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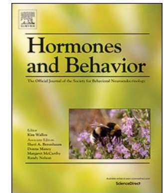

Disturbances of systemic and hippocampal insulin sensitivity in macrophage migration inhibitory factor (MIF) knockout male mice lead to behavioral changes associated with decreased PSA-NCAM levels

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a multifunctional cytokine well known for its role in inflammation enhancement. However, a growing body of evidence is emerging on its role in energy metabolism in insulin sensitive tissues such as hippocampus, a brain region implicated in cognition, learning and memory. We hypothesized that genetic deletion of MIF may result in the specific behavioral changes, which may be linked to impairments in brain or systemic insulin sensitivity by possible changes of the hippocampal synaptic plasticity. To assess memory, exploratory behavior and anxiety, three behavioral tests were applied on *Mif* gene-deficient ($MIF^{-/-}$) and "wild type" C57BL/6J mice (WT). The parameters of systemic and hippocampal insulin sensitivity were also determined. The impact of MIF deficiency on hippocampal plasticity was evaluated by analyzing the level of synaptosomal polysialylated-neural cell adhesion molecule (PSA-NCAM) plasticity marker and mRNA levels of different neurotrophic factors.

The results showed that $MIF^{-/-}$ mice exhibit emphasized anxiety-like behaviors, as well as impaired recognition memory, which may be hippocampus-dependent. This behavioral phenotype was associated with impaired systemic insulin sensitivity and attenuated hippocampal insulin sensitivity, characterized by increased inhibitory Ser^{307} phosphorylation of insulin receptor substrate 1 (IRS1). Finally, $MIF^{-/-}$ mice displayed a decreased hippocampal PSA-NCAM level and unchanged *Bdnf*, *NT-3*, *NT-4* and *Igf-1* mRNA levels.

The results suggest that the lack of MIF leads to disturbances of systemic and hippocampal insulin sensitivity, which are possibly responsible for memory deficits and anxiety, most likely through decreased PSA-NCAM-mediated neuroplasticity rather than through neurotrophic factors.

1. Introduction

The nervous, endocrine and immune systems are inseparably linked through pleiotropic actions of cytokines, affecting the most of neuroendocrine and central neurotransmitter processes (Bilbo and Klein, 2012). The behavioral changes, observed as an outcome of the central cytokine action, have been among the extensively investigated intrinsic interactions in the last decade (Bilbo and Schwarz, 2012). Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine, produced by virtually all cell types (Calandra and Roger, 2003; Kudrin and Ray, 2008). It has a pivotal regulatory role in the immune response, with an infamous contribution to the number of inflammatory and autoimmune diseases and carcinogenesis (Lue et al., 2002). Besides its role in inflammation, a growing body of evidence is emerging on the role of MIF in energy metabolism in insulin sensitive tissues such as pancreas, muscle and adipose tissue (Cvetkovic et al., 2005; Morrison and Kleemann, 2015). High expression of MIF was documented throughout the brain, especially in the hippocampus, a brain region involved in cognition, learning and memory (Nishibori et al., 1996; Ogata et al., 1998; Rubin et al., 2014). Some studies showed that experimental animals with genetic deletion of MIF may express depressive and/or anxiety-like behavior (Conboy et al., 2011; Moon et al., 2012). However, some classical tests for measuring behaviors relevant to recognition memory and anxiety, such as novel object recognition test and light-dark box, were not assessed so far in MIF knockout mice. The observed behavioral phenotypes induced by the absence of MIF may be related to the alterations in both systemic and hippocampal insulin sensitivity, as previously demonstrated in experimental models of diabetes and Alzheimer's disease (AD) (Vandal et al., 2014; Winocur et al., 2005). Some clinical studies pointed to decreased hippocampal volume

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and impaired hippocampal-based memory performance, as well as to an increased risk of developing age-related cognitive disorders in patients with diabetes (Freiherr et al., 2013; Gold et al., 2007; Manschot et al., 2006).

There are several mechanisms of insulin resistance in the cells (Pirola et al., 2004). However, at the molecular level, inhibitory phosphorylation of IRS1 at serine 307 ($pIRS1^{Ser307}$) represents an early hallmark of insulin resistance leading to impairment of IRS1 ability to activate downstream kinase and transduce insulin signal (Sykiotis and Papavassiliou, 2001). It was previously shown that "diabetogenic" factors such as fatty acids and proinflammatory cytokines may lead to increased inhibitory serine Ser^{307} phosphorylation of IRS1 (Tanti et al., 2004).

The underlying mechanism that links hippocampal insulin resistance and altered behavior could be impaired plasticity of neuronal networks and reduced hippocampal neurogenesis, since insulin is involved in regulation of these processes (Blazquez et al., 2014). Until now, several studies have linked the impairment in hippocampal declarative memory, synaptic activity and reduced dendritic plasticity with the state of insulin resistance in rats (Grillo et al., 2015; Stranahan et al., 2008). However, the involvement of insulin resistance in the relationship between neuroplasticity and behavioral alterations in mice with genetically deleted MIF was not assessed so far.

Neuroplasticity may include changes in neuron morphology, neurobiochemistry, synaptic connectivity and neurogenesis and is crucial for the maintenance of cognition, memory and behavior. Neurotrophins, a family of proteins including brain-derived neurotrophic factor (*Bdnf*), neurotrophin 3 (*NT-3*) and neurotrophin 4 (*NT-4*) are known molecular mediators of synaptic plasticity, with the involvement in neurogenesis, synapse formation, plasticity and higher cognitive functions (Gomez-Palacio-Schjetnan and Escobar, 2013; Park and Poo, 2013). It was previously shown that lower expression of neurotrophins correlate with brain insulin resistance (Blazquez et al., 2014; Park et al., 2010) and that insulin growth factor 1 (*Igf-1*) is implicated in neural plasticity in the CNS and in stimulation of hippocampal neurogenesis (Dyer et al., 2016; Llorens-Martin et al., 2009). Another group of plasticity-related proteins, neural cell adhesion molecules (NCAMs), was also found to be modified in insulin resistant animal models of type-1 diabetes (Baydas et al., 2003). NCAMs have been strongly implicated in synaptic plasticity and memory formation (Kiss et al., 2001; Sandi, 2004). The polysialylated (PSA) isoform of the NCAM is specifically important for structural neuroplasticity and its decreased level has been commonly associated with certain stress-related psychiatric disorders and impaired cognitive function (Aonurm-Helm et al., 2016; Bisaz et al., 2009; Jurgenson et al., 2010).

In this study we hypothesized that the MIF deficiency may affect insulin sensitivity and hippocampal synaptic plasticity, thus leading to behavioral changes. We analyzed the impact of the MIF absence on systemic and hippocampal insulin sensitivity, as well as on hippocampal plasticity evaluated by the level of different plasticity markers, including synaptosomal PSA-NCAM protein level, and *Bdnf*, *NT-3*, *NT-4* and *Igf-1* mRNA levels. We also applied behavioral tests to assess memory, exploratory behavior and anxiety in $MIF^{-/-}$ mice.

2. Methods

2.1. Animals

The generation of homozygous *Mif* gene-deficient ($MIF^{-/-}$) mice (background: C57BL/6J) has been described elsewhere (Fingerle-Rowson et al., 2003). The mice were further bred using homozygous $MIF^{-/-}$ animals in the Animal Facility at the Institute for Biological Research "Siniša Stanković", along with their WT C57BL/6J counterparts. Four months old WT and $MIF^{-/-}$ male mice (8 animals per group) were used in the experiments. The animals were housed 4 per cage and kept in a temperature controlled room (22 ± 2 °C) with a 12 h light/dark cycle (lights on at 07:00 h) and constant humidity. The animals had standard laboratory chow and drinking water available ad libitum. All animal procedures were in compliance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

2.2. Behavioral testing

Animals used for behavioral testing were subjected to the test battery consisting of classical tests: novel object recognition test (NOR) was applied for measuring behaviors relevant to recognition memory, whereas the anxiety-like behaviors were evaluated by elevated plus maze (EPM) and light-dark box (LDB). The behavioral tests were adapted from existing protocols. Each behavioral task was performed daily between 9 AM and 1 PM. All behavior tests were performed in the same order on a single cohort of animals. Tests were spaced at least 48 h apart to avoid the interaction between tests. These behavioral tests were performed in a separate dimly illuminated room (indirect 2 × 40 W light) with light and acoustic isolation, and the temperature maintained at 25 °C. The experiments were recorded by the camera connected to PC, positioned and operated behind the folding screen. ANY-maze software (ANY-maze Video Tracking System 4.30, Stoelting Co., USA) was used to analyze and evaluate a number of mouse activities recorded on the video. Particular behavioral parameters were identified and counted by the observations of a proficient experimenter, who was unaware of the experimental groups. After each test, the equipment was cleaned with 10% ethanol solution and dried with paper towels, to remove any trace of odor.

2.2.1. Novel object recognition test

This test was established as a valuable measure of cognition and memory retention (Antunes and Biala, 2012). The variant of NOR applied in our experiments was adapted from the published procedure by Hammond et al. (2004). Each animal was allowed a 10 min training session with the exposure to two identical, non-toxic objects (hard plastic item) placed in the two opposite corners of the arena (45 × 45 cm). After the training session, this animal was returned to its home cage for a 30-min retention interval, before it was submitted to the test, in which one familiar object was replaced with a novel object of a similar size, but with different shape. The animal was lowered into the arena, equidistant and facing away from both objects. The sessions were video recorded for 10 min and analyzed by ANY-maze. The exploration was defined when the animal's head was inside a circle ($R = 6$ cm) around the object. Each mouse was excluded from further exploratory registration after accumulating 40 s of exploration time on either of the sample objects. The novel object preference ratios (in % of time) were calculated by dividing the novel object exploratory time with the time used to explore both objects (40 s). A value above 50% suggests preference for the novel object, while a value below 50% is indicative of familiar object preference. Motor activity, presented as the percentage of total time spent in moving and by the traveled distance (in m) during 10 min, was also documented and compared between $MIF^{-/-}$ and WT mice to determine if the variation in exploration could be caused by the changes of general activity. Based upon a suggestion that the latency to approach a new object may reflect the anxiety level of an animal (Kalueff and Tuohimaa, 2004), the latency time (in *sec*) was also measured and compared between the groups.

2.2.2. Light dark box test

LDB is widely applied to evaluate anxiety-like behaviors and it is based on innate aversion of rodents to brightly illuminated areas (Bourin and Hascoet, 2003). In this study, a Plexiglas box (50 cm × 25 cm with 30 cm high walls) was divided into two compartments: dark (one-third of the box) and illuminated (two-thirds of

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