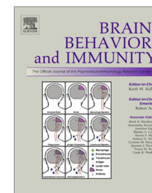
Brain, Behavior, and Immunity 64 (2017) 296–307

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi



Full-length Article

GPER activation is effective in protecting against inflammation-induced nigral dopaminergic loss and motor function impairment

Julieta Mendes-Oliveira, Filipa Lopes Campos [1], Rita Alexandra Videira, Graça Baltazar *

CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

ARTICLE INFO

Article history:
Received 17 October 2016
Received in revised form 21 April 2017
Accepted 21 April 2017
Available online 24 April 2017

Keywords:
Parkinson's disease
GPER
Microglia
Neuroinflammation
Dopaminergic neurons
Neuroprotection

ABSTRACT

Increasing evidence suggest that excessive inflammatory responses from overactivated microglia play a critical role in Parkinson's disease (PD), contributing to, or exacerbating, nigral dopaminergic (DA) degeneration. Recent results from our group and others demonstrated that selective activation of G protein-coupled estrogen receptor (GPER) with the agonist G1 can protect DA neurons from 1-methyl-4-phenylpyridinium (MPP^+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxins. However, it is not known whether modulation of microglial responses is one of the mechanisms by which G1 exerts its DA neuroprotective effects. We analyzed, in the N9 microglial cell line, the effect of G1 on microglial activation induced by lipopolysaccharide (LPS) exposure. The results revealed that G1 significantly decrease phagocytic activity, expression of inducible nitric oxide synthase (iNOS) and release of nitric oxide (NO) induced by LPS. To determine the relevance of this anti-inflammatory effect to the protection of nigral DA cells, the effect of G1 was analyzed in male mice injected unilaterally in the substantia nigra (SN) with LPS. Although G1 treatment did not decrease LPS-induced increase of ionized calcium binding adaptor molecule 1 (iba-1) positive cells it significantly reduced interleukin-1beta (IL-1β), cluster of differentiation 68 (CD68) and iNOS mRNA levels, and totally inhibited nigral DA cell loss and, as a consequence, protected the motor function. In summary, our findings demonstrated that the G1 agonist is able to modulate microglial responses and to protect DA neurons and motor functions against a lesion induced by an inflammatory insult. Since G1 lacks the feminizing effects associated with agonists of the classical estrogen receptors (ERs), the use of G1 to selectively activate the GPER may be a promising strategy for the development of new therapeutics for the treatment of PD and other neuroinflammatory diseases.

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1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by progressive and selective loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc). The loss of nigral DA neurons results in a decrease of striatal dopamine that in turn leads to the impairment of the motor functions. Although PD is considered an idiopathic disease, several evidences from human studies and experimental PD models have suggested that excessive inflammatory responses from microglia contribute significantly to DA progressive death (Lull and Block, 2010). Indeed, an increase in microglia activation occurs early in PD, simultaneously and correspondingly with the loss of DA terminals of the nigrostriatal pathway (Ouchi et al., 2005), and persists throughout the disease (Imamura et al., 2003; Langston et al., 1999). Although microglial cells are vital to normal brain functions and its mild activation promotes beneficial effects on the surrounding tissue, a chronic activation of these cells leads to exaggerated release of reactive oxygen and nitrogen species, pro-inflammatory cytokines and other pro-inflammatory factors that can trigger DA damage (Lull and Block, 2010). Studies using lipopolysaccharide (LPS), a pro-inflammatory agent that strongly activates microglial cells, demonstrated that it is capable of inducing a progressive loss of nigral DA neurons (Choi et al., 2009; Gao

Abbreviations: PD, Parkinson's disease; DA, dopaminergic; SNpc, *substantia nigra pars compacta*; LPS, lipopolysaccharide; ERs, estrogen receptors; ERα, estrogen receptors α; ERβ, estrogen receptors β; GPER, G protein-coupled estrogen receptor; MEM, Minimum Essential Medium Eagle; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; FBS, Fetal Bovine Serum; NO, nitric oxide; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; E_2, 17β-estradiol; PFA, paraformaldehyde; PBS, phosphate buffered saline; TH, tyrosine hydroxylase; iba-1, ionized calcium binding adaptor molecule 1; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α; CD68, cluster of differentiation 68.

* Corresponding author at: CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.

E-mail addresses: jucmb.oliveira@gmail.com (J. Mendes-Oliveira), filipalcampos@gmail.com (F. Lopes Campos), ritabarreiros_@hotmail.com (R.A. Videira), gbaltazar@fcsaude.ubi.pt (G. Baltazar).

[1] Present address: Department of Research and Development, BIAL – Portela & Cª, S. A., S. Mamede do Coronado, Portugal.

http://dx.doi.org/10.1016/j.bbi.2017.04.016
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et al., 2002; Hunter et al., 2009; Qin et al., 2007). In the absence of microglia LPS cannot exercise its neurotoxic effect (Gibbons and Dragunow, 2006; Liu et al., 2005, 2011) suggesting that the progressive DA loss observed after LPS exposure is a consequence of microglial activation. Since injured DA neurons release several molecules that promote microglial activation, it is difficult to understand whether excessive microglial activation is the cause or the consequence of DA neurons damage in PD. In either case, excessive responses from activated microglia appear to result in a self-perpetuating cycle of DA death (Block et al., 2007). Thereby, control microglial activation can be extremely relevant to attenuate DA progressive degeneration in PD.

Outcomes from epidemiological observations have reported a higher prevalence of the PD in men than in woman (Shulman and Bhat, 2006; Van Den Eeden et al., 2003). Furthermore, clinical studies demonstrated that women have a slower progression of the disease and a lower severity of the symptoms when compared to men (Haaxma et al., 2007; Lyons et al., 1998). These gender differences seem to be related with the neuroprotective effects of estrogens. In fact, several *in vitro* and *in vivo* experimental studies have reported the ability of estrogens to protect nigrostriatal DA system from different neurotoxins (Baraka et al., 2011; Callier et al., 2002; Campos et al., 2012; D'Astous et al., 2003; Ramirez et al., 2003; Sawada et al., 2002). A direct effect on DA neurons (Bourque et al., 2012) and a modulation of microglial responses (Liu et al., 2005; Tripanichkul et al., 2006) are mechanisms by which this hormone can exert its DA neuroprotective action. Estrogens have been reported to act in these cells through activation of the classical nuclear estrogen receptors (ERs) α (ERα) and β (ERβ). However, activation of these ERs has been associated with important side effects that result from estrogens therapy in humans. Therefore, finding an alternative target without adverse effects is crucial. Recent results showed that selective activation of the G protein-coupled estrogen receptor (GPER), a transmembrane receptor for estrogens, is able to protect DA neurons against 1-methyl-4-phenylpyridinium (MPP^+) (Bessa et al., 2015) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (Bourque et al., 2013), a widely used and extensively characterized model of PD (Blesa and Przedborski, 2014). *In vivo* studies also revealed that mice treated with G15, a GPER antagonist, exhibited a higher sensitivity to DA toxin MPTP (Bourque et al., 2013). Since GPER Knockout mice do not have an impairment of the reproductive functions (Otto et al., 2009), GPER activation seems to lack the feminizing effects associated with activation of the classical ERs. In addition to these characteristics, GPER also mediates some estrogenic anti-inflammatory effects. Studies using human and murine macrophages demonstrated that selective activation of GPER can reduce the production of pro-inflammatory cytokines induced by LPS (Blasko et al., 2009; Rettew et al., 2010). Due to the critical role of excessive microglial responses to progressive DA death in PD, it is of high relevance to understand whether the DA neuroprotective effects of GPER stimulation includes a modulation of microglial activation. To the best of our knowledge, there is only one report about the effect of GPER activation on microglial cells, demonstrating that G1, a GPER agonist, is able to attenuate the release of pro-inflammatory cytokines induced by LPS in primary microglia (Zhao et al., 2016). Therefore, the aim of the present study was to clarify the role of GPER activation on the control of microglial responses and to analyze its relevance to the protection of nigral DA neurons *in vivo*.

2. Materials and methods

Studies involving animal experimentation were previously approved by the national authorities (Direção Geral de Alimentação e Veterinária) and were performed 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 (Directive 2010/63/EU).

2.1. Microglial cell cultures

2.1.1. N9 microglia

The murine immortalized N9 microglial cell line was grown in RPMI-1640 medium (Sigma) supplemented with 5% Fetal Bovine Serum (FBS, Biochrom AG) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Sigma). For the measurement of nitric oxide (NO) levels and interleukin-1beta (IL-1β) expression, the cells were plated at a density of 1.053×10^4 cells/cm^2 and 0.526×10^4 cells/cm^2 respectively. To evaluate the phagocytic activity and inducible nitric oxide synthase (iNOS) expression they were plated at 0.395×10^4 cells/cm^2. After two days in culture the N9 cells were stimulated.

2.1.2. Primary microglia

The region of ventral midbrain of postnatal Wistar rat pups (postnatal day 2 or 3) was dissected, stripped of the meninges and transferred to iced phosphate buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.4). After mechanical dissociation the cell suspension was filtered through a 70 μm mesh, centrifuged at 1500 rpm for three minutes and resuspended in Minimum Essential Medium Eagle (MEM, Sigma) supplemented with 10% FBS, 12 U/ml penicillin plus 12 μg/ml streptomycin, 3,4 g/L D-Glucose (Thermo Fisher Scientific) and 5 mg/L Insulin from bovine pancreas (Sigma). The cells were then plated at a density of 9.6×10^4 cells/cm^2 in poly-D-lysine-coated plates and maintained at 37 °C in a 5% CO_2 and 95% air atmosphere. After seven days in culture, astrocytes were removed by mild trypsinization (0,17 g/L trypsin and 0,05 g/L EDTA in MEM). The detached astrocytes were discarded and the adherent microglial cells were kept in culture for further five to seven days. Using the microglial marker CD11b we verified, by immunocytochemistry, that 98% of the cells present in the cultured labeled for CD11b.

2.2. Treatment of microglial cells

Before starting cell treatments the culture medium of microglial cells was renewed and 17β-estradiol (E_2, 100 nM, Sigma) or the selective agonist of GPER (G1, 100 nM, Calbiochem) were added. In the experimental conditions using the selective antagonist of GPER (G15, 100 nM, Calbiochem), this compound was added thirty minutes before E_2. After a twenty-four hours incubation period the microglial cells were exposed to LPS (0.1 μg/ml, Sigma) for further twenty-four hours. The supernatants were then collected for measurement of NO levels and cells were used to evaluate the phagocytic activity and the expression of iNOS and IL-1β.

2.3. Nitrite assay

NO production was determined by measuring the total amount of nitrite, a stable oxidation product of NO by the Griess reaction. Culture supernatants (50 μl) were collected, transferred to a 96-well plate, and incubated with 50 μl sulfanilamide solution (1% sulfanilamide and 5% H_3PO_4 in water) for eight minutes at room temperature, in the dark. Then 50 μl N-1-napthylethylenediamine dihydrochloride solution (0.1% N-1-napthylethylenediamine dihydrochloride in water) were added. Eight minutes later the absorbance was measured at 550 nm in a microplate reader (Anthos Labtec LP400, Diagnostics pasteur).

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