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Early-postnatal iron deficiency impacts plasticity in the dorsal and ventral hippocampus in piglets

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ABSTRACT

In this study, we investigated whether alterations in plasticity markers such as brain-derived neurotrophic factor (BDNF), p75 neurotrophin receptor ($p75^{NTR}$) and tyrosine receptor kinase B (TrkB) are underlying iron deficiency (ID)-induced cognitive impairments in iron depleted piglets. Newborn piglets were either fed an iron-depleted diet (21 mg Fe/kg) or an iron-sufficient diet (88 mg Fe/kg) for four weeks. Subsequently, eight weeks after iron repletion (190–240 mg Fe/kg) we found a significant decrease in mature BDNF (14 kDa) and proBDNF (18 kDa and 24 kDa) protein levels in the ventral hippocampus, whereas we found increases in the dorsal hippocampus. The phosphorylation of cAMP response element binding protein (CREB) follows the mature BDNF protein level pattern. No effects were found on BDNF and CREB protein levels in the prefrontal cortex. The protein levels of the high affinity BDNF receptor, TrkB, was significantly decreased in both dorsal and ventral hippocampus of ID piglets, whereas it was increased in the prefrontal cortex. Together, our data suggest a disrupted hippocampal plasticity upon postnatal ID.

1. Introduction

Nutritional iron deficiency (ID) in neonates and toddlers negatively affects brain development leading to prolonged functional and structural deficits in the central nervous system (CNS) (Beard and Connor, 2003; Walter, 2003; Beard, 2003; Grantham-McGregor and Ani, 2001; Lozoff et al., 2000). Iron is not only an essential micronutrient required for oxygen transport, it is also a required cofactor for key enzymes in neurotransmitter production (Beard, 2003; Dunkley et al., 2004; Wang et al., 2002). From late prenatal to early postnatal life, the brain undergoes the so-called brain growth spurt. This critical time frame is featured by high demands of iron needed to orchestrate neuronal connectivity and myelination (Dobbing and Sands, 1979; Connor and Menzies, 1996). ID particularly leads to deficiencies in prefrontal cortex and hippocampal signaling (Rao et al., 2013). Interestingly, many of the ID-induced CNS deficits do not recover upon iron repletion and eventually lead to sustained impairments (Bourque et al., 2008; Ranade et al., 2013; Yehuda et al., 1986). In a longitudinal follow-up study, it was shown that children who suffered from severe ID during infancy maintained mental and motor impairment, even ten years after the onset of iron treatment (Lozoff et al., 2000).

Although ID is often studied in rodents, the brain development of piglets more closely resembles human brain development than for instance rats, in part due to the similar timing of the brain growth spurt (Dobbing and Sands, 1979; Conrad et al., 2012). Both piglets and infants are prone to develop CNS deficits due to restrained perinatal iron availability, underscoring the necessity of iron-containing nutrition (Whiteker, 1965; Lipinski et al., 2010). Furthermore, the neonatal porcine and human brain structures are similar; sharing a similar gyral pattern and distribution of gray and white matter (Lind et al., 2007; Pond et al., 2000). Recently, we showed in piglets that pre-weaning dietary ID impairs later spatial learning and memory even after restoring nutritional iron levels (Antonides et al., 2015). Moreover, we confirmed lower iron content in the CA1 and dentate gyrus regions of the hippocampus.

The hippocampus plays an essential role in spatial learning and memory: hippocampal-cortical interactions produce memory traces (Eichenbaum et al., 1996; Preston and Eichenbaum, 2013). Rodent

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models for ID have already shown that iron is required for apical dendrite formation in the CA1 region of the hippocampus (Jorgenson et al., 2003; Brunette et al., 2010). Previously, it has been shown in rats that fetal-neonatal ID downregulated long-term hippocampal brain-derived neurotrophic factor (BDNF) expression in adulthood despite complete iron repletion (Tran et al., 2009). Hippocampal BDNF signaling is strongly associated with synaptic plasticity and memory formation (Gomez-Palacio Schjetnan and Escobar-Rodriguez, 2007). In this study we investigate whether the previously found lower hippocampal iron content and impaired memory performance (Antonides et al., 2015), are linked to sustained impaired BDNF signaling in the hippocampus and prefrontal cortex in piglets. Protein levels of the plasticity markers BDNF, TrkB, $p75^{NTR}$, CREB, synaptophysin, PSD95, and NeuN in the hippocampus and prefrontal cortex were measured.

2. Materials and methods

Ethics note

This study was reviewed and approved by the local animal ethics committee of Utrecht University (DEC, DierExperimenten Commissie) and was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid suffering.

2.1. Animals and housing

The brain tissue used in this study is derived from the animals described in Antonides et al. (Antonides et al., 2015). In brief, hippocampal and prefrontal cortex samples were isolated from piglets [(Terra x Finnish landrace) x Duroc mix] bred at the Faculty of Veterinary Medicine of Utrecht University. Two male sibling piglets with a normal body weight were selected from each of ten different litters (Antonides et al., 2015). The piglets were separated from the sow after 4-6 days. One piglet of each pair was randomly assigned to the treatment group (n = 10) and the other piglet to the control group (n = 10). The piglets in the control group were given a 200 mg iron dextran injection (Gleptosil, 200 mg as gleptoferron MS Schippers, Lommel, Belgium) and fed a control milk diet (88 mg Fe/kg) for four weeks. The piglets of the treatment group were given a saline injection and were fed an iron deficient (ID) milk diet (21 mg Fe/kg). Starting after 4 weeks on the iron-deficient or control diet, all piglets received a balanced, iron-sufficient diet (190–240 mg Fe/kg) for eight weeks. They were tested in a complex spatial holeboard task to assess the effects of pre-weaning iron deficiency on cognitive performance between 3 and 8 weeks after dietary treatment (Antonides et al., 2015).

Two ID piglets were euthanized during the first week as they did not gain weight as expected. One control animal died unexpectedly at 11 weeks. At 12 weeks of age, all animals were euthanized by an intracardial injection with pentobarbital (Euthasol®, AST Farma B.V. Oudewater, The Netherlands). Directly afterwards, brains were dissected and weighed. The prefrontal cortex and both hippocampi were then carefully removed and weighed, after which the right hippocampus was cut in half. Prefrontal cortex, and the ventral and dorsal hippocampus were then snap frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. Sample preparation

Approximately 500 mg of the brain samples were homogenized in 1 ml lysis buffer (100 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.05% triton, 1 tablet complete protease inhibitor mix/20 ml buffer (Roche, Vilvoorde, Belgium), 1 tablet PhosSTOP phosphatase inhibitor cocktail/10 ml buffer (Roche) using a mini-Bead-Beater (BioSpec products, Bartlesville, OK, USA). Samples were homogenized three times for 30 s with 5 min cooling on ice between runs. After 30 min cooling on ice, samples were centrifuged at 16,000*g* for 20 min. (4 °C), and the supernatant was divided into aliquots and stored at −80 °C until further use. Protein concentrations were determined with the Bio-Rad *DC*™ protein assay (Bio-Rad Laboratories, Inc.).

2.3. Western blot

Brain homogenates were separated with SDS-PAGE. Proteins were transferred onto a PVDF membrane, which was subsequently blocked (50% Odyssey blocking buffer in PBS, Li-Cor, Lincoln, NE) for 1 h at room temperature. The membranes were incubated with primary antibody overnight at 4 °C. Antibodies used were 1:250 rabbit anti-TrkB (#4606, Cell signaling technologies Beverly, MA), 1:250 rabbit anti-pTrkB (#ABN1381, Millipore Billerica, MA), 1:1000 rabbit anti-$p75^{NTR}$ (#07-476, Millipore Billerica, MA), 1:1000 mouse anti-synaptophysin (#MAB5258, Millipore), 1:100 rabbit anti-pCREB (#9198S, Cell signaling technologies), 1:1000 mouse anti-CREB (#9104, Cell signaling technologies), 1:500 mouse anti-NeuN (#MAB377, Millipore), 1:2000 mouse anti-PSD95 (#0711, QED Bioscience inc. San Diego, CA), and 1:600 rabbit anti-BDNF (#20981, Santa Cruz Biotechnologies Santa Cruz, CA). For normalization, either GAPDH or β-actin was used: 1:2,000.000 mouse anti-GAPDH (#10R-G109A, Fitzgerald Huissen, NL), and 1:2000 mouse anti-β-actin (#F0110, Santa Cruz Biotechnologies). The membranes were washed with PBS and PBS-Tween, and subsequently incubated with secondary antibody for 1 h at room temperature: 1:5000 goat anti-rabbit IRDye 800 (#926-32211, Li-Cor), and 1:10,000 donkey anti-mouse IRDye 680 (#926-32222, Li-Cor). Membranes were washed in PBS and PBS-Tween and fluorescent protein bands were visualized using the Odyssey Infrared Imaging System (Li-Cor). ImageJ (http://imagej.nih.gov/ij/) was used to quantify the fluorescent protein bands.

2.4. Statistical analysis

All data were analyzed using the statistical software program SAS (version 9.4, SAS Institute, Cary, NC). First, residuals of all variables were tested for normality using the Shapiro-Wilk test (SAS POC UNIVARIATE). The pCREB/CREB ratio was log10 transformed for statistical analysis, and visualized as non-transformed. The effects of iron deficiency at 12 weeks of age were analyzed using a mixed model ANOVA (SAS PROC MIXED) with the fixed effect treatment and with litter as random effect. One outlier in 18 kDa proBDNF/mBDNF ratio for the dorsal hippocampus was detected using the online outlier detector QuickCalcs (GraphPad Software, Inc., La Jolla, CA) and removed.

3. Results

Whereas in the dorsal hippocampus of ID piglets mature BDNF (mBDNF) was increased ($p < 0.05$), it was decreased in the ventral part of the hippocampus of ID piglets ($p < 0.001$; Fig. 1A). No effects were found on mBDNF protein levels in the prefrontal cortex. In line with the mBDNF protein levels, the 18 kDa and 24 kDa proBDNF fragments were increased in the dorsal hippocampus of ID piglets ($p < 0.05$), while both fragments were decreased in the ventral hippocampus ($p < 0.05$ and $p < 0.01$; Fig. 1B, C).

The 18 kDa and 24 kDa proBDNF/mBDNF ratios in the dorsal hippocampus were not affected by ID. In contrast, the 24 kDa proBDNF/mBDNF decreased in the ventral hippocampus in ID ($p < 0.05$). ID did not affect the proBDNF/mBDNF ratio in the prefrontal cortex (Fig. 1D, E). Together, BDNF protein levels are shown to be disturbed in the hippocampus of ID piglets. Regarding the BDNF receptors, ID did not affect the $p75^{NTR}$ protein levels in the dorsal hippocampus, ventral hippocampus, or prefrontal cortex (Fig. 1F). In contrast, protein levels of the high affinity BDNF receptor TrkB were reduced in ID piglets in both the dorsal ($p < 0.05$) and ventral hippocampus ($p < 0.05$), while increased TrkB protein levels were