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Research Paper

Intraspinal TLR4 activation promotes iron storage but does not protect neurons or oligodendrocytes from progressive iron-mediated damage

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ABSTRACT

Iron is essential for basic cellular functions but in excess is highly toxic. For this reason, free iron and iron storage are controlled in the periphery by elaborate regulatory mechanisms. In contrast, iron regulation in the central nervous system (CNS) is not well defined. Given that excess iron is present after trauma, hemorrhagic stroke and neurodegeneration, understanding normal iron regulation and promoting iron uptake in CNS pathology is crucial. Peripherally, toll-like receptor 4 (TLR4) activation promotes iron sequestration by macrophages. Notably, iron-rich sites of CNS pathology typically contain TLR4 agonists, which may promote iron uptake. Indeed, our recent work showed impaired iron storage after acute spinal cord injury in mice with TLR4 deficiency. Here we used a reductionist model to ask if TLR4 activation in the CNS stimulates iron uptake and promotes neuroprotection from iron-induced toxicity. For this, we measured the ability of microglia/macrophages to sequester exogenous iron and prevent pathology with and without concomitant intraspinal TLR4 activation. Results show that, similar to the periphery, activating intraspinal TLR4 *via* focal LPS injection increased mRNA encoding iron uptake and storage proteins and promoted iron sequestration into ferritin-expressing macrophages. However, this did not prevent oligodendrocyte and neuron loss. Moreover, replacement of oligodendrocytes by progenitor cells – a normally robust response to *in vivo* macrophage TLR4 activation – was significantly reduced if iron was present concomitant with TLR4 activation. Thus, while TLR4 signaling promotes CNS iron uptake, future work needs to determine ways to enhance iron removal without blocking the reparative effects of innate immune receptor signaling.

1. Introduction

Iron is essential for virtually all cellular processes, including energy production, proliferation and protein synthesis. Iron is highly reactive, however, and in excess will induce oxidative radicals that directly damage proteins, DNA and lipids. Thus, excess iron in the central nervous system (CNS) following injuries such as hemorrhagic stroke or neurotrauma can exacerbate neuropathology. Iron also accumulates in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Zecca et al., 2004). Thus, excess iron within the pathological CNS may be a common mechanism for progressive neuron and glial loss (Armstrong et al., 2001; Uttara et al., 2009; Caliaperumal et al., 2012). How iron is handled in the intact and injured CNS, however, is not yet well understood.

In addition to iron, sites of CNS disease and trauma commonly contain ligands that activate toll-like receptor 4 (TLR4) (Kigerl and Popovich, 2009). This is relevant as systemic TLR4 activation promotes iron uptake and sequestration by macrophages. Indeed, iron chelation by innate immune cells (*e.g.*, macrophages) is a key mechanism for controlling infection. When pathogens bind TLR4, signaling pathways are initiated that promote iron sequestration, thereby restricting iron from microbes, which need it for proliferation and infiltration (Nairz et al., 2010). Given that TLR4-expressing microglia and macrophages accumulate at CNS injury sites, central TLR4 signaling may be an endogenous repair mechanism that limits the toxicity of excess iron at sites of neuropathology. In support of this, our prior work showed that functional recovery and intraspinal iron storage were impaired after

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spinal cord injury in mice with deficient TLR4 signaling (Kigerl et al., 2007; Church et al., 2016). Also, activation of intraspinal TLR4 by a non-pathogenic TLR4 agonist promotes myelin repair in a model of CNS demyelination (Church et al., 2017).

To test the hypothesis that TLR4 activation stimulates iron uptake in the CNS, we measured the ability of microglia/macrophages to sequester exogenous iron and prevent iron-induced pathology with and without concomitant TLR4 activation. A reductionist non-traumatic microinjection model was used to allow focusing on the specific interaction between TLR4 activation and iron, without the confounds of other pathological mechanisms present in CNS disease or trauma. Results show that TLR4 activation increases intraspinal mRNA for iron storage proteins and enhances iron uptake and storage by microglia and ferritin-positive macrophages. However, enhanced TLR4 signaling did not prevent iron-induced neurotoxicity or oligodendrocyte loss. Moreover, replacement of oligodendrocytes by progenitor cells – a normally robust response to intraspinal TLR4 activation – was significantly impaired when iron was present at the time of TLR4 activation.

Collectively, these data indicate that it is possible to co-opt intraspinal inflammation to enhance iron sequestration. However, alone, this is not a mechanism that can overcome the pathology caused by excessive inflammation and/or iron accumulation. For neurological diseases associated with intraparenchymal iron overload (*e.g.*, brain/spinal cord injury, stroke), additional strategies are needed to enhance iron uptake without also eliciting inflammatory-mediated cytotoxicity.

2. Material and methods

2.1. Neonatal spinal cord microglia

Neonatal spinal cord microglia were cultured as previously described with minor modifications (McCarthy and de Vellis, 1980; Kerstetter and Miller, 2012). Briefly, 2-day-old Sprague–Dawley rat pups were decapitated and their vertebrae were removed by cutting bilaterally along the length of their spinal columns. After the removal of meninges, the spinal cords were isolated and cut into small pieces, digested in TrypLE express (Thermo Fisher) for 30 min and neutralized with cell culture media containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamax, 100 IU/ml penicillin and 100 μg/ml streptomycin. After brief centrifugation, the spinal cord tissue was resuspended and triturated in cell culture media and the resulting cell suspension was plated into 75 cm^2 tissue culture flasks coated with 10 μg/ml poly-L-lysine. The mixed glial cultures were maintained for 10 d in cell culture media with media changes on days 3 and 6. On day 10, the mixed glial cultures were vigorously shaken for 18 h on a rotary shaker incubator (240 rpm) and the detached cells were collected. Oligodendrocyte progenitors and microglia in the cell suspension were separated by plating the cells in uncoated plastic wells, to which only microglia adhere. The following day, microglia were stimulated with $FeCl_3$ (1 mM), LPS (100 ng/ml; Sigma 0111:B4), and a combination of both with or without TAK-242 (0.5 μM; Millipore) for 24 h. Microglia were then either fixed in 4% PFA for 30 min or homogenized in TRIzol reagent (Life Technologies).

2.2. In vitro iron uptake

Fixed microglia were stained for non-heme bound iron using a modified Perls Prussian blue staining protocol. Briefly, endogenous peroxidase activity was quenched using a 4:1 mixture of methanol and 30% hydrogen peroxide for 15 min followed by an incubation of the cells in a 1:1 mixture of 4% HCl and 4% potassium ferrocyanide solution (Polysciences, Inc.) for 30 min. Finally, after 10 min incubation with 0.1% Triton X-100 in PBS, the Prussian blue signal was amplified using DAB with nickel (Vector). DAPI counterstain was used to identify the nuclei. Then, images from 50 arbitrary viewfields were taken from each well (n = 4/group) using an ArrayScanXTI High Content Analysis Reader (Thermo Fisher) and the images were analyzed using the associated HCS Studio Cell Analysis Software to identify the number of cells positive for Perls stain per viewfield. The percentage of iron positive cells per well was calculated and reported.

2.3. Glutamate and Griess assay

Adult rat bone marrow-derived macrophages (BMDMs) were generated using a modified protocol described previously (Longbrake et al., 2007). Briefly, bilateral femurs and tibias were dissected from adult Sprague Dawley rats. Using a 23 gauge needle, bone marrow was flushed into sterile conical tubes using a syringe filled with ice cold DMEM. Cells were triturated into a single cell solution, and red blood cells were lysed in lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). After washing, cells were plated at 1×10^6 cells per ml of DMEM supplemented with 10% FBS, 20% L929 supernatant, 0.5% gentamicin, 1% HEPES, 1% glutamax, and 0.001% β-mercaptoethanol. Supernatant from L929 cells contains macrophage colony stimulating factor (CSF1) required to drive bone marrow cells to differentiate into macrophages (Burgess et al., 1985). After 7 d *in vitro*, BMDMs were replated into wells with DMEM, supplemented with 10% FBS, 1% glutamax, and 0.25% gentamicin. The following day, BMDMs were stimulated with the either $FeCl_3$ (1 mM), LPS (100 ng/ml, Sigma; 0111:B4) or both LPS and $FeCl_3$ for 24 h. Supernatants were collected and spun at 13,000 × *g* to remove insoluble material. Colorimetric glutamate (Sigma) and Griess (ThermoFisher Scientific) assays were conducted according to manufacturer's instructions.

2.4. Solution preparation for in vivo microinjections

Vehicle and isosmotic ferric-citrate solutions were prepared as described by McDonald et al. (2002). First, the vehicle solution was made using sodium citrate-dihydrate (180 mM), sodium bicarbonate (11.4 mM), and tris base (0.455 M), in 0.1 M sterile PBS. A 0.75 nM ferric citrate solution was made with ferric chloride hexahydrate (100 mM) diluted with vehicle. LPS (1 mg/ml, Sigma; 0111:B4) was similarly diluted in the vehicle solution. LPS + iron solution consisted of 2 × concentrated LPS and ferric-citrate solution mixed 1:1. All solutions were filter sterilized using a 0.22 μm filter, and iron + LPS solutions were mixed immediately before use.

2.5. Intraspinal microinjections

All surgical and postoperative care procedures were performed in accordance with The Ohio State University Institutional Animal Care and Use Committee. Adult female Sprague-Dawley rats (~250 g; n = 56) were randomly assigned to treatment groups (vehicle control, iron, iron + LPS, LPS; n = 10/group), then anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Using aseptic technique, a laminectomy was performed at the T8 vertebral level. Custom pulled UV-sterilized glass micropipettes beveled to an outer tip diameter of 25–40 μm were loaded with the proper solution and positioned 0.7 mm lateral to the dorsal spinal cord midline. Using a hydraulic micropositioner (David Kopf Instruments, Tujunga, CA), pipettes were lowered 1.1 mm into the spinal cord. For histological experiments, a 500 nl bolus injection was administered to the lateral gray-white matter border using a PicoPump (World Precision Instruments). For tissue RNA experiments, a 200 nl bolus injection was administered bilaterally in the lateral gray-white matter border. Injection sites were labeled with sterile charcoal (Sigma), muscles surrounding the laminectomy were sutured, skin was stapled with wound clips, and rats were given 5 cm^3 sterile saline (subcutaneous) before being placed into a warmed recovery cage. Two rats died due to complications with anesthesia.