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AN ALTERED BLOOD–BRAIN BARRIER CONTRIBUTES TO BRAIN IRON ACCUMULATION AND NEUROINFLAMMATION IN THE 6-OHDA RAT MODEL OF PARKINSON'S DISEASE

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Abstract—Brain iron accumulation is a common feature shared by several neurodegenerative disorders including Parkinson's disease. However, what produces this accumulation of iron is still unknown. In this study, the 6-hydroxydopamine (6-OHDA) hemi-parkinsonian rat model was used to investigate abnormal iron accumulation in substantia nigra. We investigated three possible causes of iron accumulation; a compromised blood–brain barrier (BBB), abnormal expression of ferritin, and neuroinflammation. We identified alterations in the BBB subsequent to the injection of 6-OHDA using gadolinium-enhanced magnetic resonance imaging (MRI). Moreover, detection of extravasated IgG suggested that peripheral components are able to enter the brain through a leaky BBB. Presence of iron following dopamine cell degeneration was studied by MRI, which revealed hypointense signals in the substantia nigra. The presence of iron deposits was further validated in histological evaluations. Furthermore, iron inclusions were closely associated with active microglia and with increased levels of L-ferritin indicating a putative role for microglia and L-ferritin in brain iron accumulation and dopamine neurodegeneration.

Key words: brain-iron, 6-OHDA, MRI, blood–brain barrier, microglia.

INTRODUCTION

Iron import to the brain is highly regulated due to the existence of the blood–brain barrier (BBB) and mainly occurs via the transferrin receptor (Mills et al., 2010). In the brain, iron is normally intracellular and bound to ferritin in its ferric (Fe^{+3}) form. Increased levels of brain iron are commonly observed as a consequence of aging and only in particular areas of the brain such as substantia nigra, globus pallidus or putamen. Interestingly, under pathological conditions, the amount of iron in these areas is somehow enhanced and can lead to neurodegeneration (Stankiewicz et al., 2007; Nunez et al., 2012; Ward et al., 2014). The exact reasons for the increase in iron during aging or neurodegeneration are still unknown. The toxicity of free iron is owed to its participation in the Fenton/Haber–Weiss reaction which leads to the formation of reactive oxygen species (ROS) as a byproduct (Gaasch et al., 2007). Biologically available free radicals such as $O_2^{\bullet -}$ or H_2O_2 are the products of certain biological reactions, which principally occur in mitochondria (Adam-Vizi, 2005). When $O_2^{\bullet -}$ or H_2O_2 radicals are produced in excess they can react via the Haber–Weiss reaction and form the highly reactive hydroxyl radical ($HO^{\bullet}$). However, in biological systems this reaction is only favored when it is catalyzed by iron via the Fenton reaction (Kehrer, 2000), thereby providing the toxic participation of iron in oxidative stress.

In both patients and animal models of Parkinson's disease, iron accumulation has been observed in different structures of the basal ganglia (Zecca et al., 2004; Hare et al., 2009; Lv et al., 2011; Rouault, 2013; Virel et al., 2014; Hare and Double, 2016) highlighting its potential role in neurodegeneration. Indeed, unilateral injection of iron directly into the substantia nigra has been shown to be sufficient for specific degeneration of dopamine neurons in the substantia nigra (Ben-Shachar and Youdim, 1991). Moreover, oral administration of high doses of ferrous iron to neonatal mice, in which the BBB is not fully formed, results in nigral degeneration and Parkinsonism (Dal-Pizzol et al., 2001; Kaur et al., 2007). Iron accumulation in substantia nigra after 6-hydroxydopamine (6-OHDA) injections has been detected using methods such as sonography (Zhu et al., 2017) or laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) (Hare et al., 2009). The iron load in these animals has been found not only associated with dopamine neurons but also with microglia and astrocytes (He et al.,

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Abbreviations: 6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance; BBB, blood–brain barrier; CD68, cluster of differentiation 68; FOV, field of view; GFAP, glial fibrillary acidic protein; IgG, immunoglobulin G; MRI, magnetic resonance imaging; NEX, number of excitations; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; ROI, region of interest; ROS, reactive oxygen species; TH, tyrosine hydroxylase.

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1999; Blum et al., 2001). Still, the reasons why iron accumulates in certain structures of the dopamine system are not well understood and it is still debated whether it is a consequence, or a cause, of neuronal death.

Several mechanisms have been proposed for an unbalanced iron metabolism observed in Parkinson's disease. Possible causes are the dysregulation of iron transport or the abnormal expression/malfunction of iron-related proteins (Zecca et al., 2004). For instance, abnormal levels of lactoferrin in plasma or cerebrospinal fluid have recently been reported and suggested as putative biomarkers for diagnosis of Parkinson's disease (Grau et al., 2001). Another hypothesis proposed is abnormal iron homeostasis between peripheral and brain iron (Costa-Mallen et al., 2017) or alterations in the BBB (Zecca et al., 2004; Gerlach et al., 2006; Carvey et al., 2009). However, few studies are available where the permeability of the BBB in Parkinson's disease has been carefully studied (Kortekaas et al., 2005; Bartels et al., 2008). The expression of ferritin in degenerated substantia nigra has also been investigated. Ferritin represents the major storage protein for intracellular iron in our body and it exists in two major isoforms, Heavy (H) and Light (L) chain ferritin. In the brain under non-pathological conditions, L-ferritin is predominantly expressed in microglia whereas H-ferritin is expressed in neurons and other glial cell types (Berg et al., 2001). The lack of consensus in the literature regarding ferritin levels in animal models and patients of Parkinson's disease might be due to the selectivity and specificity of different antibodies used for its detection by immunohistochemistry. To date, there is no consensus on whether ferritin levels are altered in patients or animal models of Parkinson's disease. While some studies report a decrease in L-ferritin (Dexter et al., 1990; Jellinger et al., 1990) other studies suggest that ferritin levels remain unchanged (Mann et al., 1994; Faucheux et al., 2002). Therefore, a more sensitive and accurate analysis is needed to evaluate the expression of the different ferritin isoforms.

Advances regarding the causes and pathophysiology of brain iron accumulation in Parkinson's disease are needed to better understand the origin of iron accumulation and prevent its toxic effects. Magnetic resonance imaging (MRI) has been used to investigate putative biomarkers for Parkinson's disease such as the accumulation of iron or differences in diffusion tensor imaging (DTI) values (Marino et al., 2012). However, the lack of standardization among existing MRI protocols leads to discrepancies in the results (Meijer and Goraj, 2014; Pyatigorskaya et al., 2014; Dashtipour et al., 2015). The aim of this study is to detect abnormal concentrations of iron in the substantia nigra of the 6-OHDA hemiparkinsonian rat model of Parkinson's disease utilizing MRI as well as to investigate possible mechanisms behind this iron accumulation.

EXPERIMENTAL PROCEDURES

Animals and surgery

Female Sprague–Dawley rats weighting between 130 and 150 g (Charles River, Germany) were used in this study (n = 54). Umeå Ethics Committee for Animal Studies in accordance with international guidelines approved all the experimental procedures (Permit A-8 15). The animals were housed in groups of 3–4 per cage and provided with food and water *ad libitum* and were housed on a 12-h light/dark cycle. All efforts were made to minimize animal suffering. Rats were anaesthetized by 2–3% isoflurane (Baster Medical AB, Kista, Sweden) and mounted in a stereotactic frame. 6-OHDA or vehicle injections (Sham) were performed unilaterally into the right medial forebrain bundle using the following coordinates: 4.4 mm posterior, −1.2 mm lateral with respect to Bregma and −7.8 mm below the dura mater. Animals received an injection of 8 μg 6-OHDA-HCl (Sigma, Stockholm, Sweden) dissolved in 4 μl of 0.9% NaCl containing 0.02% ascorbic acid to avoid oxidation of 6-OHDA. Sham animals received an injection of 4 μl containing 0.9% NaCl and 0.02% ascorbic acid. Injections were performed at a rate of 1 μl/min using a 26-gauge Hamilton syringe with a blunt tip. After administration of 6-OHDA or vehicle, the cannula was left in place for an additional 4 min before it was slowly retracted. Animals were divided into three experimental groups. Experimental group 1: Animals were sacrificed two days after administration of 6-OHDA (n = 8) or vehicle (n = 4) and their brains were processed for histological analyses. Experimental group 2: Animals were scanned at different time points after administration of 6-OHDA (n = 8) or vehicle (n = 14) and sacrificed 4 weeks post-lesion and the brains processed for histological analyses. Experimental group 3: Animals were sacrificed 4 weeks after administration of 6-OHDA (n = 11) or vehicle (n = 11) and their brains processed for quantitative polymerase chain reaction (qPCR) analyses. Animals from experimental groups 2 and 3 were evaluated individually for dopamine cell loss by apomorphine-induced rotational behavior at two and three weeks after administration of 6-OHDA. Animals were administrated a low dose of apomorphine (0.05 mg/kg, *s.c.*, Sigma–Aldrich) and the number of contra-lateral rotations were counted during 70 min. Animals that rotated at least 10 rotations/min were selected.

In vivo MRI

Animals in experimental group 2 were scanned after 6-OHDA or vehicle injections. The images were obtained at different time points using a Bruker BioSpec 94/20 USR 9.4 Tesla Preclinical MRI System and running Paravision 6.0 software (Bruker, Ettlingen, Germany). Animals were anesthetized with 4% isoflurane in O_2 and positioned in a 40-mm quadrupolar coil (Bruker). During the scans anesthesia was maintained at 1.5% isoflurane in O_2. Respiratory frequency and core body temperature were monitored using a physiological monitoring system (SA Instruments, Inc.; Stony Brook, USA).

Gadolinium-enhanced MRI

To study BBB permeability, gadolinium (Gd-DTPA, Magnevist; Bayer Schering Pharma, Berlin, Germany)