Neurobiology of Disease 81 (2015) 108–118

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Neurodegeneration with inflammation is accompanied by accumulation of iron and ferritin in microglia and neurons

Maj Schneider Thomsen, Michelle Vandborg Andersen [1], Pia Rægaard Christoffersen [1], Malene Duedal Jensen [1], Jacek Lichota, Torben Moos *

Laboratory for Neurobiology, Biomedicine Group, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark

ARTICLE INFO

Article history:
Received 8 November 2014
Revised 3 March 2015
Accepted 12 March 2015
Available online 20 March 2015

Keywords:
Ferritin
Hepcidin
Iron
Microglia
Monocyte
Neurodegeneration

ABSTRACT

Chronic inflammation in the substantia nigra (SN) accompanies conditions with progressive neurodegeneration. This inflammatory process contributes to gradual iron deposition that may catalyze formation of free-radical mediated damage, hence exacerbating the neurodegeneration. This study examined proteins related to iron-storage (ferritin) and iron-export (ferroportin) (aka metal transporter protein 1, MTP1) in a model of neurodegeneration. Ibotenic acid injected stereotactically into the striatum leads to loss of GABAergic neurons projecting to SN pars reticulata (SNpr), which subsequently leads to excitotoxicity in the SNpr as neurons here become vulnerable to their additional glutamatergic projections from the subthalamic nucleus. This imbalance between glutamate and GABA eventually led to progressive shrinkage of the SNpr and neuronal loss. Neuronal cell death was accompanied by chronic inflammation as revealed by the presence of cells expressing ED1 and CD11b in the SNpr and the adjacent white matter mainly denoted by the crus cerebri. The SNpr also exhibited changes in iron metabolism seen as a marked accumulation of inflammatory cells containing ferric iron and ferritin with morphology corresponding to macrophages and microglia. Ferritin was detected in neurons of the lesioned SNpr in contrast to the non-injected side. Compared to non-injected rats, surviving neurons of the SNpr expressed ferroportin at unchanged level. Analyses of dissected SNpr using RT-qPCR showed a rise in ferritin-H and -L transcripts with increasing age but no change was observed in the lesioned side compared to the non-lesioned side, indicating that the increased expression of ferritin in the lesioned side occurred at the post-transcriptional level. Hepcidin transcripts were higher in the lesioned side in contrast to ferroportin mRNA that remained unaltered. The continuous entry of iron-containing inflammatory cells into the degenerating SNpr and their subsequent demise is probably responsible for iron donation in neurodegeneration. This is accompanied by only a slight increase in neuronal ferritin and not ferroportin, which suggests that the iron-containing debris of dying inflammatory cells and degenerating neurons gets scavenged by invading macrophages and activated microglia to prevent an increase in neuronal iron.

Introduction

Conditions with progressive neurodegeneration in the substantia nigra (SN) like Parkinson's disease (PD) and pantothenate kinase-associated neurodegeneration (aka neurodegeneration with brain iron accumulation (NBIA) formerly Hallervorden–Spatz disease) are accompanied by chronic inflammation (Hayflick et al., 2006; Rouault, 2013; Andersen et al., 2014; Zecca et al., 2004; Levi and Finazzi, 2014), which contributes to gradual deposition of iron in affected brain areas (Andersen et al., 2014). The chronic deposition of iron may catalyze formation of free-radical mediated damage, hence gradually exacerbating the neurodegenerative stage (Filomeni et al., 2012). A mechanism by which iron can accumulate in brain areas with neurodegeneration is through the accumulation of iron-containing inflammatory cells such as macrophages (Rathnasamy et al., 2013). The macrophages phagocytose damaged neurons and subsequently undergo apoptosis and deposit their iron in the extracellular space (Neher et al., 2011; Reif and Simmons, 1990; Wirenfeldt et al., 2007; Tabas et al., 2009; Rathnasamy et al., 2013). This may interfere with the otherwise healthy neurons causing reactive oxygen species (ROS)-mediated damages, ranging from acute interference with the neuronal cellular membrane

Abbreviations: BBB, blood–brain barrier; DAB, 3,3′-diaminobenzidine tetrahydrochloride; iNOS, inducible nitric oxide synthetase; KPBS, potassium phosphate-buffered saline; NBIA, neurodegeneration with brain iron accumulation; NO, nitric oxide; NTBI, non-transferrin bound iron; P, post-surgery; PD, Parkinson's disease; ROS, reactive oxygen species; SN, substantia nigra; SNpr, substantia nigra pars reticulata.

* Corresponding author at: Laboratory for Neurobiology, Biomedicine Group, Department of Health Science and Technology, Fr. Bajers Vej 3B, 1.216, Aalborg University, DK-9220 Aalborg East, Denmark.
E-mail address: tmoos@hst.aau.dk (T. Moos).

[1] Equal contribution.

Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.03.013
0969-9961/

and rapid cell death to chronic changes like post-translational modification of neuronal proteins e.g., propagation of alpha-synuclein oligomer formation and subsequent aggregate formation (Uversky et al., 2001; Riedlerer, 2004).

We recently reviewed the handling of iron in brain regions with extensive iron accumulation (Andersen et al., 2014). Iron is probably released into the extracellular space from dying neurons, apoptotic macrophages that have migrated into the brain, and glial cells. Iron may bind to transferrin which then facilitates the uptake of iron in healthy neurons containing transferrin receptors. This uptake mechanism is expected to cease via down-regulation of the neuronal receptor in conditions with ample iron (Moos et al., 1998). Neurons also take up non-transferrin bound iron (NTBI) that is unlikely to be lower in iron excess (Pelizzoni et al., 2011, 2012). The neuronal uptake of NTBI will induce ferritin expression as a means to scavenge the iron accumulation (Rouault, 2013). As ferroportin excretes ferrous iron across the cellular membrane, neurons may regulate their iron content by excretion. Theoretically the neurons would also increase their expression of ferroportin in conditions with iron-overloading, as the ferroportin mRNA has an iron-responsive element (Zhang et al., 2009). Ferroportin is widely expressed in neurons of the CNS and is often co-expressed with transferrin receptors in neurons thought to contain large amounts of iron, e.g., neurons of the medial habenular nucleus and neurons of the interpeduncular nucleus (Moos and Rosengren Nielsen, 2006; Moos et al., 1998). Counteracting the significance of neuronal ferroportin in pathological conditions, hepcidin, which is a hormone secreted from hepatocytes in inflammatory conditions and known to post-translationally down-regulate the expression of ferroportin (Nemeth et al., 2004), could enter inflamed brain regions with a compromised blood–brain barrier (BBB), and hence impede the function of ferroportin (Andersen et al., 2014).

The aim of the present study was to investigate the expression of ferritin and ferroportin in an experimental in vivo model of neurodegeneration with inflammation in the brain (Sastry and Arendash, 1995). In this model, ibotenic acid, a glutamate agonist, is injected into the striatum, which leads to cell death among a great number of GABAergic neurons projecting to neurons of the substantia nigra pars reticulata (SNpr). In turn, this will result in an overstimulation of these SNpr neurons by glutamate projecting from neurons of the nucleus subthalamicus. Eventually, this imbalance between glutamate and GABA leads to the death of the SNpr neurons and chronic inflammation in this site remote in distance from the injection site of ibotenic acid. The resulting pathology of the SNpr is followed by a significant 42% increase in total iron when measured as milligram iron per milligram protein after 1 month (Sastry and Arendash, 1995). We hypothesized that the time dependent increase in cellular iron in the SNpr with chronic neurodegeneration and accompanying inflammation would lead to changes in ferroportin and ferritin levels to facilitate neuronal iron export and storage, respectively.

Materials and methods

Animals

Forty-six male Wistar rats (Charles River Laboratories, Wilmington, DE) weighing 250–300 g were housed in cages at the Animal Department of Aalborg University Hospital under constant temperature and humidity conditions and a 12 hour light/dark cycle with free access to food and water. The handling of the animals in this study was approved by the Danish Experimental Animal Inspectorate under the Ministry of Food and Agriculture.

Surgical procedure

Ibotenic acid was stereotactically injected in quadruplicate into the striatum, which leads to loss of GABAergic neurons in the striatum including their fibers projecting to the SNpr (Fig. 1A) (Sastry and Arendash, 1995). The rats were anesthetized using hypnorm–dormicum (20 mg/100 g) injected subcutaneously and placed in a stereotactic apparatus. The skin was incised along the midline exposing the skull. Ibotenic acid (Sigma-Aldrich) was injected using a 10 μl Hamilton Syringe in doses of 5 μg dissolved in 1 μl PBS into the left striatum at two depths in each drill hole at coordinates 0,35; 3,05; 4,2 and 5,5 mm (anterior; lateral; ventral) and −1,2; 3,65; 4,5 and 6,2 mm (anterior; lateral; ventral) relative to bregma using a stereotactic atlas for orientation (Paxinos and Watson, 1986).

Tissue preparation

At post-surgery (P) day 3 (n = 8), day 7 (n = 5), day 28 (n = 4), and day 91 (n = 8), the rats were euthanized with an overdose of hypnorm–dormicum injected subcutaneously and transcardially perfused via the left ventricle, first with saline and then with 4% paraformaldehyde in 0.01 M potassium phosphate-buffered saline (KPBS), pH 7.4. The brains were dissected and post-fixed in 4% paraformaldehyde overnight at 4 °C. A control group of non-injected rats was also included and examined corresponding to post-surgery days 3 (n = 3) and 91 (n = 8). Serial coronal sections (40 μm) through striatum and SN were cut on a cryostat and collected free-floating in 0.1 M PBS, pH 7.4 in a sequential series of six. For RT-qPCR analyses, rats were euthanized with an overdose of hypnorm–dormicum injected subcutaneously at post-surgery interval day 91 (n = 4), and the brains rapidly dissected on ice under a dissecting microscope to isolate the ventral mesencephalon containing the SNpr from both the operated and non-operated sides. The ventral mesencephalon containing the SNpr from age-matched non-injected rats was similarly isolated bilaterally (n = 6).

Cresyl violet staining

Cresyl violet staining was used to quantify neurodegeneration. The sections were washed in KPBS three times and then immersed in a 0.5% gelatin solution, mounted on glass slides and dried. Subsequently, they were stained for 5 min in cresyl violet and rinsed in running tap water. The sections were then dehydrated in graded alcohols, dried and sealed in Pertex Mounting Media. To quantify the degeneration of the SNpr, the width was measured using the center of the interpeduncular nucleus as a reference of orientation. A horizontal line and a line at an angle of 45° through each SN were measured to divide the SNpr into a ventro-medial and a dorso-lateral part (Fig. 2A).

Immunohistochemistry

The sections were pre-incubated in blocking buffer consisting of 3% swine serum diluted in 0.01 M KPBS with 0.3% Triton X-100 (Sigma) for 30 min at room temperature to block any unspecific binding. The sections were then incubated overnight at 4 °C with primary antibodies diluted in blocking buffer: Mouse anti-rat CD11b (OX42) (Serotec) diluted 1:100, mouse anti-rat CD68 (ED1) (Abcam) diluted 1:100, rabbit anti-human ferritin (Dako) diluted 1:200, and rabbit-anti ferroportin (Abboud and Haile, 2000) diluted 1:1000. The polyclonal antibody raised against ferritin also reacts with both isotypes of rat ferritin (Hansen et al., 1999). Next day, the sections were incubated for 30 min at room temperature with biotinylated goat anti-mouse immunoglobulin (Dako) or biotinylated swine anti-rabbit immunoglobulin (Dako) both diluted 1:200 in KPBS. Binding of the antibodies was visualized using the ABC-system and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Moos et al., 2006).

Iron staining

The Prussian blue-DAB staining method was used to demonstrate ferric iron (Moos et al., 2006). In brief, sections were treated with 2%