ORIGINAL RESEARCH PAPER

The role of dietary non-heme iron load and peripheral nerve inflammation in the development of peripheral neuropathy (PN) in obese non-diabetic leptin-deficient ob/ob mice

Joanna Kosacka*, Katrin Woidt**, Klaus V. Toyka*, Sabine Paeschke†, Nora Klöting‡, Ingo Bechmann§, Matthias Blüher∥, Joachim Thiery¶, Susann Ossmann#, Petra Baum** and Marcin Nowicki***

*Department of Neurology, University of Leipzig, Leipzig, Germany; **Institute of Anatomy, University of Leipzig, Leipzig, Germany; †Department of Neurology, University of Würzburg, Würzburg, Germany; §Department of Medicine, University of Leipzig, Leipzig, Germany; ∥Integrated Research and Treatment Center (IFB) Adiposity Disease, Leipzig, Germany; ¶Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics (ILM), University of Leipzig, Leipzig, German; #Heart Center, University of Leipzig, Leipzig, Germany

ABSTRACT

Introduction: Here, we investigated inflammatory signs of peripheral nerves in leptin-deficient obese ob/ob mice and the modulating effects of the exogenous iron load.

Methods: Ob/ob and ob/+ control mice were fed with high, standard, or low iron diet for four months.

Results: We found intraepidermal nerve fiber degeneration in foot skin and low-grade neuropathic abnormalities including mildly slowed motor and compound sensory nerve conduction velocities and low-grade macrophage and T-cell infiltration without overt neuropa-thology in sciatic nerves of all ob/ob mice. Low dietary iron load caused more pronounced abnormalities than high iron load in ob/ob mice.

Discussion: Our data suggest that dietary non-heme iron deficiency may be a modulating factor in the pathogenesis of peripheral neuropathy in obese ob/ob mice with metabolic syndrome. Once the mechanisms can be further elucidated, how low dietary iron augments peripheral nerve degeneration and dysfunction via pro-inflammatory pathways and new therapeutic strategies could be developed.

Abbreviations: CMAP: compound muscle action potential; cSNCV: compound sensory nerve conduction velocity; IENFD: intraepidermal nerve fiber density; LDL: low-density lipoprotein; MetS: metabolic syndrome; MNCV: motor conduction velocity; NCV: nerve conduction velocity; PN: peripheral neuropathy; PNS: peripheral nervous system; STZ: streptozotocin; T2D: type 2 diabetes mellitus; TNF alpha: tumor necrosis factor alpha; WHO: World Health Organization

1. Introduction

The worldwide prevalence of overweight, obesity, and their metabolic complications has increased dramati-cally over the last decades. In 2016, it was estimated that more than 1.9 billion adults were overweight, and of these, over 650 million were obese (WHO, 2017). Excessive fat accumulation is often associated with chronic inflammation, insulin resistance, impaired glucose tolerance, hyperlipidemia, hypertension, metabolic syndrome (MetS), and type 2 diabetes (T2D) [1,2].

In the peripheral nervous system (PNS), obesity-, MetS-, and T2D-related alterations may all affect nerve function and cause peripheral neuropathy (PN) [2–7]. The putative pathophysiology of PN includes metabolic, vascular, and inflammatory mechanisms, but a unifying hypothesis of the culprit pathogenic factors does still not exist [2,8,9]. PN leads to degeneration and impaired regeneration of nerve axons associated with structural and functional changes in endoneurial and epineural microvessels and in Schwann cells in afflicted humans [7,10–14]. Indeed, patients with obesity and MetS have a higher prevalence of developing PN, even in the absence of hyperglycemia when compared to the lean control population [2,15,16].

Several animal models and human studies have recently shown an association between obesity, MetS, T2D, and deranged iron homeostasis [17–23]. Iron deficiency has been found in patients with advanced stages of obesity, whereas increased iron levels have been demonstrated in many patients suffering from MetS or T2D [17,18]. Increased body iron storage enhances the generation of free radicals [24]. Free radicals are highly reactive species promoting oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids [25,26] and are implicated in the pathogenesis of several neurological diseases [27,28,29–31]. Iron may also

CONTACT Marcin Nowicki Marcin.Nowicki@medizin.uni-leipzig.de Institute of Anatomy, Liebigstr. 13, Leipzig D-04103, Germany

*These authors contributed equally to this work.

© 2019 Informa UK Limited, trading as Taylor & Francis Group
act in Schwann cell differentiation, peripheral nerve myelination, and regeneration [23].

Following up on our previous studies on the negative role of low iron in a streptozotocin (STZ)-diabetes rat model [32] and in diabetic db/db mice (Paeschke, Nowicki et al., unpublished data 2018), we here investigated the effects of increased or reduced exogenous iron load on the severity of PN in leptin-deficient ob/ob mice as an animal model of obesity and MetS. Ob/ob mice may show deficits in nerve conduction velocity (NCV), nerve fiber abnormalities, and endoneurial microvessel changes in the sciatic nerve [13,33,34]. Metabolically, as a result of a mutation in the gene encoding leptin, ob/ob mice exhibit over 50% body fat, insulin resistance, hyperphagia, and mild and transient, if any, hyperglycemia which disappears around 5 months of age [35]. In line with our previous study [32], we found that chronic iron depletion rather than iron overload augments peripheral nerve pathology and pro-inflammatory activity in ob/ob mice.

2. Methods

2.1. Animals

A total number of 21 male ob/ob (Lep<sup>b/b</sup>/Lep<sup>b/b</sup>) homozygous and of 21 male ob/+ (Lep<sup>b/b</sup>+/+) heterozygous 3-month-old mice were used in this study. The experiments had been approved by the state authorities (Landesdirektion Sachsen, reg. no.: TVV 63/12). Animals were randomly assigned to three ob/ob and three ob/+ treatment groups of seven mice each. All six groups of mice were fed ad libitum (Altromin, Lage, Germany). The chows contained (1) high iron (29 g/kg – standard diet complemented with 3% carbonyl iron), (2) standard iron (178 mg/kg), or (3) low iron (5 mg/kg) concentration. Blood glucose concentrations were measured before and during the experimental period in whole blood taken from the lateral tail vein using an Optium Omega glucometer (GlucoMen, Menarini Diagnostics, Berlin, Germany). Serum iron levels were measured with Cobas® 8000 modular analyzer (Roche Diagnostics) using a colorimetric test by Roche Diagnostics (Berlin, Germany) based on the FerroZine method without deproteinization.

2.3. Electrophysiology

NCVs were analyzed in the left sciatic nerve as described elsewhere [36,37]. In brief, we measured motor and compound sensory NCVs (MNCV and cSNCV). The sciatic notch was used as the proximal stimulation point (S1) in motor NCV and as the proximal pick-up point for compound SNCV using near-nerve needle electrodes (kindly provided by Prof. C. Krarup, Copenhagen) [37]. A pair of bare steel needle electrodes was inserted at the ankle as the distal stimulation point (S2). Recording electrodes for the compound muscle action potentials (CMAP) were placed between digits 2 and 3 (active electrode) and at the base of digit 5 of the left foot. The MNCVs were calculated by dividing the distance between the two stimulation points by the differences in latencies of the CMAPs after proximal and distal supramaximal stimulation. Compound sensory nerve action potentials (cSNAP) were recorded with the near-nerve electrodes that had served as proximal stimulation electrodes for MNCVs. Stimulation electrodes at the ankle were the same electrodes as used for eliciting distal CMAPs. The cSNCVs were calculated by dividing the distance between stimulation and recording electrodes by the latency of the first positive peak of the cSNAP. All parameters were calculated semi-automatically using the Neurosoft-Evidence 3102 electromyograph software (Schreiber und Tholen, Stade, Germany).

2.4. Immunostaining

Mice (n = 5 per group) were perfused and sciatic nerves and hindfoot skin biopsies were dissected and prepared as previously described [38]. Sections were double stained by first incubating with rabbit polyclonal antibodies directed at the microglia/macrophage cytoplasmatic calcium adaptor (anti-Iba-1) for the detection of macrophages (1:200; WAKO Chemicals USA, Richmond, VA) or with rabbit polyclonal anti-CD3 antibodies for detection of T-cells (1:200; Dako Cytomation, Hamburg, Germany). Second, the mouse monoclonal antibody against neurofilament 200 (NF200; 1:500; Sigma Aldrich, Taukirchen, Germany) was used to identify nerve axons or the mouse monoclonal antibody against CD68 (ED1) for the detection of macrophages (1:200; Abcam, Cambridge, UK). For identification of intraepidermal small nerve fibers, polyclonal antibody against the axonal protein gene product (PGP 9.5; 1:1000; Abcam) was used. Next, the immunostaining was conducted as described elsewhere [38].

2.5. Quantification of the intraepidermal nerve fiber density (IENFD)

We prepared 30-µm-thick skin sections from the hindfoot of ob/ob and ob/+ mice (n = 3 per experimental group) and stained them using the immunostaining methods described above. The number of PGP 9.5-positive fibers crossing the dermal–epidermal junction and individual fibers in the dermis and epidermis was counted for each randomly selected section (five per one animal) and divided by the epidermal length measured using Zeiss software (Zeiss LSM image Browser). All sections were analyzed by an observer blinded as to the dietary treatment groups.
Biochemical and physiological parameters of ob/ob and ob/+ control mice fed with high iron, standard iron, and low iron diet. Values represent means ± SD of six animals (n = 6; blood samples were collected between 8 a.m. and 9:30 a.m.).

| Parameter               | High iron | Standard iron | Low iron |
|-------------------------|-----------|---------------|----------|
| Blood glucose (mmol/l)  | 5.45 ± 0.98 | 5.68 ± 1.84   | 5.17 ± 0.92 |
| Serum insulin (ng/ml)   | 5.03 ± 4.64 | 0.73 ± 0.55   | 12.88 ± 4.05 |
| Body weight (g) – start| 54.25 ± 2.90 | 30.33 ± 1.04  | 54.57 ± 6.67  |
| Body weight (g) – end   | 65.27 ± 2.41 | 33.30 ± 1.73  | 67.68 ± 4.38  |
| % of fat of the body weight | 51.82 ± 0.92 | 11.33 ± 2.88  | 54.86 ± 3.29  |
| Total cholesterol (mmol/l)| 5.2 ± 0.43   | 2.96 ± 0.82   | 6.32 ± 1.82   |
| LDL-cholesterol (mmol/l)| 1.77 ± 0.24   | 0.66 ± 0.51   | 2.14 ± 0.58   |
| Triglyceride (mmol/l)   | 0.56 ± 0.11   | 1.10 ± 0.48   | 0.94 ± 0.29   |
| Serum insulin (ng/ml)   | 73.50 ± 3.85  | 40.93 ± 19.6  | 45.10 ± 5.26  |

Table 1. Biochemical and physiological parameters of ob/ob and ob/+ control mice fed with high iron, standard iron, and low iron diet. Values represent means ± SD of six animals (n = 6; blood samples were collected between 8 a.m. and 9:30 a.m.).

2.6. Quantification of macrophages and T-cells in sciatic nerves

Digitized pictures were taken with an LSM 510 Meta Confocal Microscope (Zeiss). The number of Iba-1 and/or ED1-positive macrophages and CD3-positive T-cells was counted in whole sciatic nerve cross sections (n = 5 in each group). Values represent numbers of stained cells per mm².

2.7. Transmission electron microscopy

Mice (n = 3 per group) were perfused via the left heart ventricle, first with 250 ml phosphate-buffered saline (PBS, pH 7.4, 37°C) containing 6250 µl heparin (Sigma, Taufkirchen, Germany), followed by 250 ml 2% glutaraldehyde with 1% paraformaldehyde in 0.1 M PBS. Sciatic nerves were prepared and analyzed as described previously [32].

2.8. Western blot

Sciatic nerves (n = 5 per group) were lysed by ultrasonication in 60 mM Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 10% sucrose. Tissue lysates were diluted 1:1 in sample buffer (250 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% glycerol, and 2% b-mercaptoethanol) and denatured at 95°C for 5 min. Protein concentration was assessed with the bicinchoninic acid assay (BCA assay) protein assay (Pierbo Science, Bonn, Germany). Proteins (20 µg per lane) were separated by electrophoresis on a 12.5% or 15% SDS-polyacrylamide gel and transferred to nitrocellulose by electroblotting. Nonspecific binding sites were blocked with 5% dried milk powder for 45 min. The blots were incubated with rabbit polyclonal anti-TNFα (ab 6671; 1:2000; Abcam, MA, USA) at 4°C overnight. Proteins were detected by incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies at a 1:4,000 dilution; Dianova) at RT for 2 h and chemiluminescence kit (Amersham, Pharmacia, Freiburg, Germany). Integrated optical densities of the immunoreactive protein bands were measured with Gel Analyzer software (Media Cybernetics, Silver Spring, MD). Equal protein loading was verified using mouse anti-D-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH, Research Diagnostics, Flanders, The Netherlands; 1:3000).

3. Results

3.1. Biochemical and physiological parameters

In ob/ob and ob/+ control mice, blood glucose concentrations analyzed between 8 a.m. and 9:30 a.m. were below 7 mmol/l and not significantly different between experimental groups (Table 1). In agreement with the previous studies [33,35], all ob/ob animals were obese, exhibiting over 50% body fat and showing significantly higher levels of insulin, cholesterol, and low-density lipoprotein-cholesterol as compared to the ob/+ control mice. Triglyceride levels were higher only in ob/ob mice on standard and low iron diet as compared to the ob/+ control animals. Noteworthy, serum insulin concentrations were significantly lower in ob/ob mice on the high iron diet as compared to the ob/ob mice on the standard and low iron (Table 1).

Serum iron concentrations were significantly higher in all ob/ob animals than in the control groups. The highest serum iron concentration was found in ob/ob mice on a high iron diet (Table 1).

3.2. Nerve conduction studies

We performed nerve conduction studies at the beginning and at the end of the experimental period. Sciatic nerve MNCVs of all ob/+ groups increased mildly throughout the experiment as expected with further maturation in control mice. Motor NCVs (MNCVs) of ob/ob mice significantly declined with all iron diets (Figure 1(a and b)). When compared to the ob/+ control animals, sciatic MNCVs were reduced in ob/ob mice with different iron diets by up to 24% (Figure 1(e)). This indicates that conduction was abnormally slowed rather than halted by a lack of further nerve maturation. The sciatic compound sensory NCVs (cSNCVs) were already by about 30% lower in all ob/ob mice at age 3 months before any dietary treatment started as compared to the ob/+ controls (Figure 1(c and d)). At the end of the study, the sciatic cSNCVs
had significantly decreased in all ob/ob mice as compared to the respective control animals (Figure 1(f)). In contrast to MNCV, the cSNCVs did not increase over the experimental period in the control mice. The highest cSNCV decrease (by up to 45%) was observed in ob/ob animals on a low iron diet (Figure 1(f)).

3.3 Morphology of terminal skin fibers and sciatic nerves

A significant degenerative loss of intraepidermal nerve fibers as terminal branches of the sensory fiber population of the sciatic nerve was observed in all ob/ob mice.
as compared to the ob/+ control animals (Figure 2(a–c)) as expressed by a markedly reduced IENFD. This reduction was similar with high, standard, or low iron diet (Figure 2(c)).

Semithin sections showed no obvious changes in fiber morphology with regard to the distribution of myelinated vs. unmyelinated fiber bundles. The thickness of the myelin sheath appeared similar across all

Figure 2. Morphology of sciatic nerve and its sensory fiber endings in the hindfoot skin of ob/ob and ob/+ control mice fed with different iron diets. (a and b): Representative sections showing immunoreactivity (green) of PGF9.5-positive fibers (arrows). Nuclei are counterstained with DAPI (blue). Bars represent 20 µm. (c): Quantitative analysis of IENFD in the skin of the hindfoot in ob/ob and ob/+ control animals. A significant loss of intraepidermal nerve fibers forming the terminal branches of sensory nerve components of the sciatic nerve was observed in all ob/ob mice compared to the ob/+ control animals. This reduction was similar with a high, standard, or low iron diet. (d and e): Sciatic nerves were examined for pathological changes by electron microscopy to determine the pathological changes in their structure and thickness of myelin sheath in relation to axon diameters expressed as fiber area-ratio and g-ratio. The global g-ratios (axon diameter/whole fiber diameter; (d) and the global area-ratios (axon area/whole fiber area; (e) were similar between experimental groups (p > 0.05). Values represent mean ± SEM (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001, according to the one-way analysis of variance together with the Newman–Keuls test.
groups. Ultrathin sections of cross-sectioned nerve fibers allowed more detailed analyses. Here, no pathological changes of nerve fibers were found in mice of all dietary groups, neither in myelin sheaths nor in axons, and g-ratios (axon diameter/whole fiber diameter) and area-ratios (axon area/whole fiber area) also showed no difference between all experimental groups (Figure 2(d and e)).

### 3.4 Inflammatory cells and TNF-alfa expression in sciatic nerves

Several previous studies suggest a role for obesity and lipid-induced inflammation in the development of PN [3]. The number of Iba-1-positive macrophages and T-cells was increased by about 95% up to almost 150% in ob/ob mice as compared to the respective lean control animals (Figures 3–6). Overall, the inflammatory cell numbers were markedly and significantly higher in sciatic nerves of ob/ob mice on a low iron diet as compared to ob/ob and ob/+ mice on standard or high iron diet (Figures 3 and 6). With double-immunofluorescence staining, we identified co-localization of ED1 (CD68) for activated macrophages with Iba-1-positive macrophages (Figure 5). Noteworthy, the highest number of Iba-1~/ED1-positive cells (39%) was found in ob/ob mice with the low iron diet as compared to the other experimental groups indicating the highest pro-inflammatory milieu in this dietary group (Figure 3(a)).

To corroborate these findings, we tested the protein expression of the prime pro-inflammatory cytokine TNFα. Indeed, TNFα protein expression was increased up to fivefold in ob/ob mice with iron low diet and least with high iron diet (Figure 7).

### 4. Discussion

The principal results of our study are intraepidermal nerve fiber degeneration in the foot skin and functional abnormalities of the sensory component in the sciatic nerve associated with low-grade macrophage and T-cell infiltration and TNFα activation in sciatic nerve in ob/ob mice. This pattern of peripheral nerve pathology could be augmented by a partial iron deprivation induced by low dietary iron intake, while a high iron diet was an ameliorating factor. Pathogenetically relevant mild inflammatory activity has first been described in here-dodegenerative PNS disorders such as Charcot–Marie–Tooth disease and its various mouse models [39–41].

It is widely accepted that a major increase in adipose mass contributes to adipose tissue dysfunction and promotes metabolic disorders via a mild, chronic inflammation which is characterized by increased expression of pro-inflammatory factors including TNFα [3,42,43]. Our findings of a mild peripheral nerve dysfunction with more marked distal axon degeneration lead us to suggest that the inflammatory milieu as expressed by mononuclear cells and autocrine cytokines may be a pivotal pathogenic process contributing to PN. TNFα may be secreted by these intraneural inflammatory cells or by cells in adipose tissue and can alter and penetrate the blood–nerve barrier exerting neurotoxic tissue effects and attracting further immunocompetent white cells such as macrophages and T-cells into the endoneurial nerve compartment. This type of mechanism was first suggested by our earlier work in the STZ-diabetes model. As now shown here and also suggested from work by other groups, all this may happen in the absence of hyperglycemia [3,43–45]. This could, in turn, lead a pro-inflammatory vicious circle via TNFα-induced sensitization of sensory neurons potentially augmented by an array of other cytokines and chemokines [46]. An important effect of this immune activation is an increased release of further pro-inflammatory factors at peripheral nerve terminals [47].

The second important finding of this study is that this pathologic mechanism can collectively be modulated by non-heme iron. The increased number of pro-inflammatory cells together with a markedly augmented TNFα protein expression in the low iron diet fed ob/ob mice suggests a pro-inflammatory role of dietary deprivation of non-heme iron.

How this effect of iron deprivation may be related to iron metabolism in obesity and MetS is a crucial but not yet answered question. Recently, it has been shown that obesity alters adipose tissue macrophage iron content and tissue iron distribution [48]. High-fat diet feeding increased the absolute number of adipose tissue macrophages. This increase was driven by a dramatic accumulation of macrophages with low iron content, which displayed decreased gene expression of anti-inflammatory factors and increased expression of pro-inflammatory mediators [48]. There were also macrophages described with high iron content exhibiting a pro-inflammatory shift. Impaired iron handling of macrophages with high iron content coincided with adipocyte iron accumulation and hepatic iron deficiency [48]. To the best of our knowledge, there are few, if any, studies concerning the modulatory role of non-heme iron in human peripheral neuropathies potentially acting through low-grade nerve inflammation. Previously, we have shown an increased number of activated macrophages and T-cells in sciatic nerves of STZ-rats with overt hyperglycemia as in type 1 diabetes [32]. In this study, the highest number of pro-inflammatory cells was observed in the sciatic nerves of animals with low iron intake [32]. From the present data, it becomes obvious that the modulatory role of iron intake on peripheral nerve pathology is not dependent on the co-existence of hyperglycemia suggesting a blood glucose-
independent mechanism in obesity and MetS. In accordance with our findings, it has recently been shown that iron significantly reduces pro-inflammatory polarization of macrophages and, in turn, decreases the production and secretion of pro-inflammatory cytokines [49].

Another consequence of chronic adipose tissue inflammation in obese individuals is impaired insulin signaling and compromised triglyceride storage [3]. Cooksey and co-workers (2010) have shown that dietary iron restriction or iron chelation protect from loss of β-cell function and diabetes in obese ob/ob mice [20]. In contrast to these observations, we here demonstrate that insulin and triglyceride levels were significantly lower in ob/ob mice with high iron diet than with standard and low-iron-fed ob/ob mice. The obtained

Figure 3. Macrophage infiltration and distribution in the sciatic nerve of ob/ob and ob/+ control mice fed with different iron diets. (a): Quantitative analysis of macrophage infiltration in sciatic nerves of ob/ob and ob/+ control mice. Values represent means ± SEM (n = 5), *p < 0.05, **p < 0.01, according to the one-way analysis of variance together with the Newman–Keuls test. Percent values of activated (CD68- and Iba-1-positive) and total macrophages (Iba-1-positive) as a composite evaluation: 6% in ob/ob mice vs. 3% in ob/+ mice on the high iron diet; 4% in ob/ob mice vs. 1% in ob/+ mice on the standard iron diet; and 13% in ob/ob mice vs. 5% in ob/+ mice on the low iron diet. (b and c): Double immunofluorescence staining for Iba-1-positive macrophages (in green, upper panel, white arrows) and for neurofilament 200-positive nerve fibers (in red, lower panel) in the sciatic nerve of standard iron diet ob/ob and ob/+ control mice. Nuclei are counterstained with DAPI (in blue). Bars represent 100 µm.
results suggest the improvement of insulin sensitivity in this mouse group. In the study by Cooksey et al. [20], mice had hyperglycemia and were fed with a high-carbohydrate or high-fat diet with different iron content. The high-carbohydrate or the high-fat diets could be additional factors that may have influenced insulin and glucose metabolism and β-cell function of investigated ob/ob mice [20]. Bao et al. (2012) have summarized in a meta-analysis of prospective human studies that there are no significant correlations between

Figure 4. Double immunofluorescence staining for Iba-1-positive macrophages (in green, upper panel) and for neurofilament 200-positive nerve fibers (in red, lower panel) in the sciatic nerve of low iron diet ob/ob (a) and ob/+ control (b) mice as well as high iron diet ob/ob (c) and ob/+ control (d) mice. Nuclei are counterstained with DAPI (in blue). Bars represent 100 µm.
Figure 5. Double immunofluorescence staining for Iba-1-positive (in green; a) and CD68-positive (in red; b) macrophages and co-localization of both proteins (c) in the sciatic nerve of ob/ob mouse fed with low iron diet. White arrows show activated (CD68- and Iba-1-positive) macrophages and the yellow arrow show inactive (only Iba-1-positive) macrophage. Bars represent 30 µm.

Figure 6. (a): Quantitative analysis of T-cell infiltration in sciatic nerves of ob/ob and ob/+ control mice fed with different iron diets. Values represent means ± SEM (n = 5), *p < 0.05, **p < 0.01, according to the one-way analysis of variance together with the Newman–Keuls test. (b and c): Double immunofluorescence staining for CD3-positive T-cells (in green, upper panel) and for neurofilament 200-positive nerve fibers (in red, lower panel). Nuclei are counterstained with DAPI (in blue). Bars represent 100 µm.
dietary intake of total iron, non-heme, and supplemental iron with the risk of developing T2D [50]. The observed improvement of insulin signaling could have led to a decrease in the pro-inflammatory status and, in turn, to a better peripheral nerve function.

Collectively, we found a functionally relevant increase in peripheral nerve pathology with chronic iron depletion rather than with iron overload. We propose that the effects of iron may be of a wider pathogenetic relevance than hitherto accepted. Our data support the concept of potentially relevant inflammatory features that exist independent of the presence or absence of hyperglycemia in disorders associated with obesity. Since our experiments lasted only 4 months, while in human obesity and MetS PN is a very late consequence, future mouse experiments should focus on the long-term effects of obesity and MetS and of the role of iron metabolism at later time points. Once it can be confirmed that very late effects are even more pronounced than shown in the present study, the time has come to develop new therapeutic strategies halting nerve pathology and inflammation and to plan clinical trials.

Acknowledgments
We thank J. Craatz, A. Ehrlich, and C. Merkwitz for excellent technical assistance.

Disclosure statement
No potential conflict of interest was reported by the authors.

Ethical Publication Statement
We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Funding
This work was funded by the Deutsche Forschungsgemeinschaft, SFB1052: B1 (to MB) and B4 (to NK); German Diabetes Center 82DZD00601 (NK); and Federal Ministry of Education and Research (BMBF), Germany FKZ: 01E01501 (NK). KVT is funded by a Senior Research Professorship grant by the University of Würzburg.
Notes on contributors

Joanna Kosacka is a scientist at the Institute of Anatomy and at the Department of Neurology of the University of Leipzig. She has recently researched new factors accelerating peripheral nerve regeneration and the role of inflammation and angiogenesis in peripheral neuropathies. She was the main investigator of the research group, which found Ang-1 as a novel neurotrophic factor. She is also actively involved in other neuroscience and endocrinology studies. She is interested in pathological changes of peripheral nerves as well as in new therapies of peripheral neuropathy in type 1 and 2 diabetic and obese subjects.

Katrin Woidt is a licensed medical doctor. For one year she works at the emergency surgery station in a local clinic. At the Institute of Anatomy, University of Leipzig, she has carried out a doctoral thesis on peripheral neuropathy in animal models of obesity and metabolic syndrome.

Klaus Viktor Toyka is a neurologist and university lecturer. The focus of his research lies in the investigation of disease models of neuroimmunological and degenerative diseases in mice and rats, in connection with questions of humoral and cell-mediated immunopathogenesis and the development of new, mostly molecular therapeutic strategies. These research results are the basis for therapy research in humans. His most important findings include the pathogenic significance of autoantibodies in myasthenia, multiple forms of polyneuritis, multiple sclerosis, paraneoplastic diseases and their therapy, as well as the mechanisms of lesion formation in these diseases.

Sabine Paeschke studied nutrition sciences. Since 2016, she is a PhD student at the Institute of Anatomy, University of Leipzig. Her research concerns the mechanisms of neuropathy in patients with obesity and glucose metabolism disorders.

Nora Klöting is the head of the adiposity research group of Integrated Research and Treatment Center (IFB) Adiposity Disease, Leipzig, Germany. She is interested in the role of innate immune system on obesity-related inflammation in adipose tissue. She established a new congenic mouse, with an exchanged major histocompatibility complex (MHC, H2 region) region between obesity-resistant 129S6/SvEvTac and obesity-prone C57BL/6 mice. The new constructed congenic line, Bl6.MHC129, will give the possibility to analyse the role of innate immune system on obesity-induced inflammation in adipose tissue and obesity related traits.

Ingo Bechmann is a neurologist and university lecturer and since 2009 he is the head of the Institute of Anatomy, University of Leipzig in Germany. He researches mechanisms of immunological tolerance in the brain in diseases such as multiple sclerosis and Alzheimer’s disease. He discovered under what conditions immune cells are ”called” into the brain and how they are deactivated by local immunosuppressive mechanisms. In order to be able to directly observe tissue reactions he developed cut culture models of human tissues that better map the mechanisms in the treatment of cancer cells than animal experiments.

Matthias Blüher is an endocrinologist and professor at the University Hospital Leipzig. He is mainly concerned with morbid overweight (obesity), adipokine hormones, insulin resistance and diabetes (type 2 diabetes mellitus). He is universally recognized as an authority on subject of metabolic disorders especially in human patients with obesity or/and type 2 diabetes.

Joachim Thiery is a specialist in laboratory medicine. The scientific focus of Joachim Thiery is on the field of lipid metabolism and the pathophysiology of lipids in cardiovascular diseases, especially arteriosclerosis. His work covers a broad range of methods, ranging from experimental studies on the development of new biomarkers, in particular by mass spectrometry, to clinical trials. He researches the genetic causes of arteriosclerosis and lipid metabolism, carries out metabolome analyses and genome-wide association studies in metabolic and vascular diseases and develops therapy concepts and biomarkers for the prevention of vascular diseases and lipid metabolism disorders.

Susann Ossmann works as a veterinary assistant at the Heart Centre of University of Leipzig. She coordinates animal experiments.

Petra Baum works at the Department of Neurology, University of Leipzig, Germany. She is the assistant medical director (leitende Oberärztin) of the Department of Neurology and specialized in clinical neuroelectrophysiology. She is interested in pathological changes of peripheral nerves as well as in new therapies of peripheral neuropathy in type 1 and 2 diabetic as well as obese subjects.

Marcin Nowicki is a neurologist and university lecturer. The focus of his research lies in the investigation of disease models of neuroimmunological and degenerative diseases in mice and rats, in connection with questions of humoral and cell-mediated immunopathogenesis and the development of new, mostly molecular therapeutic strategies. These research results are the basis for therapy research in humans. His most important findings include the pathogenic significance of autoantibodies in myasthenia, multiple forms of polyneuritis, multiple sclerosis, paraneoplastic diseases and their therapy, as well as the mechanisms of lesion formation in these diseases.

Author contributions

Conceived and designed the experiments: JK, KW, MN, and PB. Performed the experiments: JK, KW, SP, SO, MN, and PB. Analyzed the data: JK, KW, KVT, IB, MN, and PB. Contributed reagents/materials/analysis tools: NK, IB, JT, and PB. Wrote and edited the manuscript: JK, KW, MN, KVT, and PB.

References

[1] Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. Int J Mol Sci. 2014;15(4):6184–6223.
[2] Callaghan BC, Xia R, Reynolds E, et al. Association between metabolic syndrome components and polyneuropathy in an obese population. JAMA Neurol. 2016;73(12):1468–1476.
[3] O’Brien PD, Hinder LM, Callaghan BC, et al. Neurological consequences of obesity. Lancet Neurol. 2017;16(6):465–477.
[4] Herman RM, Brower JB, Stoddard DG, et al. Prevalence of somatic small fiber neuropathy in obesity. Int J Obesity. 2007;31(2):226–235.
[5] Pop-Busui R, Boulton AJ, Feldman EL, et al. Diabetic neuropathy: a position statement by the American Diabetes Association. Diabetes Care. 2017;40(1):136–154.

[6] Callaghan B, Feldman E. The metabolic syndrome and neuropathy: therapeutic challenges and opportunities. Ann Neurol. 2013;74(3):397–403.

[7] Kennedy JM, Zochodne DW. Impaired peripheral nerve regeneration in diabetes mellitus. J Peripher Nerv Syst. JPNSS. 2005;10(2):144–157.

[8] Said G. Diabetic neuropathy – a review. Nat Clin Pract Neurol. 2007;3(6):331–340.

[9] Hozumi J, Sumitani M, Matsubayashi Y, et al. Relationship between neuropathic pain and obesity. Pain Res Manag. 2016;2016:2487924.

[10] Cameron NE, Cotter MA. Effects of an extracellular metal chelator on neurovascular function in diabetic rats. Diabetologia. 2001;44(5):621–628.

[11] Dyck PJ, Giannini C. Pathologic alterations in the diabetic neuropathies of humans: a review. J Neuropathol Exp Neurol. 1996;55(12):1181–1193.

[12] Kosacka J, Nowicki M, Kloting N, et al. COMP-angiopoietin-1 recovers molecular biomarkers of neuropathy and improves vascularisation in sciatic nerve of ob/ob mice. PloS one. 2012;7(3):e32881.

[13] Nowicki M, Kosacka J, Serke H, et al. Altered sciatic nerve fiber morphology and endoneural microvessels in mouse models relevant for obesity, peripheral diabetic polyneuropathy, and the metabolic syndrome. J Neurosci Res. 2012;90(1):122–131.

[14] Tesfaye S, Selvarajah D. Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy. Diabetes Metab Res Rev. 2012;28(Suppl 1):8–14.

[15] Callaghan BC, Xia R, Banerjee M, et al., Health ABCS. Metabolic syndrome components are associated with symptomatic polyneuropathy independent of glycemic status. Diabetes Care. 2016;39(5):801–807.

[16] Baum P, Petroff D, Classen J, et al. Dysfunction of autonomic nervous system in childhood obesity: a cross-sectional study. PloS one. 2013;8(1):e54546.

[17] Hubler MJ, Peterson KR, Hasty AH. Iron homeostasis: a new job for macrophages in adipose tissue? Trends Endocrinol Metab. 2015;26(2):101–109.

[18] Aigner E, Feldman A, Datz C. Obesity as an emerging risk factor for iron deficiency. Nutrients. 2014;6(9):3587–3600.

[19] Aso Y, Takebayashi K, Wakabayashi S, et al. Relation between serum high molecular weight adiponectin and serum ferritin or prohepcidin in patients with type 2 diabetes. Diabetes Res Clin Pract. 2010;90(3):250–255.

[20] Cooksey RC, Jones D, Gabrielsen S, et al. Dietary iron restriction or iron chelation protects from diabetes and loss of beta-cell function in the obese (ob/ob lep/-/-) mouse. Am J Physiol Endocrinol Metab. 2010;298(6):E1236–1243.

[21] Kim CH, Kim HK, Bae SJ, et al. Association of elevated serum ferritin concentration with insulin resistance and impaired glucose metabolism in Korean men and women. Metabolism. 2011;60(3):414–420.

[22] Le Blanc S, Villarreal P, Candia V, et al. Type 2 diabetic patients and their offspring show altered parameters of iron status, oxidative stress and genes related to mitochondrial activity. Biometals. 2012;25(4):725–735.

[23] Levi S, Tavecchia C. Iron homeostasis in peripheral nervous system, still a black box? Antioxid Redox Signal. 2014;21(4):634–648.

[24] Stroh M, Swerdlow RH, Zhu H. Common defects of mitochondria and iron in neurodegeneration and diabetes (MIND): a paradigm worth exploring. Biochem Pharmacol. 2014;88(4):573–583.

[25] Wrede CE, Buettner R, Bollheimer LC, et al. Association between serum ferritin and the insulin resistance syndrome in a representative population. Eur J Endocrinol. 2006;154(2):333–340.

[26] Huang X. Iron overload and its association with cancer risk in humans: evidence for iron as a carcinogenic metal. Mutat Res. 2003;533(1–2):153–171.

[27] Emerit J, Beaumont C, Trivin F. Iron metabolism, free radicals, and oxidative injury. Biomed Pharmacother. 2001;55(6):333–339.

[28] Papanikolaou G, Pantopoulos K. Iron metabolism and toxicity. Toxicol Appl Pharmacol. 2005;202(2):199–211.

[29] Hentze MW, Muckenhauser MU, Galy B, et al. Two to tango: regulation of mammalian iron metabolism. Cell. 2010;142(1):24–38.

[30] Lee DW, Andersen JK, Kaur D. Iron dysregulation and neurodegeneration: the molecular connection. Mol Interv. 2006;6(2):89–97.

[31] Urrutia PJ, Mena NP, Nunez MT. The interplay between iron accumulation, mitochondrial dysfunction, and inflammation during the execution step of neurodegenerative disorders. Front Pharmacol. 2014;5:38.

[32] Baum P, Kosacka J, Estrela-Lopis I, et al. The role of nerve inflammation and exogenous iron load in experimental peripheral diabetic neuropathy (PDN). Metabolism. 2016;65(4):391–405.

[33] Drel VR, Mashtalir N, Ilnytska O, et al. The leptin-deficient (ob/ob) mouse: a new animal model of peripheral neuropathy of type 2 diabetes and obesity. Diabetes. 2006;55(12):3335–3343.

[34] O’Brien PD, Hur J, Hayes JM, et al. BTBR ob/ob mice as a novel diabetic neuropathy model: neurological characterization and gene expression analyses. Neurobiol Dis. 2015;73:348–355.

[35] Haluzik M, Colombo C, Gavriloa O, et al. Genetic background (C57BL/6) versus FVB/N strongly influences the severity of diabetes and insulin resistance in ob/ob mouse. Endocrinology. 2004;145(7):3258–3264.

[36] Nowicki M, Baum P, Kosacka J, et al. Effects of isoflurane anesthesia on F-waves in the sciatic nerve of the adult rat. Muscle Nerve. 2014;50(2):257–261.

[37] Krieger F, Ellein N, Saenger S, et al. Polyethylene glycol-coupled IGF1 delays motor function defects in a mouse model of spinal muscular atrophy with respiratory distress type 1. Brain. 2014;137(Pt 5):1374–1393.

[38] Kosacka J, Nowicki M, Bluhmer M, et al. Increased autophagy in peripheral nerves may protect Wistar Ottawa Karlsburg W rats against neuropathy. Exp Neurol. 2013;250:125–135.

[39] Carenini S, Maurer M, Werner A, et al. The role of macrophages in demyelinating peripheral nervous system of mice heterozygously deficient in p0. J Cell Biol. 2001;152(2):301–308.
[40] Martini R, Toyka KV. Immune-mediated components of hereditary demyelinating neuropathies: lessons from animal models and patients. Lancet Neurol. 2004;3(8):457–465.

[41] Klein D, Patzko A, Schreiber D, et al. Targeting the colony stimulating factor 1 receptor alleviates two forms of Charcot-Marie-Tooth disease in mice. Brain. 2015;138(Pt 11):3193–3205.

[42] Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87–91.

[43] Anstey KJ, Cherbuin N, Budge M, et al. Body mass index in midlife and late-life as a risk factor for dementia: a meta-analysis of prospective studies. Obes Rev. 2011;12(5):e426–437.

[44] Hur J, Dauch JR, Hinder LM, et al. The metabolic syndrome and microvascular complications in a murine model of type 2 diabetes. Diabetes. 2015;64(9):3294–3304.

[45] Lupachyk S, Watcho P, Hasanova N, et al. Triglyceride, nonesterified fatty acids, and prediabetic neuropathy: role for oxidative-nitrosative stress. Free Radic Biol Med. 2012;52(8):1255–1263.

[46] Miller RJ, Jung H, Bhangoo SK, et al. Cytokine and chemokine regulation of sensory neuron function. Handb Exp Pharmacol. 2009;194:417–449.

[47] Chiu IM, von Hehn CA, Woolf CJ. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nat Neurosci. 2012;15(8):1063–1067.

[48] Orr JS, Kennedy A, Anderson-Baucum EK, et al. Obesity alters adipose tissue macrophage iron content and tissue iron distribution. Diabetes. 2014;63(2):421–432.

[49] Gan ZS, Wang QQ, Li JH, et al. Iron reduces M1 macrophage polarization in RAW264.7 macrophages associated with inhibition of STAT1. Mediators Inflamm. 2017;2017:8570818.

[50] Bao W, Rong Y, Rong S, et al. Dietary iron intake, body iron stores, and the risk of type 2 diabetes: a systematic review and meta-analysis. BMC Med. 2012;10:119.