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Research Paper

Peripheral monocyte entry is required for alpha-Synuclein induced inflammation and Neurodegeneration in a model of Parkinson disease

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ABSTRACT

Accumulation of alpha-synuclein (α-syn) in the central nervous system (CNS) is a core feature of Parkinson disease (PD) that leads to activation of the innate immune system, production of inflammatory cytokines and chemokines, and subsequent neurodegeneration. Here, we used heterozygous reporter knock-in mice in which the first exons of the fractalkine receptor (CX3CR1) and of the C-C chemokine receptor type 2 (CCR2) are replaced with fluorescent reporters to study the role of resident microglia (CX3CR1 +) and infiltrating peripheral monocytes (CCR2 +), respectively, in the CNS. We used an α-syn mouse model induced by viral over-expression of α-syn. We find that in vivo, expression of full-length human α-syn induces robust infiltration of pro-inflammatory CCR2 + peripheral monocytes into the substantia nigra. Genetic deletion of CCR2 prevents α-syn induced monocyte entry, attenuates MHCII expression and blocks the subsequent degeneration of dopaminergic neurons. These results demonstrate that extravasation of pro-inflammatory peripheral monocytes into the CNS plays a key role in neurodegeneration in this model of PD synucleinopathy, and suggest that peripheral monocytes may be a target of neuroprotective therapies for human PD.

1. Background

Parkinson disease (PD) is the most common neurodegenerative movement disorder and is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) along with the presence of Lewy bodies and Lewy neurites composed of α-syn in the SNpc and other brain regions. Missense mutations or multiplications of the α-syn gene cause aggressive forms of PD (Polymeropoulos, 1998; Polymeropoulos et al., 1997; Ross et al., 2008) while genome wide association studies (GWAS) show that the locus encoding α-syn is one of the most potent genetic factors in susceptibility to sporadic PD (Houlden and Singleton, 2012; Simon-Sanchez et al., 2009). One of the most common non-coding disease-associated polymorphisms in this region has recently been shown to lead to enhanced α-syn protein expression (Soldner et al., 2016).

There is growing evidence for a critical role of inflammation in the pathogenesis of PD (Appel, 2012; Hirsch and Hunot, 2009; Hirsch et al., 2012; Lim et al., 2016). In the human PD brain, there is prominent reactive microgliosis (McGeer et al., 1988), enhanced expression of pro-inflammatory cytokines and chemokines (Blum-Degen et al., 1995; Mogi et al., 1994a; Mogi et al., 1994b), lymphocyte infiltration (Brochard et al., 2009) and deposition of IgG (Orr et al., 2005). There is also strong evidence for alterations in the function of the peripheral immune system in PD pathogenesis including alterations in T cell subsets (Saunders et al., 2012). Many of these features are reproduced in rodent models of PD produced by overexpression or aggregation of α-syn (Allen Reish and Standaert, 2015). We have previously shown that the neurotoxicity of α-syn can be attenuated by modulation of key immune mediators, including MHCII (Harms et al., 2013) and the Fcgamma receptors (Cao et al., 2012) which mediate interactions between immunoglobins and microglia (Daeron, 1997). Genome Wide Association (GWA) studies provide additional support for the importance of immunological mechanisms driving disease, showing that polymorphisms in the HLA-DR (MHCII) locus are associated with

Abbreviations: α-syn, alpha-synuclein; CNS, central nervous system; PD, Parkinson disease; CX3CR1, fractalkine receptor; CCR2, C-C chemokine receptor type 2; CCL2, C-C chemokine ligand 2; SNpc, substantia nigra pars compacta; GWAS, genome wide association studies; MHCII, major histocompatibility complex II; GFP, green fluorescent protein; RFP, red fluorescent protein; AAV2, adeno-associated virus serotype 2; TH, tyrosine hydroxylase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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sporadic, late-onset PD (Hamza et al., 2010). Population studies show an association between the long-term use of non-steroidal anti-inflammatory medications and reduced risk of PD (Gao et al., 2011), providing proof of principal for the use of immunomodulatory strategies.

Monocytes are a subset of myeloid cells that enter tissues, including the brain, during active disease states (Ransohoff, 2011; Ransohoff and Cardona, 2010). A key regulatory mechanism for tissue entry is the interaction of the chemokine receptor CCR2, found on peripheral myeloid cells, and its ligand, CCL2 (Mahad et al., 2006). After crossing the blood-brain barrier in response to CCL2 signaling, monocytes can differentiate into macrophage and dendritic cell phenotypes and mediate pro and anti-inflammatory responses (Mahad et al., 2006; Prinz et al., 2011; Ransohoff, 2011; Ransohoff and Cardona, 2010; Saederup et al., 2010). These peripherally derived myeloid cells are distinct from microglia, the resident innate-immune cells of the brain, and can have critical differential effects on tissue injury and protection (Butovsky et al., 2014; Butovsky et al., 2012; Gaupp et al., 2003; Schmid et al., 2009; Yamasaki et al., 2014). In experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis (MS), infiltrating monocytes promote disease progression via continual destruction of myelin (Ajami et al., 2011), and blocking CCR2-dependent entry of monocytes both reduces inflammation and delays disease onset (Ajami et al., 2011; Columba-Cabezas et al., 2002; Fife et al., 2000; Izikson et al., 2000).

While relatively little is known about peripheral monocytes in human PD, preliminary studies in patients have shown that classical monocytes expressing CCL2 are enriched in blood isolated from PD patients, and CCL2 expression is elevated in the CSF (Grozdanov et al., 2014; Reale et al., 2009), while an additional study showed elevated CCR2 expression on peripheral blood monocytes (Funk et al., 2013). In addition, peripheral blood monocytes isolated from PD patients showed a hypersensitivity or inflammatory pre-disposition to an inflammatory stimulus (Grozdanov et al., 2014). Most recently, a study of circulating blood monocytes from young, healthy individuals without PD found that these cells had remarkably high expression of PD-associated genes, including α-syn, LRRK2 and others, and concluded that the inflammatory component of PD susceptibility is strongly driven by myeloid cell types (Raj et al., 2014).

In this study, we show that in a mouse model of PD that overexpression of α-syn recruits pro-inflammatory CCR2 + peripheral monocytes into the CNS. Blocking this infiltration attenuates α-syn mediated inflammation and subsequent neurodegeneration. Understanding the role of peripherally derived pro-inflammatory monocytes in PD-related inflammation and the mechanisms responsible for their entry into the brain may lead to novel therapeutic intervention strategies for human PD.

2. Methods

2.1. Animals and treatment

C57BL/6 (catalog # 000664), CX3CR1 reporter knock in (B6.129P-*Cx3cr1*^{tm1Litt}/*J* (catalog # 005582), and CCR2 reporter knock in (B6.129(Cg)-*Ccr2tm2.1Ifc*/J, catalog # 017586) mice maintained on a congenic background were used for these studies and were obtained from Jackson Laboratories (Bar Harbor, Maine). In these strains, a monomeric green fluorescent protein (GFP) (Jung et al., 2000) and red fluorescent protein (RFP) sequence replaces the coding sequence of the Fractalkine receptor (CX3CR1) and chemokine (C-C motif) receptor 2 (*Ccr2*) gene (Saederup et al., 2010), respectively, abolishing gene function.

The rAAV vectors, rAAV-CBA-EGFP (AAV2-GFP) and rAAV-CBA-Alpha-SYNUCLEIN (AAV2-SYN) were obtained from the University of North Carolina Vector Core via the Michael J. Fox Foundation research tools catalog. (https://www.michaeljfox.org/research/research-tools-catalogue.html). Male red/green and CCR2 knockout mice (8–12 weeks of age) were deeply anesthetized with isoflurane and unilaterally or bilaterally injected with 2 μL of AAV2-SYN or AAV2-GFP (1.5×10^{13} viral genome/mL diluted in sterile PBS) into the right SNpc as previously described (Harms et al., 2013; Luk et al., 2012; St Martin et al., 2007). Co-ordinates were anterior-posterior − 3.2 mm from bregma, medio-lateral − 1.2 mm from midline, and dorso-ventral − 4.6 mm from dura. All research conducted on animals was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham. The pattern and extent of α-syn expression obtained in vivo after intranigral injection of AAV2-SYN vectors has been reported previously and similar results were obtained in this study (Cao et al., 2010; Harms et al., 2013; St Martin et al., 2007; Theodore et al., 2008; Thome et al., 2016; Thome et al., 2015). These vectors produce localized transduction of neurons in the SNc ipsilateral to the injection site. The extent of transduction of SNpc neurons is typically approximately 50%, but does vary depending on the exact location of the injection site (St Martin et al., 2007). There is no significant α-syn expression observed in neurons on the contralateral side or in glia on either side. Western blot analysis of nigral tissue shows that these vectors lead to accumulation of human α-syn in both monomeric and high molecular weight forms (Harms et al., 2013).

2.2. Immunohistochemistry

At 4 weeks and 6 months post-transduction, animals were deeply anesthetized and transcardially perfused, post-fixed for 24 h, and cryoprotected as previously described (Harms et al., 2013). Brains were frozen on dry ice and cryosectioned coronally on a sliding microtome (cut thickness: 40 μm); sections were collected serially throughout the striatum and SNpc, placed into tissue collection solution (50% 0.01 M PBS, 50% glycerol), and stored at − 20 °C for immunohistochemical analysis.

For fluorescent analysis, free-floating sections were labeled with anti-MHCII (M5/114.15.2, eBiosciences, 1:100) or anti-Tyrosine hydroxylase (TH) (Millipore, 1:2000) antibodies overnight at 4 °C. Appropriate Alexa-conjugated secondary antibodies diluted 1:1000 (Life Technologies) were used at room temperature for 2.5 h. Sections were mounted onto coated glass slides, and cover slips were added using Vectashield Hard Set mounting medium.

For TH neuron quantification using unbiased stereological analysis, free floating sections were stained as previously described (Harms et al., 2013; Luk et al., 2012; St Martin et al., 2007), coded, and analyzed with an Olympus BX51 microscope and MicroBrighfield software (MicroBrightfield Inc., Williston, VT).

2.3. Imaging and quantification

Confocal images were captured using a Leica TCS-SP5 laser scanning confocal microscope. Images were processed using the Leica LASAF software, exported and processed using Adobe Photoshop. For quantification of MHCII and LB509 staining, slides were observed using a Nikon Eclipse E800M fluorescent microscope. Coded slides were scored by using a numerical scale 0 (no staining) to 4 (most intense) by a single observer blind to the treatment paradigm (Harms et al., 2013; Thome et al., 2015). TH, MHCII, and LB509 positive cells were manually differentiated under 40 × magnification to differentiate cell size, shape, and morphology. Staining within the vicinity of viral transduction (SYN) was considered for scoring while staining immediately surrounding the needle tract was ignored. Scores obtained from 6 to 8 mice per group were plotted and statistically analyzed using the Mann Whitney test.

2.4. Mononuclear cell isolation and flow cytometry

Mononuclear cells were isolated from bilaterally transduced ventral