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BLOOD WITHDRAWAL AFFECTS IRON STORE DYNAMICS IN PRIMATES WITH CONSEQUENCES ON MONOAMINERGIC SYSTEM FUNCTION

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Abstract—Iron homeostasis is essential for the integrity of brain monoaminergic functions and its deregulation might be involved in neurological movement disorders such as the restless legs syndrome (RLS). Although iron metabolism breakdown concomitantly appears with monoaminergic system dysfunction in iron-deficient rodents and in RLS patients, the direct consequences of peripheral iron deficiency in the central nervous system (CNS) of non-human primates have received little attention. Here, we evaluated the peripheral iron-depletion impact on brain monoamine levels in macaque monkeys. After documenting circadian variations of iron and iron-related proteins (hemoglobin, ferritin and transferrin) in both serum and cerebrospinal fluid (CSF) of normal macaques, repeated blood withdrawals (RBW) were used to reduce peripheral iron-related parameter levels. Decreased serum iron levels were paradoxically associated with increased CSF iron concentrations. Despite limited consequences on tissue monoamine contents (dopamine – DA, 3, 4-dihydroxyphenylacetic acid – DOPAC, homovanillic acid, L-3, 4-dihydroxyphenylalanine – L-DOPA, 5–8 hydroxytriptamin – 5-HT, 5-hydroxyindoleacetic acid – 5-HIAA and noradrenaline) measured with post-mortem chromatography, we found distinct and region-dependent relationships of these tissue concentrations with CSF iron and/or serum iron and/or blood hemoglobin. Additionally, striatal extracellular DA, DOPAC and 5-HIAA levels evaluated by *in vivo* microdialysis showed a substantial increase, suggesting an overall increase in both DA and 5-HT tones. Finally, a trending increase in general locomotor activity, measured by actimetry, was observed in the most serum iron-depleted macaques. Taken together, our data are compatible with an increase in nigrostriatal DAergic function in the event of iron deficiency and point to a specific alteration of the 5-HT/DA interaction in the CNS that is possibly involved in the etiology of RLS. © **2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: iron, ferritin, dopamine, serotonin, non-human primate, microdialysis, HPLC, actimetry, hyperlocomotion, restless legs syndrome/Willis–Ekbom disease.

INTRODUCTION

Iron homeostasis is essential for fundamental metabolic processes including the physiological functioning of the central nervous system (CNS). Its deregulation affects many aspects of brain neurotransmission (Hentze et al., 2010) and is best known to occur in the restless legs syndrome (RLS)/Willis–Ekbom disease, a prevalent and chronic sensorimotor disorder that typically manifests in a circadian pattern (Allen et al., 2014). In this condition, the interplay between iron metabolism and the dopaminergic (DAergic) transmission is increasingly recognized to be the core of the pathological process (Earley et al., 2014). In patients with RLS, reduced iron stores have been evidenced in circulating blood, in the cerebrospinal fluid (CSF) and in the substantia nigra (Earley et al., 2000; Connor et al., 2003, 2004; Pittock et al., 2004; Mizuno et al., 2005). However, the mechanism by which the reduction in blood iron concentrations impacts the DAergic metabolism and translates into RLS symptoms remains speculative (Earley et al., 2014), especially as brain-iron insufficiency does not mirror systemic iron deficiency (Ben-Shachar et al., 1986; Youdim et al., 1989; Earley et al., 2000; Mizuno et al., 2005). On one hand, compelling evidence from rodents with nutritional iron deficiency supports an increase in DAergic nigrostriatal neurotransmission (Beard et al., 1994; Chen et al.,

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Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-8 hydroxytriptamin; BW, blood withdrawal; CNS, central nervous system; CSF, cerebrospinal fluid; DA, dopamine; DAergic, dopaminergic; DOPAC, 3, 4-dihydroxyphenylacetic acid; Hgb, hemoglobin; HPLC, High- performance liquid chromatography; L-DOPA, L-3, 4-dihydroxyphenylalanine; NA, noradrenaline; NHP, non-human primates; PFC, prefrontal cortex; RBW, repeated blood withdrawals; RLS, restless legs syndrome.

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1995; Nelson et al., 1997; Bianco et al., 2009; Unger et al., 2014), which is in line with human brain autopsy and imaging findings (Connor et al., 2009; Earley et al., 2011, 2013). On the other hand, evidence of reduced DAergic transmission in RLS stems from the excellent pharmacological response to low-dose DAergic medications (Akpinar, 1987; Hening et al., 1999) and from clinical and electrophysiological data reporting enhanced spinal cord excitability (Bara-Jimenez et al., 2000; Aksu and Bara-Jimenez, 2002), which is thought to be the consequence of a reduced dopamine-mediated inhibition from descending supraspinal diencephalospinal L-3, 4-dihydroxyphenylalanine (L-DOPA) neurons, originating in the hypothalamus (A11) (Barraud et al., 2010). In small animals, reduced DAergic transmission at the level of the spinal cord and triggering hyperactive behavior has been shown in rodents following direct injection of 6-hydroxydopamine into the A11 area (Ondo et al., 2000). Combined with bilateral destruction of the A11 area, iron deprivation further significantly augments locomotor activity in comparison to mice that were only iron-deprived (Qu et al., 2007; Zhao et al., 2007). Therefore, it is likely that changes in blood iron concentrations differentially alter the DAergic system function.

Iron deficiency has also been shown to affect other monoaminergic pathways, notably the serotoninergic system (Beard et al., 1994; Chen et al., 1995; Nelson et al., 1997) either directly and/or indirectly by way of interactions between the monoaminergic systems (Fitoussi et al., 2013; Hensler et al., 2013; Engeln et al., 2014). As deregulation of serotonergic transmission also contributes to the development of RLS (Rottach et al., 2008; Jhoo et al., 2010), the causal role of iron-homeostasis breakdown in the etiology of the disorder appears increasingly established.

In this study we sought to evaluate the impact of progressive serum iron depletion on brain monoamine (dopamine (DA), serotonin – 5-8 hydroxytriptamin (5-HT) and noradrenaline – NA) levels in the macaque monkey. After documenting the circadian variations of iron and iron-related proteins (hemoglobin – Hgb, ferritin and transferrin) in both serum and CSF of normal macaques, we developed a repeated blood withdrawal (RBW) protocol (Wills and Stewart, 1935) to induce a progressive serum decrease in levels of iron and other iron-related parameters. Striatal variations in monoamine concentrations and their metabolite levels were first measured using *in vivo* microdialysis and then by high-performance liquid chromatography (HPLC) in post-mortem tissues. To evaluate the behavioral impact of iron depletion, daytime and nighttime general locomotor activity was also assessed in actimetry cages at various time points of the RBW protocol.

EXPERIMENTAL PROCEDURES

Animals

Eighteen adult male *Macaca fascicularis* (Xierxin, Beijing, People's Republic of China; weight = 6.3 ± 1.3 kg; age = 7 ± 2 years) were used in this study. Briefly, 18 control animals were used to measure basal concentrations of iron parameters allowing the homogenization of interindividual differences of physiological values. Nine of them were further submitted to the RBW protocol assorted to baseline and post-RBW locomotor assessments. Two RBW-naive animals, formerly tested for basal iron and iron-related proteins, (equivalent species and age), were added as controls for *in vivo* microdialysis and post-mortem analyses (see Fig. 1 for detailed experimental design). The remaining seven animals that did not go through the RBW protocol, locomotor tests and monoamine assessments were allowed to recover before joining other experimental protocols, thus minimizing the use of non-human primate (NHP) in the present study. All experiments were performed in accordance with the French (87-848, Ministère de l'Agriculture et de la Forêt) and European Communities Council Directive (2010/63/EU) guidelines for the care of laboratory animals. Animals were permanently housed in individual cages (L70 × W60 × H80 cm; Institute of Laboratory Animal Sciences, China Academy of Medical Sciences, Beijing, China) and under controlled conditions (temperature: 24 ± 1 °C, humidity: 50 ± 20%, light/dark cycle: 12-h/12-h, light switched on/off:8-am/8-pm) in an AAALAC-accredited facility following acceptance of the study design and the approval of the Institute of Laboratory Animal Science IACUC (Chinese Academy of Medical Sciences of Beijing, 10021, China). The use of NHP was minimized by using an experimental design that permits statistically significant changes to be demonstrated with the smallest number of animals per group and the smallest number of groups, consistent with good scientific practice. All steps were taken to improve the welfare and avoid the suffering of the animals in accordance with the "Weatherall report for

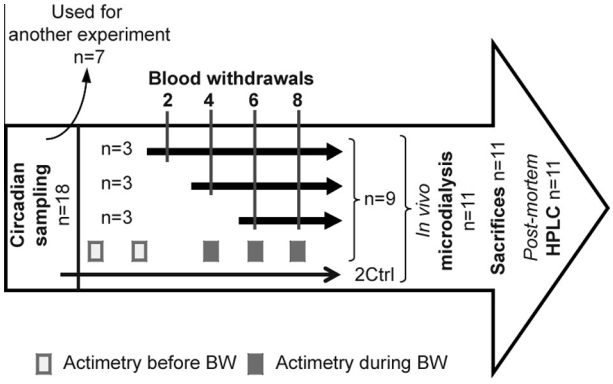

Fig. 1. Chronological experimental design. Eighteen macaques were used for physiological circadian blood sampling. Only nine of them were used for the subsequent RBW protocol: three animals were assigned to each BW condition (i.e. 4, 6, and 8 BW). Thus, all nine animals underwent 4 BW, six animals were subjected to 6 BW and the last three animals to 8 BW. Locomotor activity was assessed in each group at the baseline and throughout the BW protocol, every 2 BW, until completion of the procedure. For *in vivo* microdialysis, two control (Ctrl) animals formerly tested for physiological iron values were added to the nine experimental monkeys. After the completion of *in vivo* microdialysis, all 11 animals were euthanized and brain tissue was collected for further post-mortem analysis. BW = blood withdrawals, Ctrl = control.

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