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Adenosine A_{2A} receptor antagonism reverses inflammation-induced impairment of microglial process extension in a model of Parkinson's disease

Stefka Gyoneva [a,b,*,1], Lauren Shapiro [b], Carlos Lazo [c], Ethel Garnier-Amblard [d,2], Yoland Smith [e,f], Gary W. Miller [a,c,f], Stephen F. Traynelis [a]

[a] Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, USA
[b] Graduate Division of Biological and Biomedical Sciences, Molecular and Systems Pharmacology Program, Emory University, Atlanta, GA 30322, USA
[c] School of Public Health, Environmental Health, Emory University, Atlanta, GA 30322, USA
[d] Department of Chemistry, Emory University, Atlanta, GA 30322, USA
[e] Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329, USA
[f] Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA

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ABSTRACT

Microglia, the immune cells of the central nervous system, constantly survey the parenchyma in the healthy brain to maintain homeostasis. When a disturbance, such as cell death, results in ATP release *in vivo*, microglial processes respond by utilizing $P2Y_{12}$ purinergic receptors to trigger extension toward the site of damage. Processes ultimately surround the injury site, preventing the spread of harmful cellular constituents and assisting with tissue repair. In contrast to the healthy brain, many neurodegenerative diseases, including Parkinson's disease, are characterized by the presence of neuroinflammation. Yet, the ability of microglia to respond to tissue damage under pro-inflammatory conditions has not been well studied. To assess the ability of microglia to respond to tissue injury and localized cell death in the context of Parkinson's disease, we performed confocal imaging of acute brain slices from mice with microglia-specific green fluorescent protein expression. Microglia in coronal slices containing the substantia nigra extend processes toward a mechanical injury in a $P2Y_{12}$ receptor-dependent manner. However, microglia in mice treated for 5 days with 20 mg/kg/day 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show significantly reduced process displacement toward the injury compared to microglia in control animals. Pre-treatment of slices from MPTP-injected mice with the A_{2A} receptor-selective antagonist preladenant restores the ability of activated microglia to respond to tissue damage. These data support the hypothesis that chronic inflammation impedes microglial motility in response to further injury, such as cell death, and suggest that some aspects of the neuroprotection observed with adenosine A_{2A} receptor antagonists may involve direct or indirect actions at microglia.

Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder in the United States, is characterized by tremors, bradykinesia, rigidity, and postural instability. The cellular hallmarks of the disease are loss of dopaminergic neurons originating in the substantia nigra (SN) and subsequent loss of dopamine in the striatum (Kish et al., 1988; Rinne, 1991). Another important feature of PD is the presence of neuroinflammation. For example, some pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and others, can be found at higher levels in cerebrospinal fluid samples from PD patients compared to age-matched controls (Mogi et al., 1994a,1994b, 1996). Further supporting the involvement of inflammation, meta-analyses of several studies show that use of non-steroidal

Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; BSA, bovine serum albumin; CX_3CR1, CX_3C-type chemokine receptor 1; GFP, enhanced green fluorescent protein; IL, interleukin; i.p, intraperitoneal; s.c, subcutaneous; LPS, lipopolysaccharide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NSAIDs, non-steroidal anti-inflammatory drugs; PD, Parkinson's disease; PET, positron emission tomography; PFA, paraformaldehyde; RT-PCR, reverse transcriptase polymerase chain reaction; SN(c/r), substantia nigra (pars compacta/pars reticulata); TH, tyrosine hydroxylase; TNF, tumor necrosis factor; DAB, 3,3′-diaminobenzidine.

* Corresponding author at: Department of Pharmacology, Rollins Research Center Rm 5062, 1510 Clifton Rd. NE, Atlanta, GA 30322, USA. Fax: +1 404 727 0365.
E-mail address: stefka.gyoneva@gmail.com (S. Gyoneva).
Available online on ScienceDirect (www.sciencedirect.com).
[1] Present address: Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA.
[2] RFS Pharma LLC., Tucker, GA 30084, USA.

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anti-inflammatory drugs (NSAIDs), and specifically ibuprofen, are associated with lower risk for developing PD (Gagne and Power, 2010; Gao et al., 2011). Moreover, activated microglia, the brain's resident immune cells, can be detected in brains of living PD patients [with positron emission tomography (PET) imaging] and in post-mortem samples from people who suffered from the disease (Gerhard et al., 2006; McGeer et al., 1988). Finally, activated microglia have been observed in post-mortem samples from animal models of PD, such as monkeys intoxicated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and in humans accidentally exposed to MPTP even years after the toxin has been removed (Barcia et al., 2004; Kanaan et al., 2008; Langston et al., 1999; McGeer et al., 2003).

The ability of microglia to perform immune functions such as cytokine secretion and reactive oxygen species generation when activated renders microglia as potential contributors to PD pathology by compromising neuronal survival (Block et al., 2007). However, microglia also perform many other functions in the brain that are not directly linked to the immune response (Kettenmann et al., 2013). For example, the "resting" microglia in the healthy brain have highly motile processes that can detect disturbances of the brain parenchyma, such as rupture of brain capillaries or cell death that occur throughout life (Davalos et al., 2005; Nimmerjahn et al., 2005). The response to injury *in vivo* is mediated by ATP release by damaged cells, activation of $P2Y_{12}$ receptors on microglia, and directional process extension to surround the damaged area and promote tissue repair (Davalos et al., 2005; Haynes et al., 2006). Interestingly, microglia that are in an activated state downregulate $P2Y_{12}$ receptors and upregulate adenosine A_{2A} receptors (Haynes et al., 2006; Orr et al., 2009), the latter of which are indirectly activated by ATP after its rapid breakdown to adenosine (Zimmermann, 2000). However, unlike the ability of ATP to induce process extension in resting microglia, ATP (and adenosine) induces process retraction in activated microglia (Orr et al., 2009). This raises a question as to how activated microglia, such as those found in PD, detect and respond to the neuronal death that is characteristic of the disease.

To study the ability of activated microglia to respond to tissue damage in a PD-related context, we developed an assay that allowed us to examine microglial motility in acute slices containing the SN from MPTP-treated mice in response to mechanically induced tissue injury. Using this preparation, we demonstrate that microglia in slices from MPTP-treated mice show a diminished capacity to extend their processes to the site of damage. Blockade of A_{2A} receptors restored process extension to the damaged area in slices from MPTP-treated mice. Our findings suggest that microglia in PD might display a delayed response to the ongoing cell death, which could promote disease progression. Moreover, these data show that A_{2A} receptor antagonists have the capacity to restore normal function of microglial processes, which could be relevant to their clinical utility.

Materials and methods

Animals

All procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University. $CX_3CR1^{GFP/GFP}$ mice, which exhibit enhanced green fluorescent protein (GFP) expression from the microglia-specific CX_3C-type chemokine receptor 1 (CX_3CR1) promoter (Jung et al., 2000), were purchased from Jackson Laboratories and bred in-house with C57Bl/6 mice to generate mice heterozygous for the GFP knock-in gene ($CX_3CR1^{GFP/+}$ mice) to be used for slice preparation and imaging. To achieve microglial activation *in vivo*, mice were injected with either lipopolysaccharide (LPS, *E. coli* strain K-235, Sigma L2143) or MPTP (Sigma, cat. #M0896). The LPS injection paradigm consisted of a single treatment of 2 mg/kg intraperitoneally (i.p.), and preparation of slices or isolation of tissues two days later. MPTP-treated mice received daily subcutaneous (s.c.) injections of 20 mg/kg (free base) of the neurotoxin for 5 consecutive days for a total dose of 100 mg/kg. We chose a lower dose than what has been commonly used [30 mg/kg/day; Jackson-Lewis and Przedborski (2007)] to reduce the high mortality we observed with 30 mg/kg in $CX_3CR1^{GFP/+}$ mice. The total dose of 100 mg/kg MPTP administered here is known to induce statistically significant dopaminergic neuronal loss [Seniuk et al. (1990); see Fig. 5]. Tissues for immunohistochemistry and imaging were collected 4–6 days after the conclusion of the MPTP treatment. We chose this time point to capture the tissue early in the processes of cell death, rather than at a terminal stage after dopaminergic cells have already been lost.

Brain slicing and imaging

Acute coronal brain slices that contained the substantia nigra were prepared from $CX_3CR1^{GFP/+}$ mice at a thickness of 200 μm as described before (Gyoneva and Traynelis, 2013). After at least 1 h of rest to allow microglia to recover from the slicing procedure, the slices were moved to the pre-warmed stage (32 °C) of an inverted Olympus IX51 confocal microscope equipped with a disc scanning unit and imaged through a 20× dry objective (NA 0.50) over time with constant perfusion with oxygenated artificial cerebrospinal fluid (aCSF). The slices were centered on the stage to include the SN in the imaging field and held in place by a ring with nylon threads positioned on top of the slice. Imaging consisted of obtaining 31 optical sections through the slices (1 μm step) every 30–60 s for 20 min through a Hamatsu Orca-ER camera with the IPlab software. Following recording of baseline motility for 20 min, we induced localized tissue injury in the substantia nigra by lowering a blunted 30-gauge needle (referred to as "rod"). The rod was carefully positioned over the substantia nigra pars compacta (SNc) and lowered at a rate of 100 μm/s with a closed loop motorized micromanipulator (SD Instruments, model MC1000e) for 180 μm into the tissue and left in place until the conclusion of the experiment. A second 20-min recording was set up as soon as the injury was created in order to capture the microglial response to injury in the same slice. For some experiments, the $P2Y_{12}$ receptor antagonist clopidogrel (2 μM final concentration from DMSO stock, Tocris 2490) or the adenosine A_{2A} receptor antagonist preladenant [5 μM final concentration from DMSO stock, synthesized at Dept. of Chemistry, Emory University, using procedures described by Neustadt et al. (2007)] were included in the perfusion solution for the duration of the experiment (baseline recording, induction of injury, response to injury).

To assess the extent of damage to dopaminergic neurons induced by the mechanical injury, slices were prepared from *tyrosine hydroxylase (TH)-GFP* animals (kindly provided by Dr. David Weinshenker, Emory University) which express GFP from the TH promoter, and injured as described above. The slices were then fixed overnight in 4% paraformaldehyde (PFA) and mounted on glass slides. The GFP signal in dopaminergic neurons in the SN was imaged with a Leica SP8 multiphoton microscope through a 10x dry objective (NA 0.30) to obtain optical sections through the slices every 4.3 μm. These sections were used to generate 3D reconstructions of the slices with ImageJ software (National Institutes of Health) and identify the location of the injury. After calculating the volume of the injury, the number of TH-positive neurons that could be located within the injured tissue was estimated from non-damaged areas in injured slices ($n = 8$) and sham-injured slices ($n = 3$).

Analysis of microglial motility

The optical sections along the z-axis at each time point of the time-lapse recordings (baseline and response to injury) from $CX_3CR1^{GFP/+}$ mice were used to generate 2D maximum intensity projections, which were later used to quantify microglial movement with the Imaris software v7.6 (Bitplane AG, Switzerland). The software detected objects with diameter larger than 2 μm, which mostly represent microglial processes. (Cell bodies, identified as objects larger than 5 μm-diameter in