Neuroscience 246 (2013) 179–185

EFFECTS OF IV IRON ISOMALTOSIDE-1000 TREATMENT ON REGIONAL BRAIN IRON STATUS IN AN IRON-DEFICIENT ANIMAL

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Abstract—*Background:* Iron deficiency has been documented to affect human cognitive function and conditions with brain iron compromise such as the restless legs syndrome (RLS). Intravenous (IV) iron treatment is used to reduce iron deficiency but its effects on brain iron are not known. It is not known if IV iron is effective in correcting regional brain iron deficiencies nor if it poses a risk of producing iron overload in some brain regions. Preclinical study of IV iron in the iron-deficient (ID) murine model is needed to evaluate and develop IV iron treatments for brain iron deficiency.

***Methods:* Response to tail vein injections of iron (iron isomaltoside-1000, dose equivalent to 1000 mg for 75 kg adult) or vehicle were evaluated for ID mice by microdialysis assessing non-transferrin bound (NTB) iron in the ventral midbrain (VMB) and autopsy at 3 and 10 days post-injection assessing iron content in critical brain regions.**

***Results:* The ID mice showed marked circadian variation in NTB extracellular iron. After iron injection, NTB iron was rapidly increased in the VMB and then decreased over 12 h to the levels observed for vehicle. Regional brain iron content at 3 and 10 days post-injection in the iron- compared to vehicle-treated group showed significantly more iron for the VMB and nucleus accumbens but not for the other regions (i.e. prefrontal cortex, caudate–putamen, cerebellum, and pons), which also did not show decreased iron content with the ID diet.**

***Conclusion:* Iron isomaltoside-1000 given IV corrects the regional brain iron deficiency in these ID mice without producing iron overload in any of the brain regions studied. This is the first demonstration of effects of IV iron in the brain and it provides a useful preclinical model for this assessment, particularly relevant for developing iron treatments for conditions with problematic iron deficiency, e.g. RLS.**

Key words: restless legs syndrome, Willis-Ekbom disease, intravenous iron isomaltoside 1000, circadian brain iron, regional brain iron, microdialysis.

INTRODUCTION

Dietary iron deprivation has long been used in mice and rats to evaluate the effects both on brain iron homeostasis and on the relation of the experimentally-induced brain iron deficiency to changes in neurotransmitter systems (Erikson et al., 2000). Many of these studies focused on iron deficiency in the striatum and ventral midbrain (VMB), which contains the substantia nigra (Erikson et al., 2000). The brain-iron features of iron deficient (ID) rodents are similar to clinical data from patients or autopsy cases with restless legs syndrome (RLS), also known as the Willis-Ekbom disease, where brain iron insufficiency has been implicated in the pathology of the disease (Earley et al., 2000; Allen and Earley, 2003; Connor et al., 2011). Patients with RLS have decreased brain iron reported for several regions (Godau et al., 2008) but most consistently for the substantia nigra documented by magnetic resonance imaging (MRI) (Allen et al., 2001; Earley et al., 2006), ultrasound (Schmidauer et al., 2005; Godau et al., 2007) and autopsy (Connor et al., 2003).

Given the brain iron insufficiency state in RLS, it had been assumed that a large intravenous (IV) dose of iron would overcome the intestinal limits on absorbing oral iron and therefore provide a large iron supply to the brain, thus reduce the brain iron insufficiency and thereby reduce RLS symptoms. The clinical studies of IV iron in RLS, however, showed limited if any response to iron–sucrose (Earley et al., 2009; Grote et al., 2009) but some good responses to the iron-formulations with the tighter iron–carbohydrate bound formulation (Earley et al., 2004; Ondo, 2010; Allen et al., 2011). This may indicate differential iron bioavailability between the compounds with respect to the brain that could produce different effects on the availability of iron to critical brain regions. Curiously, there have been no prior evaluations of changes in brain iron status with IV iron treatment in

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Abbreviations: CB, cerebellum; CP, caudate–putamen; Hgb, hemoglobin; ID, iron deficient; IV, intravenous; NA, nucleus accumbens; NTB, non-transferrin bound; PBS, phosphate-buffered saline; PFC, prefrontal cortex; RLS, restless legs syndrome; TIBC, total iron binding capacity; Tsat, transferrin saturation; VMB, ventral midbrain.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.04.049

rodents or man. The implicit assumption that a large IV dose of a tightly bound iron–carbohydrate formulation would increase brain iron without causing problems of brain-iron overload has never been tested. Iron transport is tightly regulated throughout the body with priority generally given to erythropoiesis, but it is not clear that a single large IV dose would produce significant immediate or long-lasting increase in brain iron. Moreover, iron concentration varies considerably across brain regions in iron-replete and -deficient rodents (Pinero et al., 2000) and similarly, in humans (Allen et al., 2001). Thus, there is a need to determine if IV iron would reduce experimental brain iron deficiency in areas with greater iron loss (e.g. VMB) without producing iron overload in other regions such as the prefrontal cortex (PFC).

IV iron treatments of humans for iron deficiency and for RLS have progressed without first conducting the preclinical animal studies for efficacy and safety of the brain. This study was designed to assess efficacy of IV iron for reducing experimentally induced brain iron deficiency in mice and to evaluate for possible risk of brain iron overload. It used a specific mouse strain (BXD 40 females), which has previously been shown to respond to an ID diet with a significantly greater decrease in VMB iron compared to the decrease in hemoglobin (Hgb) (Jellen et al., 2012; Yin et al., 2012). These provide a dietary murine model of brain iron deficiency that closely mimics the clinical features of RLS, where brain iron insufficiency occurs without complications of significant anemia. With this study, we attempt to address the fundamental question of what brain iron changes occur with IV iron treatment in these mice having been fed an ID diet. The study focused on the effect of IV iron treatment on iron concentrations in the VMB and also areas rich in iron and dopamine (i.e. caudate–putamen (CP) and nucleus accumbens (NA)) that have been considered in some studies to be related to RLS pathophysiology (Connor et al., 2009; Earley et al., 2011). We also broadened our assessment to include other brain regions possibly involved in brain iron deficiency and RLS, namely, the cerebellum (CB), and pons (Bucher et al., 1997). We added one area not known to be involved in RLS or sensitive to iron loss, the PFC) to better evaluate the specificity of IV treatment on regional brain iron concentrations.

EXPERIMENTAL PROCEDURES

Animals

Female mice from Strain 40 of the BXD/Ty RI recombinant inbred strain panel were used in this study. All mice were bred at the Pennsylvania State University. Female strain 40 mice were fed a pelleted, iron-deficient diet (5 mcg/g iron; Teklad TD 8096) beginning on postnatal day (P) 21 (weaning) until they were euthanized. Mice were housed in an isolated environment in groups of two-per cage in a temperature-controlled (22 °C) and humidity-controlled (50%) room with an automatic 12/12-h light/dark cycle (light 0600–1800 h). All mice received food and deionized distilled water *ad libitum.* Experimental protocols followed the National Institutes of Health Animal Care Guidelines and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Drugs

Iron isomaltoside-1000, a new IV iron matrix formulation, was obtained from Pharmacosmos A/S, Holbaek Denmark. It consists of iron and a carbohydrate moiety with tightly bound iron in the iron–carbohydrate formulation. The carbohydrate isomaltoside-1000 is a purely linear chemical structure as shown by ^{1}C NMR of repeating α1–6 linked glucopyranose residues with an average size of 5.2 glucose units, and the aldehyde residues are reduced to less than 1.5% (Jahn et al., 2011) The control vehicle consisted of sterile phosphate-buffered saline (PBS).

IV treatments by tail vein injections. Mice were assigned to one of two treatments, either iron isomaltoside-1000 or vehicle that was injected into the tail vein 3 h before the end of the dark (active) period (06:00 h) at 90 days post weaning (P90). For injections, mice were warmed with a heating blanket and then mildly restrained to locate the tail vein using a red lamp. Iron isomaltoside-1000 was prepared in sterile PBS in an amount scaled to match that used in humans at a dose of 1000 mg iron per person (assumed average size person of 75 kg), which results in a total murine dose of about 0.27 mg.

Experimental conditions and measurements

Two experiments were conducted in this study: (1) Sequential evaluation of extracellular iron in the VMB during 24 h before and 48 h after treatment using microdialysis techniques and (2) analyses of brain and peripheral iron concentrations at the times of euthanasia which were 3 h before the end of the dark period at either 3 or 10 days after iron injection. The microdialysis measures non-transferrin-bound, extracellular iron only in the VMB, while the post-euthanasia studies measure total tissue iron concentrations in multiple brain regions.

Microdialysis. A CMA microdialysis probe with a 60-kd MW cutoff was implanted into the right VMB 4 days prior to iron injections according to previously described procedures (Bianco et al., 2008). All placements were verified in Cresyl Violet-stained brain tissue slices after euthanasia. Mice with probe placement outside of the substantia nigra were excluded from the study. Starting 24 h before iron or vehicle injection, dialysate was collected at a rate of 1.3 μl/min, and individual collection periods were 180 min long to allow for adequate sample volumes for iron analysis. Sampling continued for 48 h post-injection, resulting in a total of 24 samples per mouse. Dialysate was diluted 1:3 in ultrapure nitric acid and iron measured by atomic absorption spectroscopy (Perkin Elmer AAnalyst 600) according to established laboratory procedures (Pinero et al., 2001). All standard curves exceeded $r^2 > 0.99$,