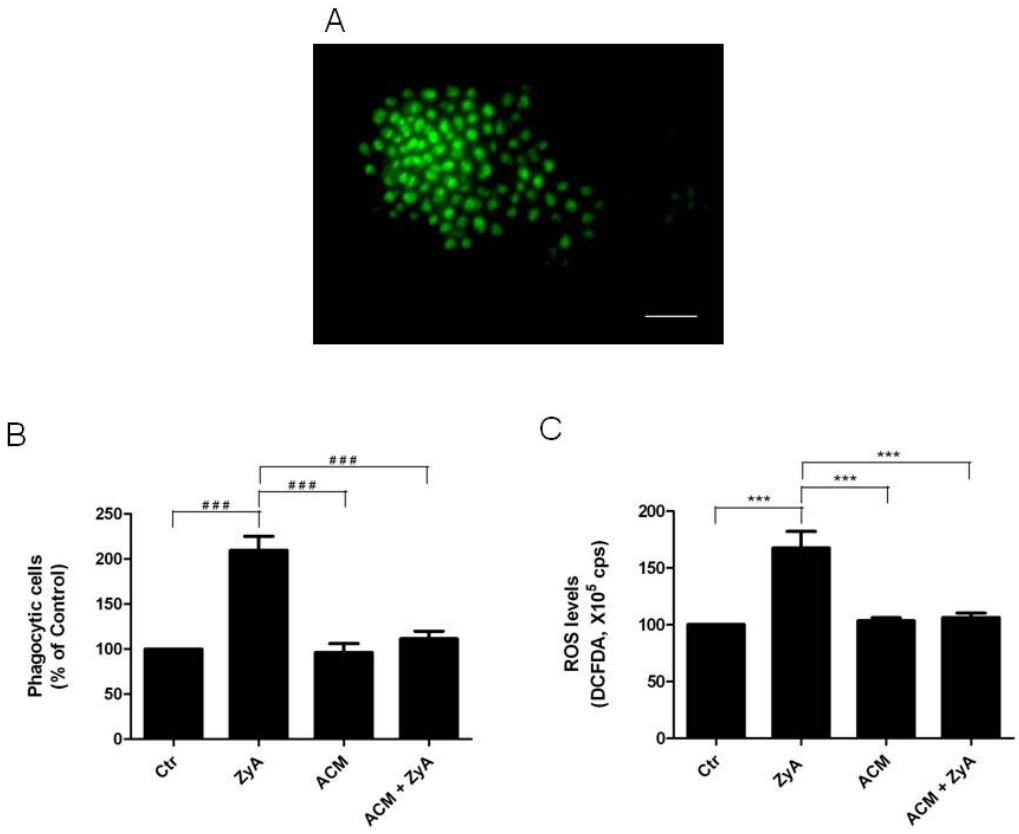


Figure 1: ***ACM suppresses microglia activation promoted by Zymosan A.*** **(A)** Fluorescence image of cultures exposed to fluorescent microspheres (magnification: X63). **(B)** Quantification of cells with punctuated fluorescence cells after a 24 hours incubation with Zymosan A **(C)** ROS levels produced by microglial cells exposed for 6 hours to Zymosan A were measured using DCFDA. Results are expressed as count per second (cps). The data represent the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{\#\#\#}$ p<0,0001 e *** p<0,0001).

4.2 GDNF, CDNF and BDNF are express by astrocytes

GDNF, CDNF and BDNF are three neurotrophic factors proposed as possible targets in PD therapies since they exert neuroprotective effects in the nigrostriatal pathway (Chang, Fang et al. 2006; Lindholm, Voutilainen et al. 2007; Nagatsu and Sawasa 2005). We started by assessing GDNF, CDNF and BDNF mRNA and protein levels. The results obtained showed that astrocytes expressed mRNA for GDNF, CDNF and BDNF whit specific bands of 368 pb, 63 pb and 343 pb, respectively (figure 2A). The presence of primers dimmer observed in figure 2A indicates that this procedure needs further optimization. Western blot results clearly showed that GDNF, CDNF and BDNF proteins are

expressed by astrocytes (figure 2B). Immunocitochemistry analysis further confirmed the presence of the neurotrophic factors and allowed us to observe that GDNF, CDNF and BDNF staining is uniformly distributed all over the cells (figure 2C and E, respectively) whereas CDNF staining was punctuated (figure 2D) After verifying the expression of GDNF, CDNF and BDNF by astrocytes we evaluate its effect on the activity of microglial.

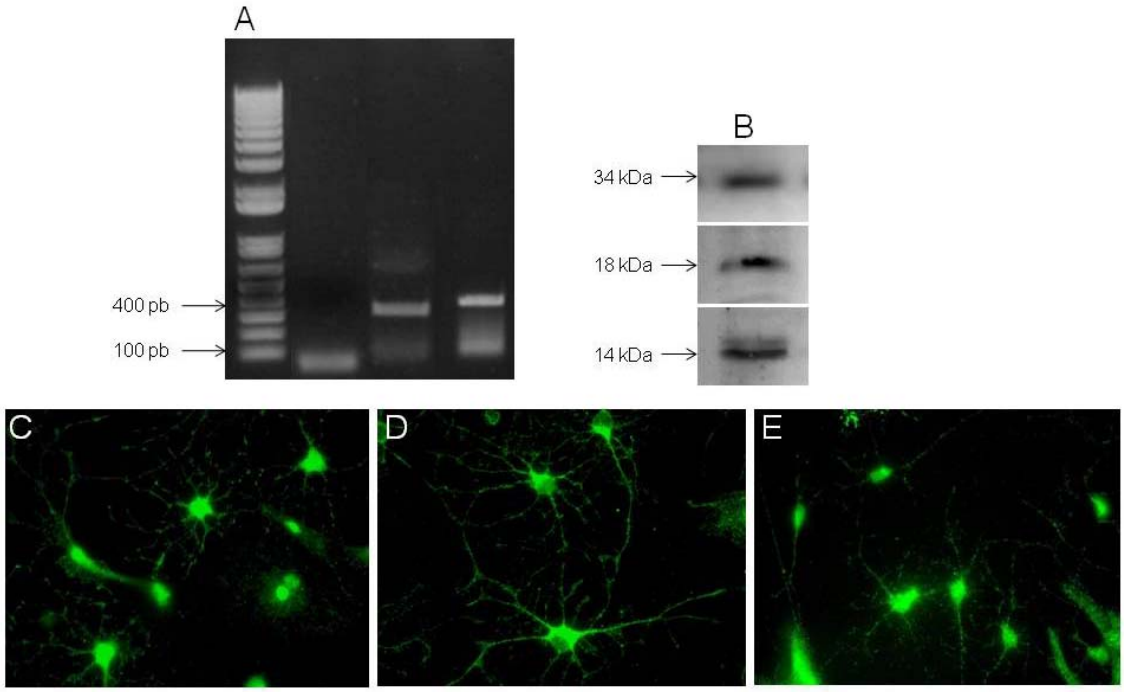


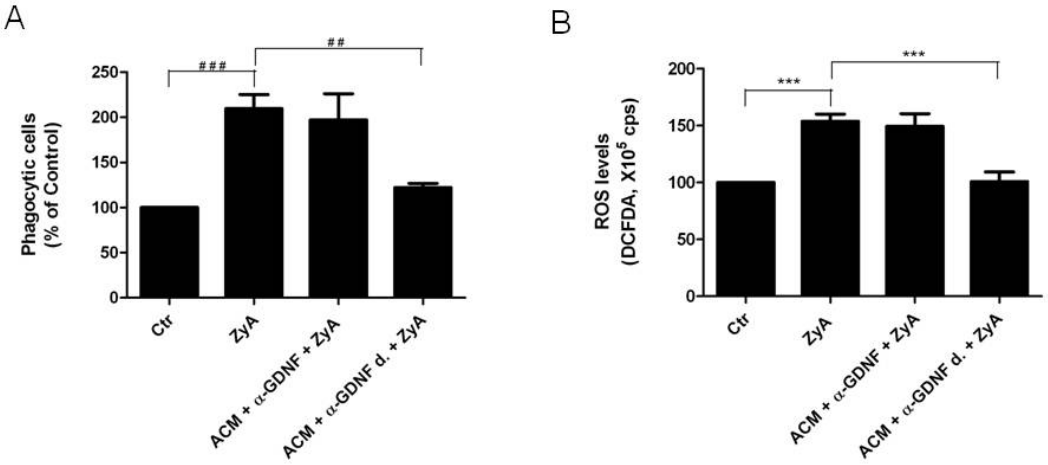
Figure 2: ***Expression of GDNF, CDNF and BDNF by astrocytes.*** Primary astrocytes was collect for analyze in RT-PCR, western blot and immunocytochemistry. **(A)** Product of RT-PCR for GDNF, CDNF and BDNF, **(B)** protein expression for GDNF, CDNF and BDNF, **(C) (D) (E)** images obtained using a Zeiss inverted microscope (Axiobserver Z1, Zeiss) whit marking for GDNF, CDNF and BDNF, respectively. Magnification: X63. Both results of RT-PCR and western blot were carried in three experiences independent. Data of immunocytochemistry were obtained of one experience.

4.3 Effect of neurotrophic factors in microglial activation

To determine if GDNF, CDNF or BDNF contributed to modulate microglial activation, the neurotrophic factors present in the ACM were blocked with specific antibodies before application of the media to microglia cultures. The results obtained showed that the blocked promoted an increase in Zymosan A

induced phagocytic activity by 108% ± 29,576 and of ROS levels by 53% ± 6,356 (figure 3A and B) , levels similar to the obtained in cells incubated with non-conditioned media and treated with Zymosan A (97% ± 28,900 increase in phagocytic cells and 49% ± 10,955 increase of ROS levels). Heat inactivation of the anti-GDNF antibody before being added to the ACM, with the aim of annulling GDNF binding, reverted the changes induced by the presence of the antibody (figure 3). In contrast with the results obtained when the GDNF present in the ACM was blockade with the specific antibody, the heat inactivation of antibodies against CDNF (figure 3C) or BDNF (figure 3D) did not affect the protection from Zymosan A induced microglial activation. The inhibition of microglial activation mediated by ACM in the presence of anti-BDNF or anti-CDNF is probably due to the presence of GDNF in the ACM.

To determine if the results observed in the presence of the anti-GDNF antibody were due to GDNF binding and not to a other unspecific effects we evaluated the effect of adding an antibody not related with microglial cells in the parameters of microglial activation. The presence of anti-MAP_2 antibody in the ACM did not any affect microglial activity induced by Zymosan A (figure 3E).



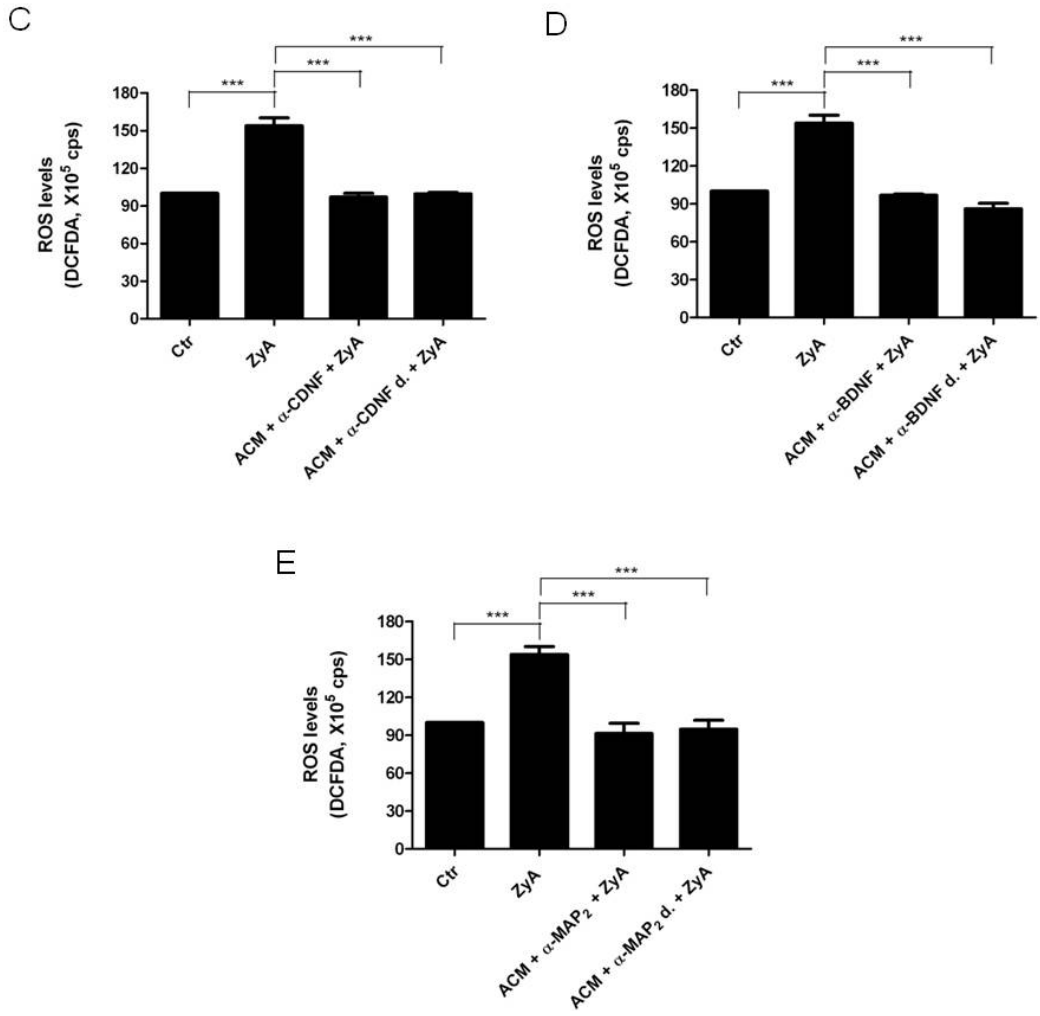


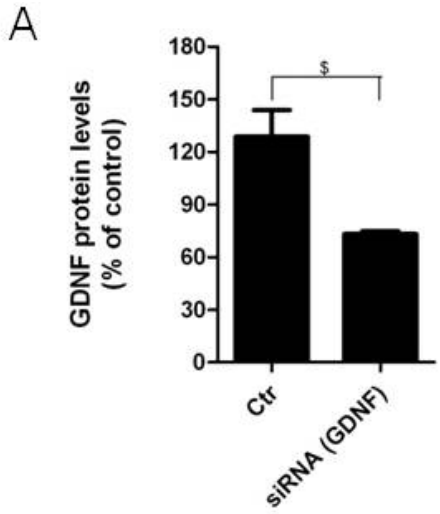
Figure 3: ***Contribution of neurotrophic factors present in the ACM to the control of microglial activity induced by Zymosan A.*** Microglial cells were pretreated for 24 hours with ACM previously incubated with anti-GDNF, anti-CDNF, anti-BDNF or anti-MAP_2, and then exposed to ZyA . **P**hagocytic activity induced by a 24 hour incubation with ZyA **(A)** and ROS levels induced by a 6 hours exposition to ZyA **(B)**. Effect of CDNF **(C)** and BDNF **(D)** in ROS levels induced by ZyA. Effect of the anti-MAP2 antibody in the control of microglial activity. Data shown represent the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{\#\#}$ p<0,0086 e *** p<0,001)

4.4 Conditioned media from astrocytes silenciated for GDNF was unable to prevent Zymosan A induced microglia activation

To further confirm the role of GDNF in controlling microglia activation this neurotrophic factor was down-regulated by siRNA. Previous, results from our group showed that the sequence used in this work was the most efficient, among three sequences tested, for silencing GDNF. The results presented in

figure 4 showed that in cells treated with media conditioned by astrocytes silenced for GDNF Zymosan A increase phagocytic activity by 89% ± 9,939 and ROS levels by 88% ± 12,2.These results suggest that the reduction of 56% in GDNF proteins levels (figure 4C) is critical for the control of microglial activation since the number of phagocytic cells and of ROS levels achieved in this situation were similar to the observed for Zymosan A in cells exposed to non-conditioned media (109% ± 15,709 in phagocytes and 76% ± 15,373 of ROS levels).

In a different approach we quantified the GDNF present in the ACM by ELISA. On average 200 pg/mL of GDNF was detected in samples from different ACM. Knowing the amount of GDNF in the ACM allowed us to determine if non-conditioned media supplemented with 100-400 pg/mL of GDNF was capable exerting the same protection from Zymosan A induced microglia activation as the ACM. Adding the exogenous GDNF to DMEM simulated the effect of ACM (Figure 5). No significant differences between the effect of the three different concentrations of GDNF added (100, 200 and 400 pg/mL) in phagocytic activity and reactive oxygen species levels produced by microglia suggesting that the lower concentration tested is sufficient to induce maximal effects.



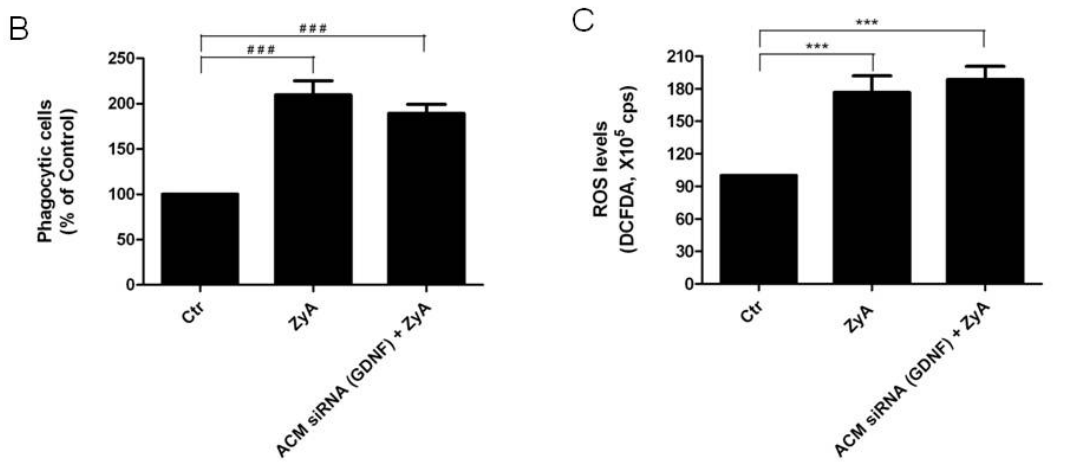


Figure 4: ***Effect of media conditioned by astrocytes silenced for GDNF in microglia activation induced by Zymosan A.*** Microglia was pretreated for 24 hours with media conditioned by astrocytes silenciated for GDNF. **(A)** GDNF protein levels determined by immunoblot analysis. **(B)** Phagocytic activity evaluated after a 24 hours incubation with Zymosan A and **(C)** ROS levels measured after an incubation of 6 hours with Zymosan A. The results are expressed as percentage of control. Data shown represent the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test and Unpaired t Test ($^{\#\#\#}$ p<0,0001; *** p<0,0001 and $^{\$}$ p<0,05).

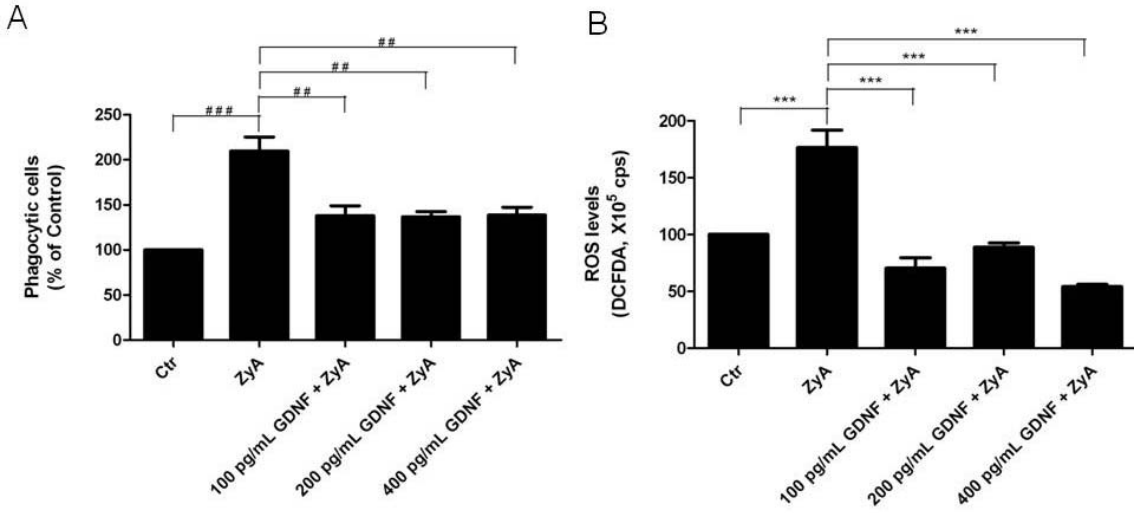


Figure 5: ***Effect of adding GDNF to the culture media in preventing ZyA induced microglial activation.*** Phagocytic activity **(A)** and ROS levels **(B)** in microglial cells pretreated for 24 hours with different concentrations of GDNF diluted in DMEM and then stimulated with Zymosan A . Data shown represent the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{\#\#\#}$ p<0,0001and *** p<0,0001).

4.5 Protection from Zymosan A induced microglia activation by ACM: Effect of blocking GFRα1 receptor

The results suggesting that astrocytes-derived GDNF modulates microglial activation, and knowing that GDNF binds preferentially to the GFRα1 receptor, lead us to evaluate the effect of blocking this receptor in microglial activity. Before adding the ACM microglial cells were pre-incubated for 15 minutes with antibody anti-GFRα1. The same antibody was added to the ACM during the 24h incubation. At the end of this incubation period we evaluated the phagocytic activity of microglia (figure 6). The number phagocytic cells remained similar to the observed in control cells exposed to Zymosan A (104% ± 10,912). This result indicated that the protection induced by the ACM involves the activity of the GFRα1 receptor, therefore preventing GDNF binding impedes the intracellular signaling cascade required to control microglial activation induced by Zymosan A.

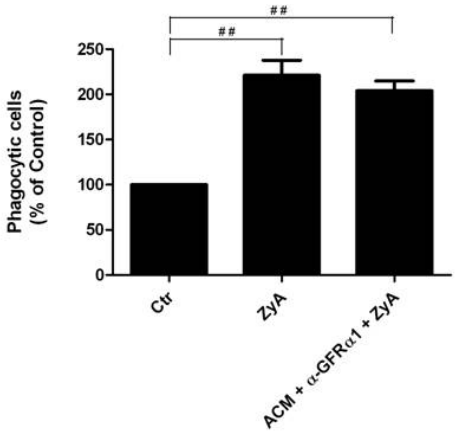


Figure 6: ***Blocked of GFRα1 receptor- effect in the control of ZyA induced microglia activation.*** Microglial cells pretreated with an anti- GFRα1 antibody were incubated with ACM, also in the presence of the same anti- GFRα1, and stimulated with ZyA for 24 hours for quantification of phagocytic cells. Data represent the mean ± SEM of one independent experiment performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{###}$ p<0,0001).

5. Discussion

Accumulating evidences indicate that brain inflammation could be a risk factor for the onset and progression of neurodegenerative diseases (Maes 1995; Gonzalez-Scarano and Baltuch 1999). Even though several studies have proved brain inflammation using, separately microglia and astrocytes cultures, it is well accepted that neuroinflammation results from a complex set of integrated signaling process occurring within all the brain cells. Therefore, in this study we examined the cross talk between astrocytes and microglia by determining whether soluble factors released by astrocytes could modulate microglial inflammatory responses. We started by preparing enriched microglia cultures. Preparation of microglial cultures can be achieved by several methods, including a Percoll gradient (Ford, Goodsall et al. 1995), nutritional deprivation (Hao, Richardson et al. 1991) or the method more often used, the orbital agitation (Frei, Bodmer et al. 1986; Giulian and Baker 1986). More recently, Cristovão *et al*. characterized primary microglia cultures from rat ventral midbrain. This study disclosed that ten days after the isolation process microglial cells remains in the activated state. However, upon 13 days *in vitro*, cells achieve properties of non-activated microglia and present the characteristic response to a pro-inflammatory agent (Cristovao, Saavedra et al. 2010). The isolation process constitutes a strong stimulus to microglia, inducing its activation and consequently its round morphology (Fujita, Tanaka et al. 1996). However, the use of microglia ramified is a prerequisite for the molecular investigation of microglial activation, in order to describe the process of transformation the quiescence cells to inflammatory cells (Rosenstiel, Lucius et al. 2001). In our study, the method used to obtain microglial cells is baseed on the knowledge that when mixed glial cultures are shaked they easily detach from the culture plate, whereas astrocytes remain attached (Giulian and Baker 1986; Sugita, Becerra et al. 1997). After obtaining the primary microglia culture from the rat ventral midbrain, all experiences were realized upon 13 days the isolation process, to let microglial cells reach the resting state.

To further activate microglial cells and to evaluate its inflammatory responses we used the inflammatory agent Zymosan A at a concentration of 5 µg/mL.

Zymosan A was used as inflammatory stimulus due to its selectivity for the microglia with no reports of efects on astrocytes or neurons (Sweitzer, Colburn et al. 1999). This agent is a powerful activator of the respiratory burst system (McGeer, Klegeris et al. 1994), thereby inducing the production of mediators that in turn are toxic for neurons (Giulian, Vaca et al. 1993). Zymosan A is capable of activating microglia through the CD11b subunit of the CR3 (Le Cabec, Cols et al. 2000). We verifyed that Zymosan A increased the number of microglial cells exhibiting phagocytic activity and also the levels of reactive oxygen species produced by this cells. The dopaminergic neurons are extremely sensitive to oxidative stress, and in addicion the NADPH oxidase, a primary producer of reactive oxygen species is upregulated in PD, been its increased expression levels also related with activated microglia (Reynolds, Laurie et al. 2007).

Pre-incubation of microglial cells with ACM was able to prevent the increase of microglial phagocytic activity and of ROS levelsinduced by Zymosan A stimulation. This result suggest that soluble factors release by astrocytes are capable of modulating microglial activation, hindering an increase in the phagocytic activity and reactive oxygen species levels in answer to the inflammatory stimulation Zymosan A. DeWitt *et.al.* evaluated the role of astrocytes as potential modulators of microglia phagocytic capacity and showed that microglial cells exposed to media conditioned by astrocytes have a reduced capacity to phagocyte latex beads, and present increased ramification (DeWitt, Perry et al. 1998). Thus supporting the role of astrocytes in the regulating microglial activity.

Astrocytes express both cytokines and neurotrophic factors. Cytokines are a class of signaling proteins that are used extensively in cellular communication and the neurotrophic factors, in a general way, promote the survival, differentiation and maintenance of neurons in the nervous system. In an attempt to identify the possible soluble factors release by astrocytes that are playing a role in the modulation of the microglial activity, we focused our study in three neurotrophic fators known to have a protective role in the nigrostriatal dopaminergic system (Hong, Mukhida et al. 2008; Gyarfas, Knuuttila et al.

2009; Tansey and Goldberg 2009). A previous study from our group, using molecular fractionation of the ACM, showed that fraction with ability to prevent ZyA-induced microglia activation is > 10k Da and < 50 kDa . The molecular weight of the neurotrophic factors studied, GDNF, CDNF and BDNF fits in this range.

BDNF is an autocrine/paracrine factor for SNpc dopaminergic neurons and diminished levels of this neurotrophic factor may increase the risk of developing PD (Howells, Porritt et al. 2000). This neurotrophic factor is expressed by nigral neurons, astrocytes, and ramified and amoeboid microglia (Knott, Stern et al. 2002). Our results obtained with RT-PCR (343 pb), Western Blot (14 kDa) and immunocytochemistry data showing BDNF expression by astrocytes are in accordance with those findings. Recently, CDNF was identified as a neurotrophic factor for dopaminergic neurons. CDNF presents high homology with MANF, is expressed in several tissues of mouse and human, including the mouse embryonic and postnatal brain and has been shown to prevent 6-OHDA-induced degeneration of dopaminergic neurons in a rat experimental model of PD (Lindholm, Voutilainen et al. 2007). However, there is no data concerning the expression of CDNF by astrocytes. In this study we demonstrate, for the first time, using RT-PCR, Western Blot and immunocytochemistry that the CDNF protein is present in postnatal midbrain astrocytes.

The ability of GDNF to protect degenerating dopamine neurons in PD as well as to promote regeneration of the nigrostriatal dopamine system is largely reported (Schaller, Andres et al. 2005; Hong, Mukhida et al. 2008). GDNF has also been reported to regulate microglia activity. The information on the effect of the GDNF in the microglia is still scarce. Our results demonstrate that GDNF released by astrocytes is capable of controlling microglia activation. Blocked of the GDNF present in the ACM or silencing GDNF expression in astrocytes reverted the ability of ACM to modulate microglia activation induced by Zymosan A. Our results also showed that the action of the GDNF present in the ACM in microglial cells depends on the GDNF family receptor alpha 1 (GFRα1), a multi-component receptor complex comprising the transmembrane Ret tyrosine kinase, also localized in microglia (Sariola and Saarma 2003).

Apparently, the binding of GDNF-GFRα1 receptor activate several intracellular signaling cascade responsible for inhibiting of microglial activation. As perspective future, it would be interesting to study the signaling pathways involved in this process.

In conclusion, this study it allowed to investigate the effect of soluble mediators release by the astrocytes of microglial activation induced for inflammatory agent Zymosan A and to evaluate the nature of these mediators. Taken together the results presented in this work point out to a major role of astrocytes-derived GDNF in controlling microglia activation induced by a pro-inflammatory stimulus, the results also suggest that the neuroprotective action of GDNF in the nigrostriatal system could result not only from a direct effect on dopaminergic neurons, but could result from the inhibition of the neuroinflammatory cascade occurring in PD.

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