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Low brain iron effects and reversibility on striatal dopamine dynamics

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ABSTRACT

Iron deficiency (ID) in rodents leads to decreased ventral midbrain (VMB) iron concentrations and to changes in the dopamine (DA) system that mimic many of the dopaminergic changes seen in RLS patient where low substantia nigra iron is a known pathology of the disease. The ID-rodent model, therefore, has been used to explore the effects that low VMB iron can have on striatal DA dynamics with the hopes of better understanding the nature of iron–dopamine interaction in Restless Legs Syndrome (RLS). Using a post-weaning, diet-induced, ID condition in rats, the No-Net-Flux microdialysis technique was used to examine the effect of ID on striatal DA dynamics and it reversibility with acute infusion of physiological concentrations of iron into the VMB. This study replicated prior findings by showing that the ID condition is associated with increased extracellular striatal DA, reduced striatal DA uptake, and blunted DA-2-receptor-agonist feedback enhancement of striatal DA uptake. Despite the increase in extracellular striatal DA, intracellular striatal DA, as determined in tissue homogenates, was decrease in the ID rat. The study's key finding was that an infusion of physiological concentrations of iron into the VMB reversed the ID-induced increase in extracellular striatal DA and the ID-induced decrease in intracellular striatal DA but had no effect on the ID-induced changes in DA uptake or on the blunted DA-uptake response to quinpirole. In summary, the ID-rodent model provides highly reproducible changes in striatal DA dynamics that remarkably parallel dopaminergic changes seen in RLS patients. Some but not all of these ID-induced changes in striatal DA dynamics were reversible with physiological increases in VMB iron. The small changes in VMB iron induced by iron infusion likely represent biologically relevant changes in the non-transferrin-bound labile iron pool and may mimic circadian-dependent changes that have been found in VBM extracellular iron.

Introduction

Restless Legs Syndrome (RLS) is a neurological disease with well defined pathology (Earley, 2003). Brain autopsy, MRI and ultrasound studies have demonstrated reduced iron stores in substantia nigra (Allen et al., 2001; Connor et al., 2003; Godau et al., 2007; Rizzo et al., 2013; Schmidauer et al., 2005). Given the sensitivity of the RLS symptoms to levodopa and to dopamine agonists (Earley, 2003) and the fact that dopamine neurons in the substantia nigra have low iron levels (Connor et al., 2003), RLS studies have also focused on the dopaminergic (DAergic) system and its relation to low substantia nigra iron. Both autopsy assessment and PET imaging have revealed changes in the striatal DAergic system (Connor et al., 2009; Earley et al., 2011, 2013). Therefore to better understand the nature of iron–dopamine interaction in RLS, investigations have focused on understanding the effects of low substantia nigra iron on striatal DAergic dynamics.

Using a post-weaning, diet-induced iron deficiency (ID) condition in rodents to reduce VMB (i.e., substantia nigra) iron, studies have demonstrated that ID increases extracellular striatal dopamine (DA) (Beard et al., 1994), decreases striatal dopamine-2 receptor (D2R) density (Erikson et al., 2001) and diminishes in vitro dopamine transporter (DAT) density and function but not DA release (Erikson et al., 2000). Similar to the findings in the ID rodent, RLS striatal dopaminergic pathology has shown diminished D2R receptor (Connor et al., 2009), decreased membrane-bound DAT (Earley et al., 2011) and increased synaptic DA levels (Earley et al., 2013). Thus the post-weaning, diet-induced ID condition appears to provide a biological model to understanding the iron–dopamine connection in RLS.

Studies using the diet-induced, ID condition in rodents have demonstrated a strong association between VMB iron concentrations, gene expression of monoamine transporters, and regional monoamine levels (Erikson et al., 2000, 2001). MRI studies in RLS have shown not only lower iron concentrations in the substantia nigra but also that iron concentrations correlated negatively with RLS symptoms severity (Lee et al., 2007). This finding suggests that small variations in substantia nigra iron concentrations may be pertinent to, at least, disease severity. It has been shown in mice that small but significant changes in

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extracellular VMB iron occur in a circadian manner (Unger et al., 2013). The physiological significance of these small circadian variations in VMB iron is unknown because studies using low physiological concentrations of iron have not been done. Understanding how small physiological changes in extracellular iron, similar to those found with circadian variation, impact DAergic function may be pertinent to our understanding of the role of iron dynamics in influencing DA dynamics in RLS, especially given the distinct circadian nature of RLS symptoms. The primary goals of the current study were to validate and extend previous findings on changes in the striatal DAergic system under ID conditions and to examine the effects of perfusing physiological concentrations of iron directly into the VMB on these ID-induced changes in striatal DA dynamics.

Materials and methods

Animals

Male weanling (postnatal day 21) Sprague–Dawley rats (Harlan, Indianapolis, IN) were randomly divided into a Control group fed an iron-sufficient diet (80 μg iron/g diet) and an ID group fed a low iron diet (4 μg iron/g diet). The 4 μg iron/g diet maintained for 5–6 weeks has been shown in prior studies to produce a decrease in VMB iron in Sprague–Dawley rats (Bianco et al., 2008; Unger et al., 2008). Control and ID diets were prepared in our laboratory as described previously (Reeves et al., 1993). The ID diet contained all components of the control diet with the exception of ferric citrate. Rats consumed control or ID diets for 5 weeks, and were provided food and deionized distilled water ad libitum throughout the study. All rats were housed 2 per cage in a temperature (21 ± 2 °C) and humidity (40%) controlled room maintained on a 12:12 h light/dark cycle (lights on at 0600) until surgeries were performed. Post-surgery, rats were housed 1 per cage and continued on a 12:12 h light/dark cycle throughout the microdialysis experiments. Experimental protocols were in accordance with the NIH Animal Care guidelines and were approved by the Pennsylvania State Institutional Animal Care and Use Committee.

Surgery

Rats were anesthetized with 4% isoflurane, and the top of the head was shaved and sterilized with providone/iodine and 70% ethanol. After placement on a stereotaxic frame (Stoelting, Wood Dale, Il), the skull was exposed and bregma identified. A hole was drilled through the skull, and a CMA 11 guide cannula was implanted into the striatum of all control and ID rats as previously described (Bianco et al., 2008). The coordinates used were: A −0.2 mm, L −2.8 mm, and V −5.3 mm. In a subset of rats from each diet group, a second guide cannula for iron infusions was implanted in the substantia nigra following the same protocol. The coordinates used for substantia nigra were: A 2.0 mm, L −6.5 mm, and V −5.7 mm. All rats received a sham surgery in the region of iron infusion in the contra-lateral side of the brain.

After 3–5 day recovery time, rats were lightly anesthetized with 25.0/2.0/0.5 mg/kg ketamine/xylazine/acepromazine. A CMA 11 (2 mm) probe was then inserted into the guide cannula implanted in the striatum of all rats. In rats with a guide cannula implanted in substantia nigra, an infusion cannula (2 mm) was inserted into the guide cannula. The rats were allowed to recover overnight with a slow perfusion of artificial cerebrospinal fluid (aCSF; 1.3 μL/min) into the microdialysis probe (Bianco et al., 2008).

NNF/quinpirole NNF

The No-Net-Flux (NNF) microdialysis technique provides quantitative real-time in vivo determination of extracellular neurotransmitter concentrations by utilizing the principle of diffusion, which takes into account both diffusion and catabolism (Mefford, 1981; Refshauge et al., 1974). In this study, two primary measures of extracellular DA are determined from NNF technique: extraction fraction and the extracellular DA concentration (Bungay et al., 2003; Chefer et al., 2006). The extraction fraction has been shown to reflect primarily DAT-dependent neurotransmitter uptake (Smith and Justice, 1994) and has been used as an estimate of DAT function (Bianco et al., 2008). For greater details of the NNF technique and its application, see our prior publication (Bianco et al., 2008) but in brief, artificial CSF samples containing 100, 50, 20, and 10 nM DA were infused into the brain via the microdialysis probe as described previously (Bianco et al., 2008; Chefer et al., 2006). Each concentration of DA was perfused for 75 min, with a random ordering for each rat. The perfusate was collected continuously into a loading loop on the HPLC and injected in 15 min intervals. After the last concentration of DA for NNF was perfused, a potent DA-2/3 receptor agonist, quinpirole (5 μM), was added to the perfusate. NNF was performed after equilibration (1.5 h) using 100, 50, 20, and 10 nM DA + 5 μM quinpirole in artificial CSF. This equilibration time for quinpirole was chosen based on previous studies, which demonstrated that the maximal effect of quinpirole occurs 1–2 h after perfusion is initiated (Mao et al., 1996; See et al., 1991). Quinpirole through its agonist effects on DA-2/3 receptors and subsequent feedback to pre-synaptic mechanism will decrease DA release and increase membrane-bound DAT and extraction of DA from the extracellular space (Bolan et al., 2007; Cooper et al., 2008; Robinson, 2002). Used within the context of the NNF techniques, it provides an assessment of the integrity of the feedback-loop mechanism that can modify pre-synaptic functions like DAT density and DA release.

Iron infusion

Ferrous sulfate ($FeSO_4$, 1 μM) was infused as described previously at a rate used in previous studies (Prikhojan et al., 2002; Santiago et al., 2000) of 1.3 μL/min for 20 min resulting in the delivery of 26 picomole (pmol) of iron into the extracellular space. This concentration of extracellular iron is within the physiological range seen with the circadian peak-to-trough differences in extracellular VMB iron (Unger et al., 2013). In a previous study, infusion of 10–100 μM $FeCl_3$ into the striatum did not cause lipid peroxidation or affect neuronal health (Santiago et al., 2000). The infusion of iron into the substantia nigra was started at 2400 h and into the striatum at 0600 h, with the NNF/quinpirole NNF experiment being performed starting at 0600 h. In the contra-lateral brain region, sterile saline was perfused under the same conditions. As has been previously described (Bianco et al., 2008; Connor et al., 1992), verification of cannula placement and distance of iron diffusion was determined with cytology and iron histochemistry, respectively. The distance of iron diffusion was on average about 350 μm from the infusion site.

High performance liquid chromatography

Dialysate samples (10 μL) were injected every 15 min onto an ESA MD-150 narrow-bore HPLC column 150 × 2 mm (ESA Inc., Chelmsford, MA) for separation followed by detection by an ESA 5014B microdialysis cell (+300 mV; ESA Coulochem III, ESA, Inc., Chelmsford, MA). A guard cell (ESA 5020) placed in line before the injection loop was set at a potential of +350 mV. The mobile phase consisted of 75 mM sodium phosphate monobasic (EMD Chemical, Gibbstown, NJ), 1.7 mM 1-octanesulfonic acid (EMD Chemical), 25 μM EDTA (Acros, Morris Plains, NJ), 10% acetonitrile (EMD), and 0.01% triethylamine (Sigma Aldrich, St. Louis, MO) in a volume of 1 L (pH 3.0). The neurotransmitter and metabolite peak areas were integrated using EZ Chrom Elite software (Scientific Software Inc., Pleasanton, CA) and quantified against known standards of dopamine (DA) (ESA Inc., Chelmsford, MA), DA