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# Metal bashing: Iron deficiency and manganese overexposure impact on peripheral nerves

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## Abstract

Iron (Fe) deficiency (FeD) and manganese (Mn) overexposure (MnOE) may result in several neurological alterations in the nervous system. Iron deficiency produces unique neurological deficits due to this elemental role in central nervous system (CNS) development and myelination, which might persist after normalization of Fe in the diet. Conversely, MnOE, is associated with diverse neurocognitive deficits. Despite these well-known neurotoxic effects on the CNS, the influence of FeD and MnOE on the peripheral nervous system (PNS) remains poorly understood. The aim of the present investigation was to examine the effects of developmental FeD and MnOE or their combination on the sciatic nerve of young and adult rats. The parameters measured included divalent metal transporter 1 (DMT1), transferrin receptor (TfR), myelin basic protein (MBP) and peripheral myelin protein 22 (PMP22) expression, as well as Fe levels in the nerve. Our results showed that FeD produced a significant reduction in MBP and PMP22 content at P29, which persisted at P60 after Fe-sufficient diet replenishment regardless of Mn exposure levels. At P60 MnOE significantly increased sciatic nerve Fe content and DMT1 expression. However, the combination of FeD and MnOE produced no marked motor skill impairment. Evidence indicates that FeD appears to hinder developmental peripheral myelination, while MnOE may directly alter Fe homeostasis. Further studies are required to elucidate the interplay between these pathological conditions.

### Keywords

iron; manganese; peripheral nervous system; myelination

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## Introduction

Iron (Fe) and manganese (Mn) are essential dietary elements whose levels are strongly regulated under physiological conditions. Consequently, numerous investigators examined the effects of Fe deficiency (FeD) and Mn overexposure (MnOE) on the central nervous system (CNS) (Park et al., 2007; Seo et al., 2013). Fe deficiency, the most common nutritional deficiency, affects approximately two billion people globally (Beard, 2004; Lee and Okam, 2011). When FeD occurs during the first two years of life, this disorder leads to irreversible damage in the nervous system (Youdim, 2008) even after the reintroduction of an Fe-sufficient (FeS) diet (Beard and Connor, 2003; Badaracco et al., 2008; Ortiz et al., 2004). As shown in developing rats, the peak in Fe uptake in the brain coincides with the point of greatest myelination, with FeD thus leading to alterations in myelin sheath production (Connor, 1994; Morath and Mayer-Pröschel, 2001). For this reason, the majority of neurological disorders associated with FeD have been attributed to hypomyelination (Ortiz et al., 2004; Erikson et al., 2000). Human studies reported a decrease in auditory and visual evoked potentials (Algarin et al., 2003), while investigations in rodents demonstrated a persistent change in resting energy status, neurotransmission and myelination (Rao et al., 2003), as well as altered dopaminergic functions (Nelson et al., 1997) and locomotor activity (Hunt et al., 1994).

Manganese overexposure was reported to be neurotoxic in children (Bouchard et al., 2011; Khan et al., 2012; Lucchini et al., 2012) and associated with cognitive deficits, behavioral disinhibition, decreased IQ and poor school performance (Zoni and Lucchini, 2013; Haynes et al., 2015). Common routes of exposure are contaminated drinking water (Oulhote et al., 2014), contaminated air from smelting factories (Menezes-Filho et al., 2014), tropical fruit acai diet (Santos et al., 2014) and soy-based infant formulas (Tran et al., 2002a). Studies in rodent models of MnOE on CNS developmental effects such as impairment in monoamine levels, learning, memory, motor activity and coordination (Amos-Kroohs et al., 2015; 2016; 2017; Tran et al., 2002b), as well as a reduction in fine motor control (Beudin et al., 2013; 2015) were previously reported.

FeD usually exacerbates tissue Mn absorption due to similarities in uptake mechanisms (Park et al., 2013; Kim and Park, 2014; Meltzer et al., 2010). Both metals possess a classical transferrin (Tf)-bound uptake route through the transferrin receptor (TfR) and an alternative Tf-independent route through divalent metal transporter-1 (DMT1; Tuschl et al., 2013). Both these proteins have high affinity for Mn and Fe (He et al., 2006; Wang et al., 2008) and can be found in various tissues (Chen et al., 2015; Wu et al., 2015). In the CNS, TfR facilitates poliovirus permeation (Mizutani et al., 2016) and enhances macromolecular drugs to permeate the blood-brain barrier (Li et al., 2012), while DMT1 is involved in the pathophysiology of neurodegenerative disorders such as Parkinson's disease (Lee et al., 2010; Salazar et al., 2008). In summary, neurobehavioral experiments demonstrate that the combination of FeD and MnOE during developmental stages was found to (1) decrease Fe in plasma, (2) increase Mn, TfR, and DMT1 expression at multiple ages in the rat brain (Amos-Kroohs et al., 2015) and (3) produce behavioral deficits in adult rats (Amos-Kroohs et al., 2016; 2017; Tran et al., 2002b).

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Despite the well-known neurotoxic effects of FeD on the CNS, the influence of this metal deficiency on peripheral myelination remains poorly understood. Salis et al (2002; 2012) previously demonstrated the pro-differentiating effect of Fe on Schwann cell (SC) maturation and survival. Kabakus et al (2002) noted that FeD is associated with peripheral neuropathies, but whether it is a causative factor or a consequence of the degenerating process remains to be established (Levi and Taveggia, 2014). Some investigators postulated that children with FeD exhibit electrophysiological impairment (Kabakus et al., 2002), while others reported no significant alterations in otherwise healthy children (Akyol et al., 2003). Further, patients with β-thalassemia (Stamboulis et al., 2004) and those submitted to bariatric surgery (Thaisetthawatkul et al., 2004) may develop peripheral neuropathies as a consequence of FeD. In addition, DMT1 is involved in the remyelination process following Wallerian degeneration (Martinez-Vivot et al., 2013; 2015). Regarding the role of Mn, significant amounts were detected in the peripheral nervous system (PNS), although at lower concentrations than in brain (Bourre et al., 1987).

Considering the plethora of effects reported for FeD and MnOE in the CNS, the transport mechanisms shared by Fe and Mn through the nervous system and the role established for Fe in PNS remyelination, it was postulated that FeD and MnOE or their combination may impact PNS myelination similar to that noted in the CNS. The aim of this study was to determine myelin basic protein (MBP), peripheral myelin protein 22 (PMP22) –two markers of peripheral myelin–, Fe levels and transporters DMT1 and TfR in the sciatic nerve in both young and adult rats. In addition, the influence of FeD and MnOE or their combination was examined on motor coordination at both ages using a Rotor-Rod test.

## Materials and methods

### Animals

Male and nulliparous female Sprague Dawley CD (IGS) rats (Charles River Laboratories, Raleigh, NC; strain #001), approximately 60 days old and weighing approximately 350 and 290 g on arrival, respectively, were acclimatized for no less than one week in the vivarium (AAALAC International accredited) before breeding. Rats were maintained on a 14–10 hr light-dark cycle (lights on 6 am) with controlled temperature (19 ± 1 °C) and humidity (50% ± 10%) throughout the experiment. Rats were housed in a Modular Animal Caging System (Alternative Design, Siloam Spring, AR). HEPA-filtered air was supplied to each cage (Alternative Design, Siloam Spring, AR) with 30 air changes/hr. Reverse osmosis filtered water (SE Lab Group, Napa, CA) and NIH-07 diet (250 ppm Fe and 80 ppm Mn) were provided *ad libitum*, except during the FeD feeding period. A semicircular stainless steel enclosure was placed in cages for enrichment (Vorhees et al., 2008). Females were separated from males the day a sperm plug was detected (embryonic day 0, E0). Birth was counted as post-natal day 0 (P0). On P1, litters were culled to 10, 5 per gender, using a random number table. On P28, pups were removed from dams into same gender cages (4/cage) until P42, when animals were re-housed (2/cage/gender). All protocols were approved by the Institutional Animal Care and Use Committee and adhered to the NIH Guide on the Care and Use of Laboratory Animals in Research.

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### Iron deficiency (FeD)

Pregnant females were kept on a standard NIH-07 diet until E15. From E15 to P28, dams were provided one of two purified diets differing only in Fe content (Land O' Lakes Purina Feed, Evansville, IN). Half the dams were provided an Fe-sufficient diet (350 ppm, FeS, 99 ppm Mn) while the other half received an Fe-deficient diet with a 90% reduction (35 ppm, FeD, 99 ppm Mn). Offspring were breastfed from P0 to P28, placed back on the standard NIH-07 diet at P28, and maintained on this diet throughout the remainder of the experiment (Figure 1). This previously published FeD paradigm (Amos-Kroohs et al., 2015; 2016; 2017) was adapted from Fitsanakis et al. (2009; 2011) and effects on hematological parameters similar to clinical FeD, but not anemia were found (Amos-Kroohs et al., 2015).

### Manganese (Mn) overexposure

For these experiments, a split-litter design was used. Within each litter, two males were gavaged 0.01 M anhydrous sodium chloride (VEH, to achieve $MnCl_2$ osmolarity), while three male pups were administered orally 100 mg/kg Mn chloride ($MnCl_2$, MnOE) at a dose of 3 ml/kg (Amos-Kroohs et al., 2016; 2017; Graham et al., 2011) every other day from P4 to P28. The extra male MnOE pup supplemented for anticipated mortality only. Gavage was used to avoid maternal MnOE and its potential influence on maternal-pup interaction. Graham et al (2011) showed that this regime did not increase corticosterone levels above that of untreated littermates. This experimental protocol attempts to replicate oral intake as a common route for exposure during development in humans (Menezes-Filho et al., 2009). Previously investigators reported that these concentrations produce elevated brain and blood Mn values (Amos-Kroohs et al., 2015; Vorhees et al., 2014).

### Sciatic nerve collection and preparation

Gender-dependent behavioral and cognitive outcomes were observed in this rat model (Amos-Khroos et al., 2015). However, no gender-dependent differences in myelination or remyelination were detected in the PNS (Aquino et al., 2006; Setton-Avruj et al., 2002; Usach et al., 2011). For these experiments, only sciatic nerves from male offspring were collected to avoid gender-dependent metabolic differences in Mn homeostasis (Carvalho Da Silva et al., 2017) as previously noted in humans. In the light of this evidence, it was decided to experiment only with male sciatic nerves, leaving the analysis in female sciatic nerves for future studies. Male rats in each experimental group (FeS-VEH, FeS-MnOE. FeD-VEH, and FeD-MnOE, Figure 1) were weighed and sacrificed for nerve collection at either P29 or P60 (weights are summarized in Table 1). These sciatic nerves were prepared for immunohistochemistry as previously described (Setton-Avruj et al., 2007; Usach et al., 2011). The number of animals employed in each experimental group is indicated in the corresponding figure legends. Personnel carrying out analyses were blind with respect to group enrollment.

### Tissue preparation and immunofluorescence analysis

Briefly, tissue was frozen after fixation and cryopreservation and cut at 16 μm thickness in a cryostat (Zeiss Microm). The sections were mounted on gelatin-precoated glass slides, allowed to dry for at least 1 hr, and rinsed twice in PBS and twice in PBS-Triton X-100

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0.1% solution. Sections were incubated in 5% fetal calf serum in PBS for 2 hr at room temperature. Slides were incubated for 18–24 hr in a humid chamber at 4 °C with: anti-rat MBP (1:500, IgG rabbit polyclonal, a kind gift of Dr. Campagnoni, UCLA Neuroscience Research Building Department of Psychiatry and Biobehavioral Sciences. LA, USA), anti-PMP22 (1:200, rabbit polyclonal, Santa Cruz Biotech), anti-rat CD71 (1:100, mouse monoclonal IgG (clone Ox-26) (BD) or anti-DMT1 (1:200 goat polyclonal, Santa Cruz Biotech). Goat anti-rabbit Cy3 (1:500) or donkey anti-goat Dylight 488 (1:200) secondary antibodies (Jackson Lab) plus Höechst 32258 (2 μg/ml, Sigma) were used accordingly. Controls were incubated without primary antibodies following Dr. Sapper's suggestions (Saper, 2005) and validated previously by Usach et al., (2011; 2017). Analysis was performed using an Olympus BX100 epifluorescence microscope.

## Iron staining

Sections were processed utilizing a modification of the Perls' staining method to detect ferric Fe (Bishop and Robinson, 2001; Moos and Mollgard, 1993). Intensification of Perls' reaction was performed with DAB-Ni sulphate (3´–3´-diaminobenzidine-nickel sulphate; 0.05%:0.01%; Guardia Clausi et al., 2010).

## Image analyses and quantification

Microscope images were obtained using a CoolSnap digital camera and Image Pro Plus 5.1 software was used for image analysis. At least 4 images per nerve were analyzed in order to cover all the length of the tissue; in each experimental group and survival time, between 5 and 8 independent nerves (derived from 5–8 different animals) were considered. In each image, the integrated optical density (IOD) was measured in 10 randomly selected fields (33 × 33 μm each). IOD values were expressed in arbitrary units (AU). All data were analyzed and quantified by experimenters who were blind to the experimental design. Figures show representative images from each experimental group and survival time.

## Rotor-Rod analysis

From a separate cohort of treated animals, male rats were tested on a San Diego Instruments (SDI, San Diego, CA) Rotor-Rod™ System to determine whether diet, treatment or their combination brought about changes in motor skills. At P29 or P60 time points, rats were first habituated to the non-moving Rotor-Rod for 30 sec and were then conditioned to the moving Rotor-Rod at 12 rpm. Twenty-four hr later, rats were tested once a day for 4 days in a 300 sec program in which the Rotor-Rod continuously accelerated from 12 rpm to 50 rpm at a rate of 0.25 rpm to measure latency to fall. The number of animals used in each experimental group is indicated in the corresponding figure legends. Personnel doing tests were blind with respect to group enrollment.

## Quantification and statistical analysis

Sciatic nerve Fe content and DMT1, MBP and PMP22 expression were analyzed by two-way analysis of variance (ANOVA) with diet (FeS or FeD) and treatment (Veh or MnOE) as independent variables. Briefly, and considering the image analysis described in the previous section, an average of all the images obtained from the same sciatic nerve was calculated

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(between 5–8, depending on the experimental group and the age) and used for the two-way ANOVA. For Rotor-Rod analysis, a two-way ANOVA was employed considering testing days as repeated measures of each rat followed by analyses of covariance for MnOE and body weight. Significant results were then analyzed by Bonferroni post-test. The statistical analysis was re-run using the SAS package and included weight as a covariate. In all cases significance was considered at $P<0.05$.

## Results

### Weight and iron content

In agreement with previous findings, both FeD and MnOE in the current study produced a reduction in animal weight at young ages (Table 1; Unger et al., 2007). In accordance with previous observations, FeD-MnOE animals weighed less compared to all other experimental groups, regardless of age (Table 1; Garcia et al., 2006; Amos-Kroohs, 2015). With respect to Fe content in the sciatic nerve, no significant differences were found between groups at younger ages (Figure 2A and 2B). Conversely, at P60, FeD rats exhibited significantly lower Fe levels than FeS rats, which were compensated by MnOE (Figure 2C and 2D). For comparison, an unstained sciatic nerve is also shown (Figure 2E).

### Myelin protein levels and distribution

MBP expression levels, a major myelin protein and a reliable index of myelination status, were assessed in both young and adult rats. Although the spatial distribution appeared comparable across groups at P29, MBP levels were significantly lower in the FeD group. No overall changes in MBP were induced by MnOE. At P60, MBP levels were also significantly decreased by FeD and further reduced by MnOE, with FeD-MnOE group displaying the lowest MBP content (Figure 3C and D). For reference, a negative control of MBP staining is also illustrated (Figure 3E). In agreement with MBP results, PMP22 values were significantly diminished at P29 in FeD animals with the FeD-MnOE group exhibiting the lowest levels (Figure 4A and B). An image of negative samples for PMP22 is presented as control (Figure 4E).

### Fe transporter levels and distribution

At P29, DMT1 spatial distribution and levels were unaffected by FeD or MnOE (Figure 5A and B). Similarly, no marked interaction between FeD and MnOE was observed in P60 rats, as DMT1 expression in MnOE rats was significantly higher regardless of diet (Figure 5C and D). An image of negative samples for DMT1 is shown as control (Figure. 5E). Finally, no significant differences were detected in TfR values across groups either at P29 (Figure 6A and B) or at P60 (Figure 6C and D). An image of negative samples for TfR is shown as control (Figure 6E).

### Rotor-Rod performance

In terms of observable motor coordination and performance, P29 animals were not markedly affected by FeD. However, MnOE rats exhibited a significant rise in amount of time spent on the Rotor-Rod (Table 2), particularly skewed by results obtained on day 4. An analysis of covariance demonstrated that weight did not exert a significant influence on performance in

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the P29 pups. At P60, motor coordination and performance were not markedly affected by FeD, MnOE or their combination.

## Discussion

Approximately two billion people worldwide suffer from FeD or anemia (de Benoist et al., 2008) and are hence more likely to accumulate Mn in several tissues including the brain (Erikson et al., 2004; Park et al., 2007; Kim et al., 2013). Despite the considerable amount of evidence available regarding FeD-mediated adverse effects on the CNS (Thompson et al., 2007; Ruvin et al, 2012; Kim et al., 2012), few studies are available on the impact on peripheral myelination.

FeD animal models presented with a decrease in blood hematocrit, body weight and locomotor activity (Amos-Kroohs et al., 2016), as well as neurological disorders predominantly attributed to hypomyelination (Rosato-Siri et al., 2018). Regarding MnOE, excessive embryonic Mn intake may be harmful to neural and skeletogenic cell differentiation in vertebrates (Pinsino et al., 2011). Further, high concentrations of Mn in human placenta correlate with enhanced risk of neural tube defects occurrence (Liu et al., 2013). Amos-Kroohs et al (2015) observed increased offspring mortality rate as a consequence of MnOE. For these reasons, the pre-weaning MnOE paradigm was selected, as this results in elevated Mn concentrations in blood and brain (Amos-Kroohs et al., 2015; Vorhees et al., 2014) which is considered to result in learning and memory deficits (Amos-Kroohs et al., 2017). Given that alterations in Fe transport mechanisms may elevate Mn uptake, our experimental model employed a combination of FeD-induced effects and consequent exacerbation of MnOE toxicity (Park et al., 2013; Kim et al., 2014; Meltzer et al., 2010).

Peripheral myelination is a long-spanning process in both rodents and humans. In rodents it is a postnatal event starting 3 days after birth and covering the first 3 weeks of life. In humans, peripheral myelination starts during embryonic life and ends by puberty (Berthold et al., 2005). This information provided the basis for the current study, which was conducted at two distinct time points of PNS development, i.e. P29, a key point in peripheral myelinogenesis, and P60, a steady-state point when myelinogenesis is complete.

FeD promoted a significant reduction in younger age rat MBP levels, which remained low in adulthood despite the re-introduction of an FeS diet. The interaction observed between FeD and MBP levels during development reinforces the notion that nerve damage is associated with high MBP sensitivity and may reflect a blockade of SC progress to a myelinating phenotype and myelin gene expression, indicating a key role for Fe in peripheral myelinogenesis (Martínez-Vivot et al., 2013; 2015). These *in vivo* findings are in agreement with those reported *in vitro* showing that Fe plays a role in SC maturation and prevents SC dedifferentiation in culture through an elevation in cyclic adenosine monophosphate (cAMP) and cAMP response element-binding (CREB) phosphorylation, both essential for myelin protein expression (Salis et al., 2012). Dysfunction of human DMT1 is associated with FeD anemia (Mims et al., 2005; Priwitzerova et al., 2005), Fe overload disorders (Hediger et al., 2002; Rolfs et al., 2002), neurodegenerative diseases (Salazar et al., 2008; Zheng et al.,

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2009), cancer (Brookes et al., 2006; Boult et al., 2008) and inflammation (Martini et al., 2008; Gaudet et al., 2011). Martinez-Vivot et al (2015) demonstrated a positive correlation between DMT1 protein expression and Fe levels and a negative correlation between DMT1 and MBP protein levels in a model of peripheral Wallerian degeneration. FeD constitutes a direct insult on peripheral myelin, while Wallerian degeneration indirectly affects myelin by interrupting SC-axon cross talk. However, both pathological scenarios induce a fall in MBP levels, which promote a rise in DMT1 to enhance Fe uptake for remyelination/regeneration. Interestingly, MnOE effects on MBP, Fe and DMT1 levels were most evident in the FeD rats, which may reflect heightened sensitivity to Mn-induced toxicity triggered by this common developmental nutritional deficiency.

Finally, and despite significant neurocognitive and behavioral impact on the CNS (Amos-Kroohs et al., 2015; 2017; Fitsanakis et al., 2009; 2011) and the influence on peripheral MBP levels, our data showed little impact of FeD on motor coordination and balance. Of note, rotor-rod performance appeared to reflect weight levels in P29 rats with the MnOE groups displaying the longest time spent at the task. In contrast, P60 motor performance appeared to respond to rat age rather than diet, treatment or weight.

These results are by no means inclusive, as more comprehensive behavioral testing would assist in determining whether this decreased sensitivity extends to areas other than gross motor function. Further studies are needed to seek corroboration of these effects in female rats exposed developmentally to this model. In addition, our results suggest vulnerability in myelination during development after FeD and its combination with MnOE.

## Conclusions

FeD treatment during development reduced sciatic nerve MBP levels both at young and adult ages, an effect enhanced by MnOE and not reversed by re-introduction of an FeS diet. Further elucidating the interaction between these two pathological conditions and their effects on myelin-dependent neurological processes may render new targets for therapeutic strategies.

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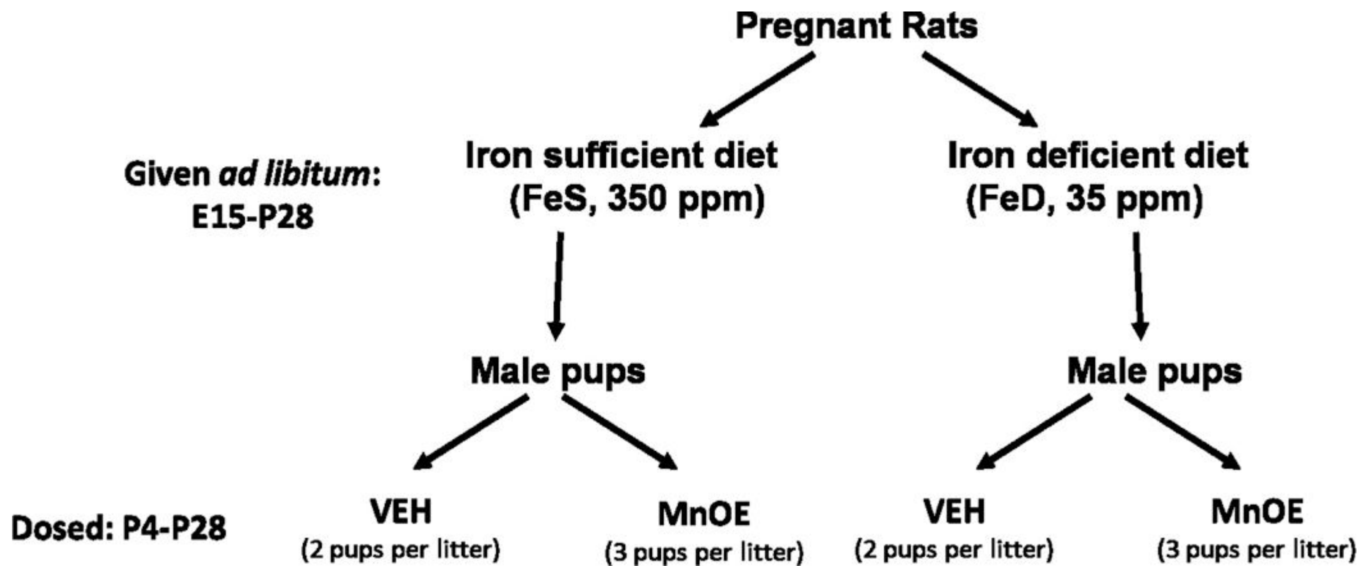


**Figure 1: Study design.**

At E15, dams were removed from the NIH-07 diet and placed on a purified FeS or a purified FeD diet, which differed only in Fe content (350 ppm vs 35 ppm). Male offspring were exposed via gavage to either 100 mg/kg $MnCl_2$ or isotonic VEH every other day from P4 to P28. MnOE and FeD diet were continued until P28, when offspring were placed back on an NIH-07 diet for the remainder of the experiment. Two or three male pups per litter were included in each experimental group, with a total of 3 litters included.

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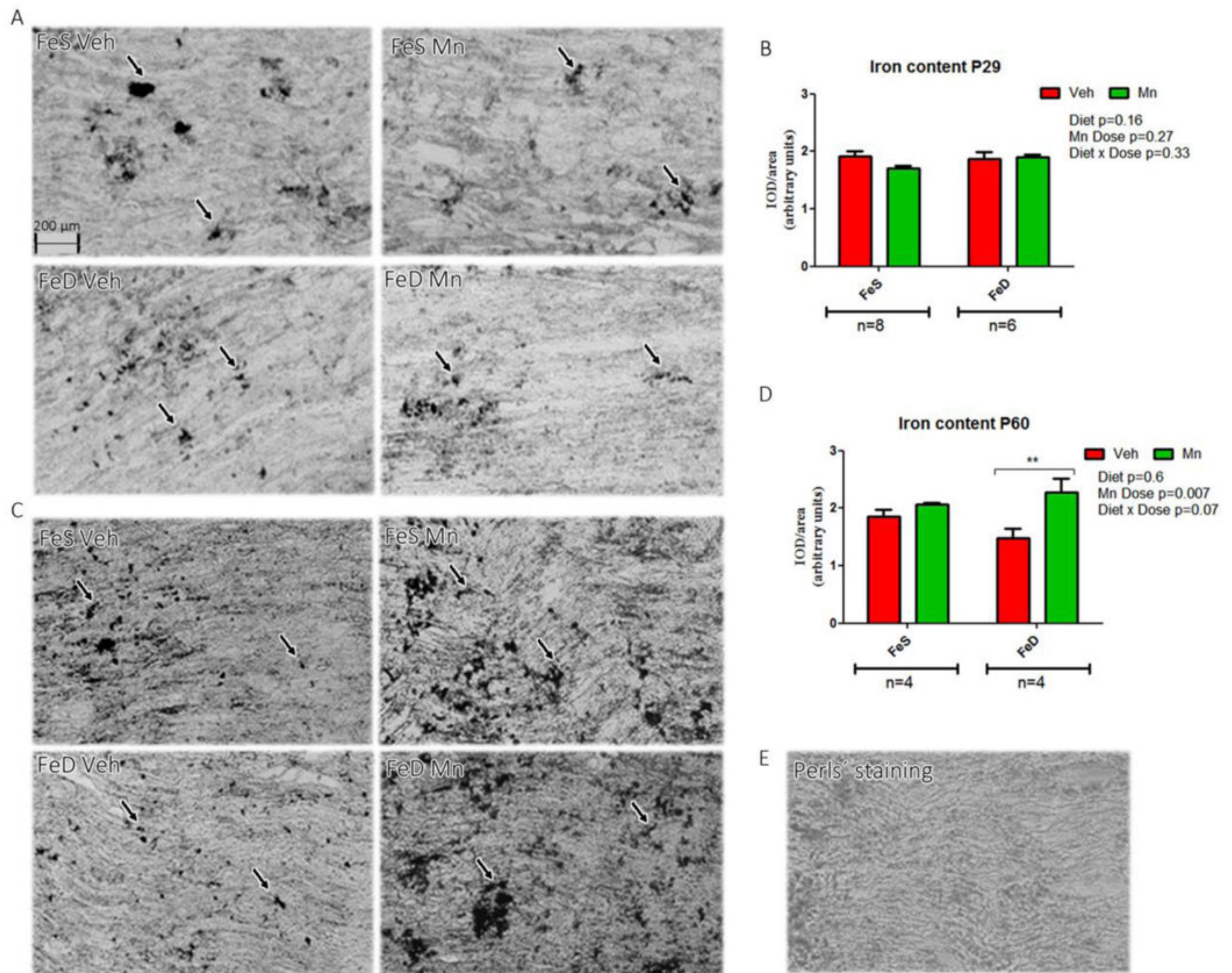


**Figure 2: Iron content in the sciatic nerve.**

A, Perls' staining of sciatic nerve from P29 in the FeS (n=8) and FeD (n=6) groups. C, Perls' staining of sciatic nerve from P60 rats (n=4) in both groups in each experimental condition. B, D: Relative Fe quantification. E: Negative control image of a Perls' staining. Values are expressed as the mean ± SEM in arbitrary units. Statistical analysis was performed through two-way ANOVA followed by Bonferroni post-test (*p< 0.05).

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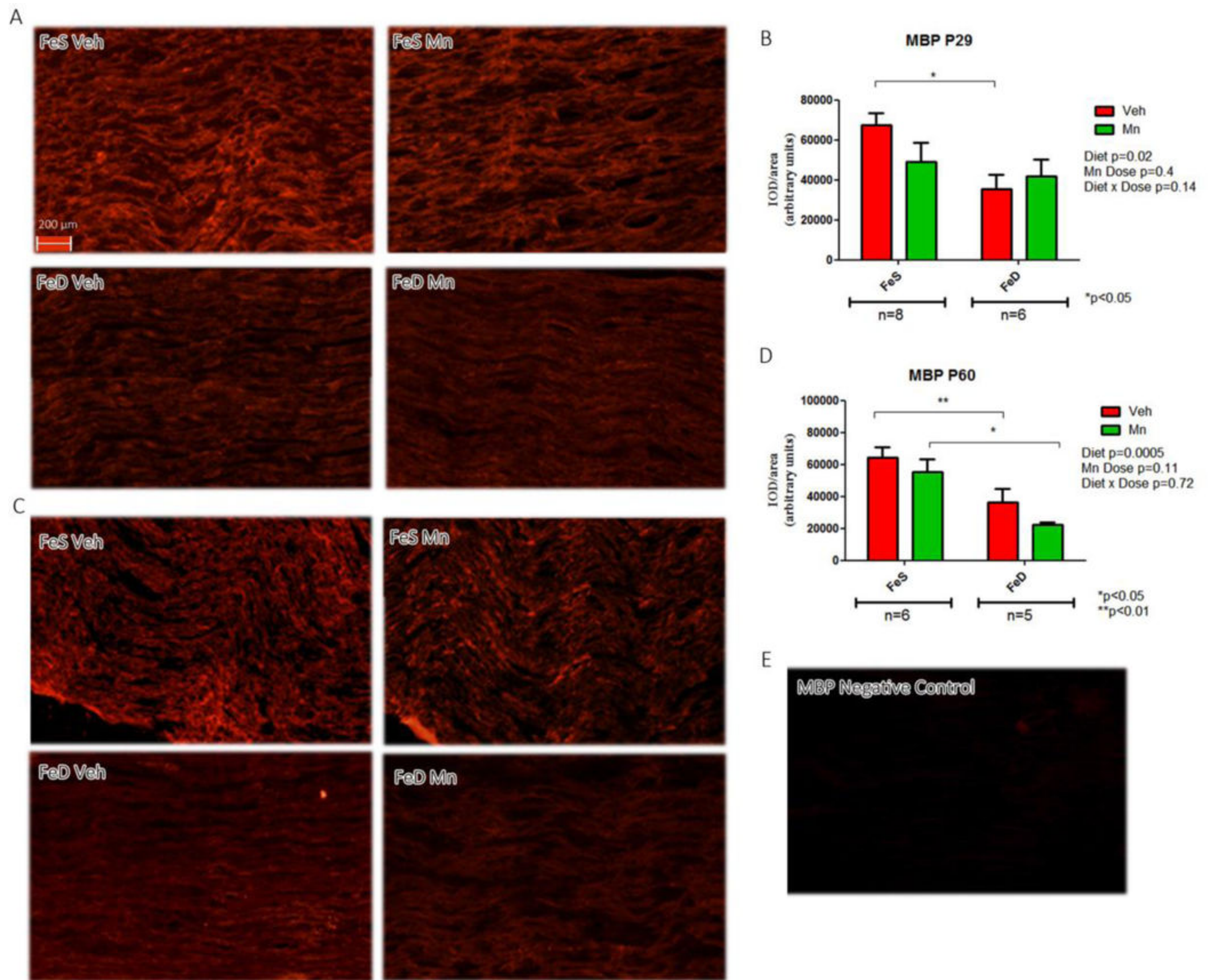


**Figure 3: MBP levels and distribution in the sciatic nerve.**

A, MBP immunofluorescence in sciatic nerve slices from P29 in the FeS (n=8) and FeD (n=6) groups. C, MBP immunofluorescence in sciatic nerve slices from P60 rats in the FeS (n=6) and FeD (n=5) groups in each experimental condition. B, D: IOD quantification for MBP. Values are expressed as the mean ± SEM in arbitrary units. Statistical analysis was performed through two-way ANOVA followed by Bonferroni post-test (*p<0.05,). E: Negative control staining for MBP.

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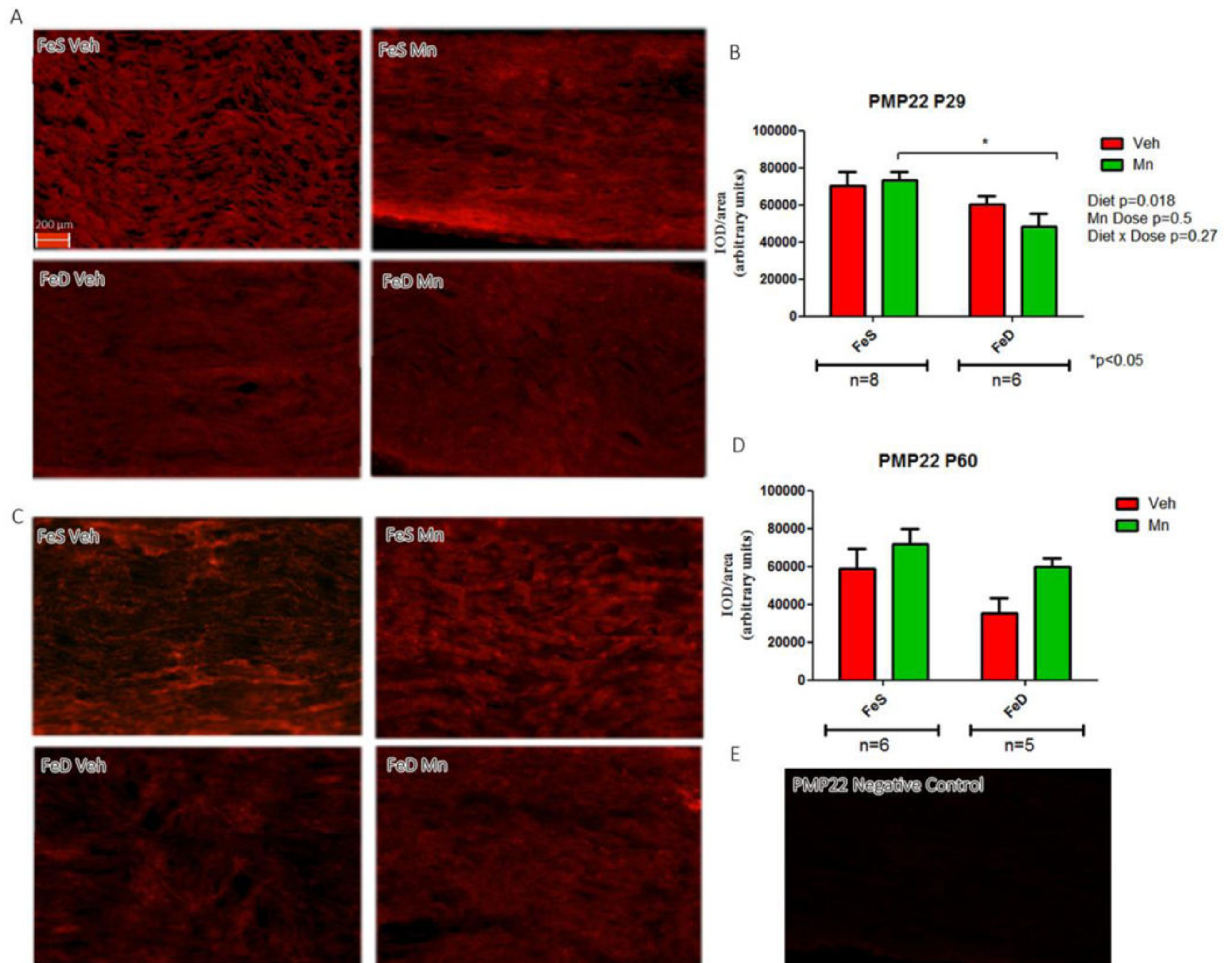


**Figure 4: PMP22 levels and distribution in the sciatic nerve.**

A, PMP22 immunofluorescence in sciatic nerve slices from P29 in the FeS (n=8) and FeD (n=6) groups. C, PMP22 immunofluorescence in sciatic nerve slices from P60 rats in the FeS (n=6) and FeD (n=5) groups in each experimental condition. B, D: IOD quantification for PMP22. Values are expressed as the mean ± SEM in arbitrary units. Statistical analysis was performed through two-way ANOVA followed by Bonferroni post-test (*$p<0.05$). E: Negative control staining for PMP22.

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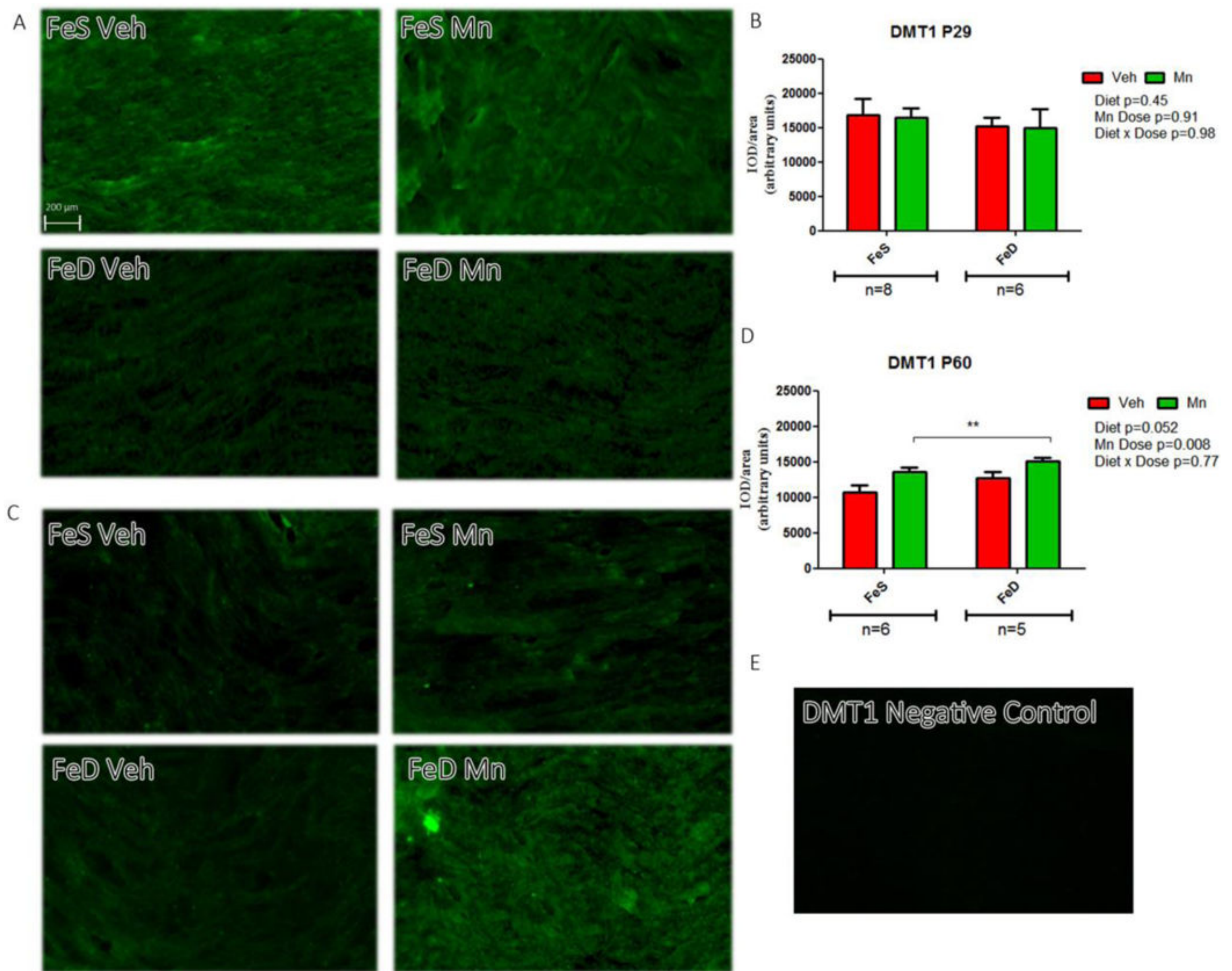


**Figure 5: DMT1 levels and distribution in the sciatic nerve.**

A, DMT1 immunofluorescence in sciatic nerve slices from P29 in the FeS (n=8) and FeD (n=6) groups. C, DMT1 immunofluorescence in sciatic nerve slices from P60 rats in the FeS (n=6) and FeD (n=5) groups in each experimental condition. B, D: IOD quantification for DMT1. Values are expressed as the mean ± SEM in arbitrary units. Statistical analysis was performed through two-way ANOVA followed by Bonferroni post-test (*$p < 0.05$). E: Negative control staining for DMT1.

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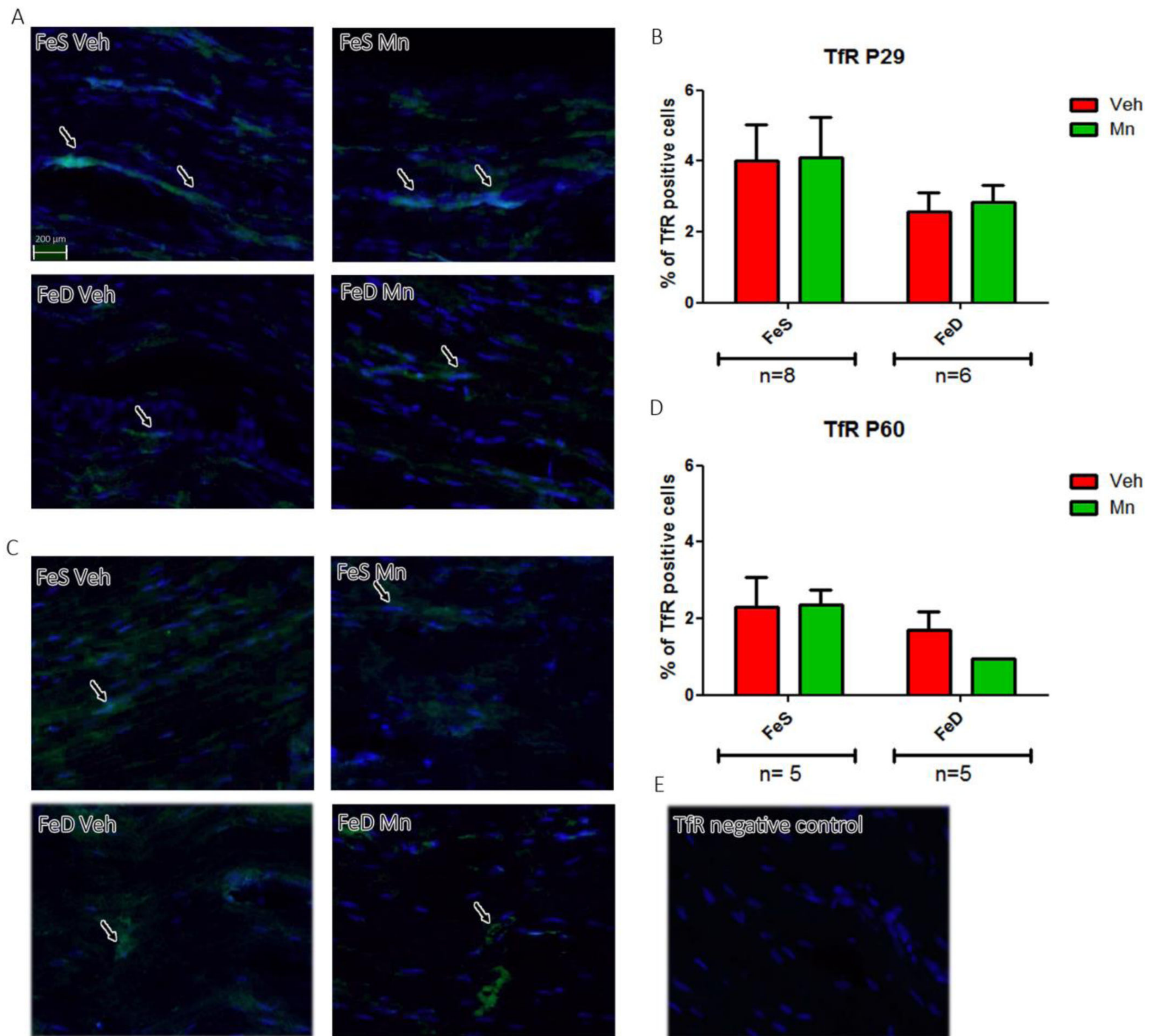


**Figure 6: TfR levels and distribution in the sciatic nerve.**

A, TfR immunofluorescence in sciatic nerve slices from P29 in the FeS (n=8) and FeD (n=6) groups. C, TfR immunofluorescence in sciatic nerve slices from P60 rats (n=5) in both groups in each experimental condition. B, D: IOD quantification for TfR. Values are expressed as the mean ± SEM in arbitrary units. Statistical analysis was performed through two-way ANOVA followed by Bonferroni post-test (*$p < 0.05$). E: Negative control staining for TfR.

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## Table 1:

**Body weight** of male pups at P29 (n=19 per group) and at P60 (n=6 per group) submitted to different diets and Mn exposure during development. Values are expressed as the mean ± SEM. Statistical analysis was performed through two-way ANOVA followed by Bonferroni post-test, where different letters indicate significant differences (p<0.05).

| | **P29 male rats** | **P60 male rats** |
|---|---|---|
| **FeS-Veh** | 98.54 ± 5.03 g A | 359.70 ± 16.67 g A |
| **FeS-MnOE** | 81.86 ± 4.16 g B | 333.93 ± 20.50 g A |
| **FeD-Veh** | 83.59 ± 2.67 g AB | 354.00 ± 17.85 g AB |
| **FeD-MnOE** | 64.28 ± 3.61 g C | 272.20 ± 27.77 g B |

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**Table 2:**

**Rotor-Rod** of male pups at P29 (n=9–11 per group) and at P60 (n=6 per group) submitted to different diets and Mn exposure during development. Values are expressed as the mean ± SEM. Statistical analysis was performed through two-way ANOVA; different letters indicate significant differences ($p<0.05$).

| | P29 male rats | P60 male rats |
|---|---|---|
| **FeS-Veh** | 113.6 ± 9.3 s A | 75.13 ± 7.13 s A |
| **FeS-MnOE** | 152.2 ± 24.5 s B | 85.38 ± 7.09 s A |
| **FeD-Veh** | 100.0 ± 13.1 s AB | 95.47 ± 7.33 s A |
| **FeD-MnOE** | 114.8 ± 9.9 s AB | 86.09 ± 7.47 s A |