THE EFFECTS OF PRENATAL IRON DEFICIENCY AND RISPERIDONE TREATMENT ON THE RAT FRONTAL CORTEX: A PROTEOMIC ANALYSIS.

Lorna Farrelly[1#], Maria Victoria Rosato-Siri[2#], Melanie Föcking[1#], Martin Codagnone[3], Analia Reines[3], Patrick Dicker[4], Kieran Wynne[4], Michael Farrell[5], Mary Cannon[1], Gerard Cagney[4], Juana Maria Pasquini[2*], David R Cotter[1*]

[1]Department of Psychiatry, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland. [2]Department of Biological Chemistry and IQUIFIB, School of Pharmacy and Biochemistry University of Buenos Aires, Buenos Aires, Argentina. [3]De Robertis Institute, University of Buenos Aires, Buenos Aires, Argentina, [4]School of Biomolecular and Biomedical Research, Conway Institute, University College Dublin, Dublin, Ireland, [5]Department of Neuropathology, Beaumont Hospital, Dublin, Ireland.

[#]These authors contributed equally to this work

[*]Both are senior authors of this paper

Key words

environmental risk factor, schizophrenia, antipsychotics, proteomics, myelination

ABSTRACT

Prenatal iron deficiency (pID) has been described to increase the risk for neurodevelopmental disorders such as autism and schizophrenia; however, the precise molecular mechanisms are still unknown. Here, we utilized high throughput mass spectrometry to examine the proteomic effects of pID in adulthood on the rat frontal cortex area (FCA). In addition, the

FCA proteome was examined in adulthood following risperidone treatment in adolescence to see if these effects could be prevented. We identified 1501 proteins of which 100 were significantly differentially expressed in the FCA at post-natal day 90. Pathway Analysis of proteins affected by pID revealed changes in metabolic processes, including the tricyclic acid cycle, mitochondrial dysfunction, and P13K/Akt signaling. Interestingly, most of these protein changes were not present in the adult pID offspring who received risperidone in adolescence. Considering the link between prenatal iron deficiency and several neurodevelopmental disorders such as autism and schizophrenia these presented results bring new perspectives to understand the role of iron in metabolic pathways and provide novel biomarkers for future studies of prenatal iron deficiency.

1. INTRODUCTION

Prenatal exposure to environmental insults are known to adversely affect neurodevelopment and considered risk factors for developmental disorders [1, 2]. For example, Brown and colleagues have explored the hypothesis that prenatal nutritional deficiency is related to the development of schizophrenia, with iron being a potential risk factor [3]. Since the pioneering work by Dallman demonstrating that iron deficiency (ID) in rats induces lower brain iron concentration, much has been learned about the neurochemical, neurometabolic, genomic/proteomic and behavioral effects of early ID on the developing brain through the use of animal models varying in methodology, timing and severity [4-13].

Adequate iron supply is essential for sufficient oxygen transport, optimal enzyme activity, neurotransmitter synthesis and correct myelination [4, 6, 7, 9, 14-18]. In addition, Insel and colleagues have shown that decreased maternal hemoglobin concentrations during pregnancy increase the susceptibility to schizophrenia spectrum disorders (SSDs) among offspring [19]. Furthermore, McGrath et al have associated prenatal, postnatal and childhood ID with motor, cognitive and behavioral abnormalities similar to those observed in children who later develop schizophrenia [20]. Of interest to us, postmortem analyses have revealed myelin abnormalities in the prefrontal cortex (PFC) of schizophrenia patients in some, but not all studies [21-24].

The model of prenatal iron deficiency has been extensively used to evaluate iron deprivation in oligodendrogenesis, myelinogenesis and iron metabolism. Developmental ID negatively impacts oligodendrocyte maturation and thus causes hypomyelination [13] [4, 12].We have previously demonstrated, using the model of prenatal inflammation, dysregulation of metabolic and myelin associated protein expression in the prefrontal cortex, and a preventative effect of adolescent risperidone treatment on these changes [25]. Prenatal inflammation, another well documented environmental risk factor model for schizophrenia, demonstrates a reduced bioavailability for iron and in turn has been shown to negatively affect white matter development [26]; a prominent pathology in postmortem schizophrenia as described previously.

Previous studies have utilized global approaches to identify altered molecular and cellular in brain induced by early-life ID anemia [27-31]. However, in the current study we applied discovery-based proteomic methods to the prenatal iron deficiency model to examine the potentially preventative effects of treatment with the antipsychotic risperidone in the crucial neurodevelopmental time window of adolescence. The present study aims to examine the global proteomic consequences of pID in adulthood while hypothesizing, based on our previous work, that early administration of risperidone in adolescence may reverse or prevent proteomic changes following pID.

2 MATERIALS and METHODS

2.1 *Animal model of Iron Deficiency*

Wistar rats on the fifth day of pregnancy were randomly assigned to two dietary treatment groups: control (Con) diet (40 mg Fe/kg diet) or iron deficient (ID) diet (4 mg Fe/kg diet). Litters were culled to eight pups per litter in order to level off nutritional availability. Mothers and pups were maintained on their respective diets throughout gestation and lactation. After weaning (PND21), a normal diet was reinstated until PND90. Two-way analysis of variance (ANOVA) did not reveal significant gender-dependent differences for the parameters analyzed at the time points selected. Rats had free access to food and water 24 h/day. Light was turned off between 7:00 PM and 7:00 AM and room temperature was maintained at 21-

23°C. Ethical approval was obtained from the Ethics Committee for Animal Research of the University of Buenos Aires.

2.2 Adolescent Risperidone treatment

A set of animals from both the Con and ID groups (n=9 per group) were intraperitoneally injected daily with saline (Con-sal; ID-sal; n=4) or risperidone (0.045mg/kg) (Con-risp; ID-risp; n=5) from PND21 to PND35**. Figure1A** illustrates the model and experimental groups. At PND90, animals were sacrificed by decapitation for proteomic studies.

Another set of animals (n=12, Con and ID groups) were intracardiacally perfused for histochemical procedures (n=3, per group, at PND21; n=9, per group, at PND90) (**Figure1B)**.

2.3 Behavioral testing

Locomotor and exploratory activities, in addition with social behavior were all explored in adult rats to confirm an altered neurodevelopmental phenotype consistent with pID (n=9, per group). Detailed protocols of both of these procedures can be found in the supplementary material.

2.4 Luxol Fast blue staining and immunohistochemistry

Hypomyelination is a reliable confirmation of iron deficiency and thus was investigated in adulthood following prenatal ID. Luxol fast blue identified hypomyelination in various brain regions and an antibody for adenomatous polyposis coli (APC) was utilized to mark mature oligodendrocytes. Detailed protocols of both of these procedures can be found in the supplementary material.

2.5 Sample preparation for LC-MS/MS analysis

Label free mass spectrometry (LC-MS) was performed on the frontal cortex area (FCA) of saline and risperidone treated adult offspring from both Con and ID dams. After decapitation, brains were placed on an ice surface and dissected under a Leica Stereozoom EZ4 dissecting microscope. The olfactory bulb was removed and the FCA was sonicated (Sonics© Newtown,

CT, USA) in triethyl-ammonium-bicarbonate buffer containing protease inhibitors and protein concentrations were determined using the Bradford assay [32]. Fifty micrograms of protein homogenate from each sample was denatured in 10 µl 2% RapiGestTM solution (Waters, United Kingdom) at 80°C for 10 min. Samples were subsequently reduced in the presence of 50mM TCEP (tris2-carboxyethylphosphine; Sigma Aldrich, Ireland) at 60°C for 60 min and alkylated in the dark with 200mM iodoacetic acid (Sigma Aldrich, Ireland). Protein was digested with 5 µg of sequence grade modified trypsin (Promega, United Kingdom), overnight in a 37°C shaking incubator. Digestion was stopped and the RapiGest precipitated, with formic acid (0.1% v/v). After digestion, peptides were resuspended in 0.5% trifluoroacetic acid, dried in an Eppendorf VacufugeTM (Eppendorf©, USA) and desalted using 5 µg capacity, C18 resin ZipTips (Millipore, USA) [33].

2.7 Label free Mass Spectrometry

LC-MS was performed on a Thermo Scientific LTQ Orbitrap XL mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Further information regarding LC-MS settings and data processing for analysis can be found in the supplementary methods. Ethical approval (application-no. REC-585) was granted by the Royal College of Surgeons in Ireland Research Ethics Committee for all proteomic experiments performed using the pID animal model.

2.8 Proteomic and statistical analyses

The label free quantitation (LFQ) mass spectrometry scores for each protein across the four groups analyzed were $\log_2$ transformed to remove the possible influence of skew in the data. Under-represented proteins in a treatment-sequence group were excluded in cases where less than three LFQ intensities per group were available. Regression normalization was performed to remove technical errors across the samples [34]. An FDR of 5% as advocated by [35] was used to determine the filtered protein set of interest. Group comparisons were performed using a 5% level of significance. Data were analyzed in MaxQuant software and group differences of log-LFQ scores were exponentiated, giving fold changes as measures of treatment effects. ANOVA (SAS software) was performed with pID and risperidone conditions as independent variables. Data management and analysis was done using SAS ®

version 9.1. Further information regarding the MaxQuant data processing can be found within the supplementary information. An ANOVA was used to provide a list of proteins differentially expressed in adult offspring by prenatal iron deficiency compared to saline controls where the main effect analysis of pID was utilized to filter the dataset to proteins significantly affected by the pID diet. Ingenuity Pathway Analysis (IPA) was used to identify the most relevant signaling and metabolic pathways, molecular networks and biological functions within these proteins. To study these protein changes of the IPA pathways within individual group, (for example Con compared to pID), independent sample t-tests were also utilized.

2.9 Western blotting

Based on the *a priori* assumptions of altered myelination following pID, western blotting was used to investigate and confirm reduced myelin basic protein (MBP) expression. For additional western blotting details, please see supplementary methods. Western blotting was undertaken on 4-5 samples per group. Densitometry was performed with ImageJ software (http://imagej.nih.gov/ij/) and statistical analyses were handled in GraphPad Prism Version 5. Independent sample t-tests for pairwise comparisons were used to analyze differences in the *groups of interest* with $p<0.05$ as the significance criterion, and a minimum of at least three experiments were performed.

3 RESULTS

3.1 Confirmation of Iron deficiency

From PND0 to PND9, Con and ID animals showed no significant differences in body weight (Supplementary Figure 1A). ID animals exhibited a lower body weight from PND9 (Con=15.8±1.09; ID=13±0.63), although a normal diet was restored after weaning (e.g. PND35: Con=131±0.71; ID=92±5.35). Significant differences were maintained until PND90 (Con=255±39; ID= 189±16). Risperidone injection had no significant effect on body weight values, neither in Con nor in ID animals (Supplementary Figure 1B). Moreover, to confirm the iron deficient status, luxol fast blue staining and immunohistochemistry for APC were

analyzed. ID animals showed marked hypomyelination, most evident in the anterior section of the corpus callosum (CC), at the level of the FCA, and a retraction of cortical myelin fibers was also observed in the ID group as compared to Con. Additionally, APC immunohistochemistry demonstrated a lower number of mature oligodendrocytes in ID animals in comparison to Con. Further microscopy magnification demonstrated marked differences in the nuclei of these cells where longitudinal orientation of nuclei rows within the CC was observed in the Con; a pattern lost in ID animals (Supplementary Figure 2). Blood parameters measured in ID and C animals at PND90 demonstrated that ID systemic features were reversed to control values upon normal diet reinstatement. This was undertaken to omit the possibility of future findings being confounded by an persistent iron deficiency in adulthood (Table 1).

3.2 Behavioral evaluation

Behavioral tests were performed in order to confirm the abnormal behavioral phenotype following pID. Adult ID animals showed a marked decrease in social activities when compared with controls. In particular, latency to mounting activities ($p<0.05$) and number of mounting events ($p<0.05$) were clearly altered and these animals also showed decreased exploration in comparison with Con ($p< 0.01$) (Supplementary Figure 3).

3.3 *Prenatal iron deficiency induced changes in core metabolic pathways*

A total of 1501 proteins were identified with 1% FDR by mass spectrometry after data input to the MaxQuant bioinformatics software (full data available in PRIDE accession number PXD004975). A total of 100 proteins were found to be significantly altered in adulthood with a main effect of pID (FDR; $p< 0.05$). The 100 proteins found to be significantly altered in adulthood with a main effect of pID were inputted to Ingenuity Pathway Analysis (IPA; Ingenuity Systems; http://www.ingenuity.com) to identify the key biological pathways. The top three pathways are listed in Table 1, together with genes identified from each pathway. These pathways involved metabolic processes, including the tricyclic acid (TCA) cycle, mitochondrial dysfunction, and P13K/Akt signaling (**Figure 2**).

Proteins identified from the implicated pathways were assessed across the groups using a 2x2 ANOVA (Table 2). The results of the independent t-test comparisons within the context of

this analysis provide insight into the effects of iron deficiency and risperidone. Offspring from iron deficient dams exhibited significant alterations in proteins in metabolic function compared to controls. In total, ten metabolic proteins were identified, 4 of which were significantly down-regulated and 6 significantly up-regulated by the main effect of ID compared to controls. Interestingly, in ID-risp adults the majority of these changes were no longer significant which means that some of these effects were prevented by the risperidone in adolescence. Seven proteins belonging to the P13K/AKT pathway (**Table 2C**) were identified, 6 of which were significantly down-regulated by the main effect of ID-sal compared to Con-sal. Once again, most of the significant differences were not present in ID-risp animals.

3.5 Myelin and myelin-related protein changes

Based on *a priori* assumptions of altered myelination following pID, we specifically aimed to quantify the expression of myelin proteins. Six myelin proteins were identified and quantified by LC-MS/MS; 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin oligodendroglial glycoprotein (MOG), myelin basic protein (MBP) and oligodendrocyte myelin glycoprotein (OMG). pID was associated with a decrease in CNP ($p<0.0005$; -1.4 fold), PLP ($p<0.005$; -1.2 fold), MAG ($p<0.005$; -1.4 fold), MOG ($p<0.005$; -1.3 fold) and MBP ($p<0.05$; -1.2 fold). Adult offspring who experienced pID but underwent risperidone treatment during adolescence still exhibited significantly lower values in these major myelin proteins with the exception of MBP and PLP, which displayed non-significant decreases in expression (**Table 3**).

Western blot analysis confirmed a significant decrease in MBP expression in ID offspring compared to Con offspring ($p< 0.005$) (**Figure** 3). Although not found by LC-MS/MS, an increase in MBP in ID-risp compared to ID-sal offspring was found via western blotting ($p< 0.05$).

4 DISCUSSION

Animal models of prenatal risk factors, such as that of pID provide an opportunity to gain insights into related neurodevelopmental disorders such as schizophrenia. Previous proteomic work from our group has suggested that prenatal inflammation may contribute to an increased

risk for schizophrenia through mechanisms involving metabolic function and myelin formation. Surprisingly, we demonstrated that risperidone in adolescence could reverse or rescue such changes during this neurodevelopmental time point compared to animals who did not receive treatment.

Here, we employed a similar study design and utilized label free mass spectrometry and bioinformatics analyses to assess the global effect of prenatal iron deficiency on the proteome of the frontal cortex. We initially confirmed the pID status with histochemical and behavioral changes in the affected adult mice.

Using label free LC-MS/MS, we observed 100 proteins to be differentially expressed following pID and pathway analysis of these implicated abnormal metabolic processes. We observed a pattern of dysregulation of proteins involved in the tricyclic acid cycle (TCA), mitochondrial dysfunction and Akt signaling following prenatal ID (Table 1). The TCA cycle was the most significantly altered pathway and is a major component of cellular metabolism, involving the oxidative metabolism of glucose and the production of carbon dioxide and oxygen. As the mammalian brain depends on glucose as its main source of energy through the generation of ATP, tight regulation of glucose metabolism is critical for brain physiology and its disruption may precede perturbations in myelination [36]. Evidence of altered expression of proteins involved in mitochondrial function and P13K/Akt signaling may reflect cellular stress. The P13K/Akt pathway plays an important role in cell survival and has previously been shown to be enhanced in hippocampal neurons exposed to different levels of iron-induced oxidative stress [37, 38]. Oxidative stress and hypoxia have consistently been implicated as risk factors for the development of schizophrenia [39-42].

In line with our hypothesis based approach we identified, and quantified, six myelin proteins in total; CNPase, PLP, MAG, MOG, MBP and OMG via LC MS/MS. pID was associated with decreased expression of CNPase, PLP, MAG, MOG and MBP. These findings are in line with the requirement of iron for optimal oligodendrocyte and myelin function, consistent with the literature [12, 13, 17, 43] and in keeping with the decreased gene expression of oligodendrocyte markers OLIG 1, OLIG2, MBP, MAG, PLP observed previously in schizophrenia [44]. Down regulation of genes related to OLG and myelin have also been observed in microarray studies of schizophrenia [21, 45-49]; among others, these genes

included MAG, MBP, PLP and MOG [50]. The importance of ID in myelin development is supported by evidence that ID increases the proliferation of oligodendrocyte progenitor cells [13, 43].

We were interested on the effect of adolescent treatment with antipsychotic drugs on pID induced proteins changes. For the most part risperidone treated ID offspring exhibited no change in the identified TCA cycle proteins compared to saline treated ID offspring suggesting that the administration of risperidone in adolescence elicited a preventative effect on the pID induced dysregulation of these proteins. Additionally, risperidone treated ID offspring did not exhibit altered expression of ATP subunits, Parkin (PARK), Synuclein-alpha (SNCA) (see Table 1B), suggesting a preventative and perhaps anti-oxidative effect of antipsychotic treatment. This data is in close agreement with our group's previous work demonstrating that the risperidone administration in adolescence reverses or prevents the altered expression of myelin and metabolic associated protein expression in a prenatal inflammation model of schizophrenia [25].

We observed a decrease in MAPK1 protein expression following pID. An interaction between iron and the MAPK signaling pathway has been noted previously and while some have explored MAPK signalling in schizophrenia, no consistent findings have emerged [51-58]. Mitogen-activated kinases are core components of the ERK/MAPK signaling pathway, which, in the context of the cellular environment, play diverse roles in cell growth, differentiation, apoptosis and response to stress, such as pID (Rubinfeld and Seger 2005; Younes-Rapozo et al. 2009. Findings suggest a role for ERK/MAPK proteins in oligodendrocyte differentiation [59].

Several models have examined how demyelination might induce the hyperdopaminergic state found in schizophrenic patients, involving a misbalance in glutamate [60]. Altered myelination could correlate with an aberrant wiring network, which in turn triggers alterations in dopamine levels [61]. Our results suggest that the timing of demyelination significantly influences the development of schizophrenia-relevant behavior, particularly in adult males.

It should also be appreciated that pID is also associated with autism and ADHD, disorders which also demonstrate white matter changes within the brain [62, 63]. These findings underscore both the importance of pID to neurodevelopmental disorders generally and the need for other additive and/or interactive genetic and environmental influences which lead to these diverse outcomes following pID. Indeed in relation to disorders of white matter, it has been suggested that antipsychotics may exert therapeutic effects by altering metabolic pathways and thus increasing myelin formation [64]. Noto et al. [65] analyzed oxidative stress (OS) and antioxidant status in healthy subjects, drug-naïve and risperidone-treated first-episode psychotic (FEP) patients to show that untreated FEP patients demonstrated marked increases in lipid peroxidation for example. In parallel, risperidone acted to decrease markers of oxidative stress by manipulating antioxidative enzymes. Recent studies from our group have demonstrated that microglial cultures derived from ID animals exhibit poor inflammatory potential [66]. On the basis of these parallel findings, more thorough bioassays will be carried out to study risperidone's effects in the ID model. Furthermore, treatment with quetiapine in animal models of white matter lesions has shown repopulation of mature oligodendrocytes, remyelination and improvements in spatial working memory [67-69]. As adolescence represents a critical time period for neurodevelopment it may represent an ideal time point for treatment intervention in schizophrenia [51, 70].

There are some limitations that should be addressed in this study. Firstly the proteomic analyses were undertaken at only one time point and short-term changes in protein expression closer to the beginning of the ID diet or following drug administration were not assessed. Furthermore, different durations and doses of risperidone treatment may produce different effects. Future studies will consider looking at proteomic analyses at more time-points and can consider other classes and doses of antipsychotic drugs along with their behavioral outputs.

Overall, however, our data provide novel insights into the dysregulating effects of pID on both myelin and metabolic associated protein expression, and into the preventative action of the antipsychotic, risperidone, on these protein changes. These findings open new avenues for potential drugs targets in ID related disorders which promote metabolic and mitochondrial functions and enhance myelin recovery.

pmic201600407-sup-0001-SuppMat.docx

Supplimentary Information.

pmic201600407-sup-0002-figure1.docx

Supplementary Figure 1.

pmic201600407-sup-0002-figure2.docx

Supplementary Figure 2.

pmic201600407-sup-0002-figure3.docx

Supplementary Figure 3.

ACKNOWLEDGEMENTS

This study was supported by a grant from Science Foundation Ireland (grant No. RFP1304) and a Health Research Board Clinician Scientist Award to DR Cotter and a grant from the University of Buenos Aires (UBA 20020100100395) to JM Pasquini. Access to the mass spectrometry equipment in the Conway Institute and the input of Dr. Jane A. English is gratefully acknowledged.

Disclosure Statement: The authors declare no conflict of interest.

Significance of the study

It is well established that environmental factors may increase the risk of schizophrenia and other several neurodevelopmental disorders. Prenatal exposure to insults such as iron deficiency is one such factor although the precise molecular mechanisms are still not well elucidated. Here, we utilized high throughput mass spectrometry to examine the proteomic effects of pID in adulthood on the rat frontal cortex area (FCA). In addition, the FCA proteome was examined in adulthood following risperidone treatment in adolescence to see if these effects could be prevented. Pathway Analysis of proteins affected by pID revealed changes in metabolic processes, including the tricyclic acid cycle, mitochondrial dysfunction, and P13K/Akt signaling. Interestingly, most of these protein changes were not present in the

adult pID offspring who received risperidone in adolescence. Considering the link between prenatal iron deficiency and several neurodevelopmental disorders such as autism and schizophrenia these presented results bring a new perspectives to understanding the role of iron in metabolic pathways and provide novel biomarkers for future studies of prenatal iron deficiency.

REFERENCES

[1] Meli, G., Ottl, B., Paladini, A., Cataldi, L., Prenatal and perinatal risk factors of schizophrenia. *J Matern Fetal Neona* 2012, *25*, 2559-2563.

[2] van Os, J., Kenis, G., Rutten, B. P., The environment and schizophrenia. *Nature* 2010, *468*, 203-212.

[3] Brown, A. S., Susser, E. S., Prenatal nutritional deficiency and risk of adult schizophrenia. *Schizophrenia bulletin* 2008, *34*, 1054-1063.

[4] Badaracco, M. E., Ortiz, E. H., Soto, E. F., Connor, J., Pasquini, J. M., Effect of transferrin on hypomyelination induced by iron deficiency. *Journal of neuroscience research* 2008, *86*, 2663-2673.

[5] Beard, J., Recent evidence from human and animal studies regarding iron status and infant development. *The Journal of nutrition* 2007, *137*, 524S-530S.

[6] Beard, J., Erikson, K. M., Jones, B. C., Neonatal iron deficiency results in irreversible changes in dopamine function in rats. *The Journal of nutrition* 2003, *133*, 1174-1179.

[7] Beard, J. L., Connor, J. R., Iron status and neural functioning. *Annual review of nutrition* 2003, *23*, 41-58.

[8] Beard, J. L., Unger, E. L., Bianco, L. E., Paul, T*., et al.*, Early postnatal iron repletion overcomes lasting effects of gestational iron deficiency in rats. *The Journal of nutrition* 2007, *137*, 1176-1182.

[9] Beard, J. L., Wiesinger, J. A., Connor, J. R., Pre- and postweaning iron deficiency alters myelination in Sprague-Dawley rats. *Developmental neuroscience* 2003, *25*, 308-315.

[10] Georgieff, M. K., The role of iron in neurodevelopment: fetal iron deficiency and the developing hippocampus. *Biochemical Society transactions* 2008, *36*, 1267-1271.

[11] Lozoff, B., Georgieff, M. K., Iron deficiency and brain development. *Seminars in pediatric neurology* 2006, *13*, 158-165.

[12] Ortiz, E., Pasquini, J. M., Thompson, K., Felt, B.*, et al.*, Effect of manipulation of iron storage, transport, or availability on myelin composition and brain iron content in three different animal models. *Journal of neuroscience research* 2004, *77*, 681-689.

[13] Rosato-Siri, M. V., Badaracco, M. E., Ortiz, E. H., Belforte, N.*, et al.*, Oligodendrogenesis in iron-deficient rats: effect of apotransferrin. *Journal of neuroscience research* 2010, *88*, 1695-1707.

[14] Badaracco, M. E., Siri, M. V., Pasquini, J. M., Oligodendrogenesis: the role of iron. *BioFactors* 2010, *36*, 98-102.

[15] Beard, J. L., Why iron deficiency is important in infant development. *The Journal of nutrition* 2008, *138*, 2534-2536.

[16] Beard, J. L., Connor, J. R., Jones, B. C., Iron in the brain. *Nutrition reviews* 1993, *51*, 157-170.

[17] Todorich, B., Pasquini, J. M., Garcia, C. I., Paez, P. M., Connor, J. R., Oligodendrocytes and myelination: the role of iron. *Glia* 2009, *57*, 467-478.

[18] Todorich, B., Zhang, X., Connor, J. R., H-ferritin is the major source of iron for oligodendrocytes. *Glia* 2011, *59*, 927-935.

[19] Insel, B. J., Schaefer, C. A., McKeague, I. W., Susser, E. S., Brown, A. S., Maternal iron deficiency and the risk of schizophrenia in offspring. *Archives of general psychiatry* 2008, *65*, 1136-1144.

[20] McGrath, J., Brown, A., St Clair, D., Prevention and schizophrenia--the role of dietary factors. *Schizophrenia bulletin* 2011, *37*, 272-283.

[21] Hakak, Y., Walker, J. R., Li, C., Wong, W. H.*, et al.*, Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America* 2001, *98*, 4746-4751.

[22] Uranova, N. A., Vikhreva, O. V., Rachmanova, V. I., Orlovskaya, D. D., Ultrastructural alterations of myelinated fibers and oligodendrocytes in the prefrontal cortex in schizophrenia: a postmortem morphometric study. *Schizophrenia research and treatment* 2011, *2011*, 325789.

[23] Matthews, P. R., Eastwood, S. L., Harrison, P. J., Reduced myelin basic protein and actin-related gene expression in visual cortex in schizophrenia. *PloS one* 2012, *7*, e38211.

[24] Mitkus, S. N., Hyde, T. M., Vakkalanka, R., Kolachana, B.*, et al.*, Expression of oligodendrocyte-associated genes in dorsolateral prefrontal cortex of patients with schizophrenia. *Schizophrenia research* 2008, *98*, 129-138.

[25] Farrelly, L., Focking, M., Piontkewitz, Y., Dicker, P.*, et al.*, Maternal immune activation induces changes in myelin and metabolic proteins, some of which can be prevented with risperidone in adolescence. *Developmental neuroscience* 2015, *37*, 43-55.

[26] Lieblein-Boff, J. C., McKim, D. B., Shea, D. T., Wei, P.*, et al.*, Neonatal E. coli infection causes neuro-behavioral deficits associated with hypomyelination and neuronal sequestration of iron. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2013, *33*, 16334-16345.

[27] Carlson, E. S., Stead, J. D., Neal, C. R., Petryk, A., Georgieff, M. K., Perinatal iron deficiency results in altered developmental expression of genes mediating energy metabolism and neuronal morphogenesis in hippocampus. *Hippocampus* 2007, *17*, 679-691.

[28] Clardy, S. L., Wang, X., Zhao, W., Liu, W.*, et al.*, Acute and chronic effects of developmental iron deficiency on mRNA expression patterns in the brain. *Journal of neural transmission. Supplementum* 2006, 173-196.

[29] Rao, R., Tkac, I., Townsend, E. L., Gruetter, R., Georgieff, M. K., Perinatal iron deficiency alters the neurochemical profile of the developing rat hippocampus. *The Journal of nutrition* 2003, *133*, 3215-3221.

[30] Tran, P. V., Dakoji, S., Reise, K. H., Storey, K. K., Georgieff, M. K., Fetal iron deficiency alters the proteome of adult rat hippocampal synaptosomes. *American journal of physiology. Regulatory, integrative and comparative physiology* 2013, *305*, R1297-1306.

[31] Tran, P. V., Kennedy, B. C., Pisansky, M. T., Won, K. J.*, et al.*, Prenatal Choline Supplementation Diminishes Early-Life Iron Deficiency-Induced Reprogramming of Molecular Networks Associated with Behavioral Abnormalities in the Adult Rat Hippocampus. *The Journal of nutrition* 2016, *146*, 484-493.

[32] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 1976, *72*, 248-254.

[33] Farrelly, L. A., Dicker, P., Wynne, K., English, J.*, et al.*, Adolescent Risperidone treatment alters protein expression associated with protein trafficking and cellular metabolism in the adult rat prefrontal cortex. *Proteomics* 2014, *14*, 1574-1578.

[34] Callister, S. J., Barry, R. C., Adkins, J. N., Johnson, E. T.*, et al.*, Normalization approaches for removing systematic biases associated with mass spectrometry and label-free proteomics. *J Proteome Res* 2006, *5*, 277-286.

[35] Benjamini, Y., Hochberg, Y., Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society* 1995, *Series B*, 289-300.

[36] Uranova, N. A., Vostrikov, V. M., Orlovskaya, D. D., Rachmanova, V. I., Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. *Schizophrenia research* 2004, *67*, 269-275.

[37] Fretham, S. J., Carlson, E. S., Georgieff, M. K., Neuronal-specific iron deficiency dysregulates mammalian target of rapamycin signaling during hippocampal development in nonanemic genetic mouse models. *The Journal of nutrition* 2013, *143*, 260-266.

[38] Uranga, R. M., Katz, S., Salvador, G. A., Enhanced phosphatidylinositol 3-kinase (PI3K)/Akt signaling has pleiotropic targets in hippocampal neurons exposed to iron-induced oxidative stress. *The Journal of biological chemistry* 2013, *288*, 19773-19784.

[39] Clarke, M. C., Harley, M., Cannon, M., The role of obstetric events in schizophrenia. *Schizophrenia bulletin* 2006, *32*, 3-8.

[40] English, J. A., Pennington, K., Dunn, M. J., Cotter, D. R., The neuroproteomics of schizophrenia. *Biological psychiatry* 2011, *69*, 163-172.

[41] Pennington, K., Beasley, C. L., Dicker, P., Fagan, A*., et al.*, Prominent synaptic and metabolic abnormalities revealed by proteomic analysis of the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder. *Molecular psychiatry* 2008, *13*, 1102-1117.

[42] Prabakaran, S., Swatton, J. E., Ryan, M. M., Huffaker, S. J*., et al.*, Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. *Molecular psychiatry* 2004, *9*, 684-697, 643.

[43] Morath, D. J., Mayer-Proschel, M., Iron deficiency during embryogenesis and consequences for oligodendrocyte generation in vivo. *Developmental neuroscience* 2002, *24*, 197-207.

[44] Konradi, C., Sillivan, S. E., Clay, H. B., Mitochondria, oligodendrocytes and inflammation in bipolar disorder: evidence from transcriptome studies points to intriguing parallels with multiple sclerosis. *Neurobiology of disease* 2012, *45*, 37-47.

[45] Aston, C., Jiang, L., Sokolov, B. P., Transcriptional profiling reveals evidence for signaling and oligodendroglial abnormalities in the temporal cortex from patients with major depressive disorder. *Molecular psychiatry* 2005, *10*, 309-322.

[46] Dracheva, S., Davis, K. L., Chin, B., Woo, D. A*., et al.*, Myelin-associated mRNA and protein expression deficits in the anterior cingulate cortex and hippocampus in elderly schizophrenia patients. *Neurobiology of disease* 2006, *21*, 531-540.

[47] Katsel, P., Davis, K. L., Haroutunian, V., Variations in myelin and oligodendrocyte-related gene expression across multiple brain regions in schizophrenia: a gene ontology study. *Schizophrenia research* 2005, *79*, 157-173.

[48] Shimizu, S., Koyama, Y., Hattori, T., Tachibana, T*., et al.*, DBZ, a CNS-specific DISC1 binding protein, positively regulates oligodendrocyte differentiation. *Glia* 2014, *62*, 709-724.

[49] Tkachev, D., Mimmack, M. L., Ryan, M. M., Wayland, M*., et al.*, Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *Lancet* 2003, *362*, 798-805.

[50] Zhu, H., Zhao, L., Wang, E., Dimova, N*., et al.*, The QKI-PLP pathway controls SIRT2 abundance in CNS myelin. *Glia* 2012, *60*, 69-82.

[51] Huang, X., Dai, J., Huang, C., Zhang, Q*., et al.*, Deferoxamine synergistically enhances iron-mediated AP-1 activation: a showcase of the interplay between extracellular-signal-regulated kinase and tyrosine phosphatase. *Free radical research* 2007, *41*, 1135-1142.

[52] Mardini, L., Gasiorek, J., Derjuga, A., Carriere, L*., et al.*, Antagonistic roles of the ERK and p38 MAPK signalling pathways in globin expression, haem biosynthesis and iron uptake. *The Biochemical journal* 2010, *432*, 145-151.

[53] Rathnasamy, G., Ling, E. A., Kaur, C., Hypoxia inducible factor-1alpha mediates iron uptake which induces inflammatory response in amoeboid microglial cells in developing periventricular white matter through MAP kinase pathway. *Neuropharmacology* 2014, *77*, 428-440.

[54] Funk, A. J., McCullumsmith, R. E., Haroutunian, V., Meador-Woodruff, J. H., Abnormal activity of the MAPK- and cAMP-associated signaling pathways in frontal cortical areas in postmortem brain in schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 2012, *37*, 896-905.

[55] Kranz, T. M., Goetz, R. R., Walsh-Messinger, J., Goetz, D*., et al.*, Rare variants in the neurotrophin signaling pathway implicated in schizophrenia risk. *Schizophrenia research* 2015, *168*, 421-428.

[56] Swatton, J. E., Sellers, L. A., Faull, R. L., Holland, A*., et al.*, Increased MAP kinase activity in Alzheimer's and Down syndrome but not in schizophrenia human brain. *The European journal of neuroscience* 2004, *19*, 2711-2719.

[57] Walsh, T., McClellan, J. M., McCarthy, S. E., Addington, A. M*., et al.*, Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. *Science* 2008, *320*, 539-543.

[58] Yuan, P., Zhou, R., Wang, Y., Li, X*., et al.*, Altered levels of extracellular signal-regulated kinase signaling proteins in postmortem frontal cortex of individuals with mood disorders and schizophrenia. *Journal of affective disorders* 2010, *124*, 164-169.

[59] Younes-Rapozo, V., Felgueiras, L. O., Viana, N. L., Fierro, I. M*., et al.*, A role for the MAPK/ERK pathway in oligodendroglial differentiation in vitro: stage specific effects on cell branching. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience* 2009, *27*, 757-768.

[60] Takahashi, N., Sakurai, T., Davis, K. L., Buxbaum, J. D., Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. *Progress in neurobiology* 2011, *93*, 13-24.

[61] Valeiras, B., Rosato Siri, M. V., Codagnone, M., Reines, A., Pasquini, J. M., Gender influence on schizophrenia-relevant abnormalities in a cuprizone demyelination model. *Glia* 2014, *62*, 1629-1644.

[62] Doom, J. R., Georgieff, M. K., Gunnar, M. R., Institutional care and iron deficiency increase ADHD symptomology and lower IQ 2.5-5 years post-adoption. *Developmental science* 2015, *18*, 484-494.

[63] Schmidt, R. J., Tancredi, D. J., Krakowiak, P., Hansen, R. L., Ozonoff, S., Maternal intake of supplemental iron and risk of autism spectrum disorder. *American journal of epidemiology* 2014, *180*, 890-900.

[64] Bartzokis, G., Neuroglialpharmacology: myelination as a shared mechanism of action of psychotropic treatments. *Neuropharmacology* 2012, *62*, 2137-2153.

[65] Noto, C., Ota, V. K., Gadelha, A., Noto, M. N.*, et al.*, Oxidative stress in drug naive first episode psychosis and antioxidant effects of risperidone. *Journal of psychiatric research* 2015, *68*, 210-216.

[66] Rosato-Siri, M. V., Marziali, L., Guitart, M. E., Badaracco, M. E.*, et al.*, Iron Availability Compromises Not Only Oligodendrocytes But Also Astrocytes and Microglial Cells. *Molecular neurobiology* 2017.

[67] Xiao, L., Xu, H., Zhang, Y., Wei, Z.*, et al.*, Quetiapine facilitates oligodendrocyte development and prevents mice from myelin breakdown and behavioral changes. *Molecular psychiatry* 2008, *13*, 697-708.

[68] Xu, H., Yang, H. J., Rose, G. M., Li, X. M., Recovery of behavioral changes and compromised white matter in C57BL/6 mice exposed to cuprizone: effects of antipsychotic drugs. *Frontiers in behavioral neuroscience* 2011, *5*, 31.

[69] Zhang, Y., Zhang, H., Wang, L., Jiang, W.*, et al.*, Quetiapine enhances oligodendrocyte regeneration and myelin repair after cuprizone-induced demyelination. *Schizophrenia research* 2012, *138*, 8-17.

[70] Taylor, S. J., Barker, L. A., Heavey, L., McHale, S., The typical developmental trajectory of social and executive functions in late adolescence and early adulthood. *Developmental psychology* 2013, *49*, 1253-1265.

Figure 1. Experimental groups and brain dissections. A: Blue bars represent normal diet while the periods when animals were fed an iron deficient diet are shown in red. For each experimental condition, siblings were sacrificed at PND90. Behavioral tests were performed at PND90 Con-sal and ID-sal animals (n=9). **B**: Animals were sacrificed and brains were dissected (n=9; Con/ID-sal, n=4; Con/ID-risp, n=5). The olfactory bulb was removed and proteomic analysis was performed on the area named as Frontal Cortex Area (FCA) (left panel).
Specific slices from PFA-fixed brains (n=9) were chosen for histochemical analyses (right panel); plate numbers correspond to those analogous within Paxinos and Watson Atlas.

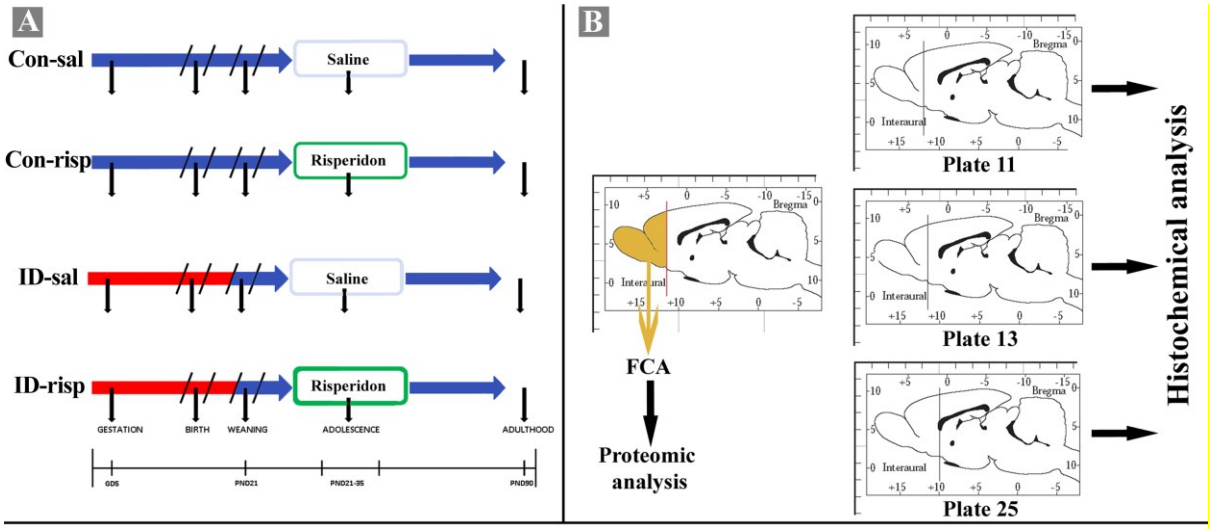


Figure 2: Top five pathways implicated by the main effect of iron deficiency compared to controls with Ingenuity Pathway Analysis. The –log(p-value) is presented and further bioinformatic analyses was performed on the top three significant pathways, namely the "tri cyclic acid cycle', "mitochondrial dysfunction" and "P13k/AKT signaling".

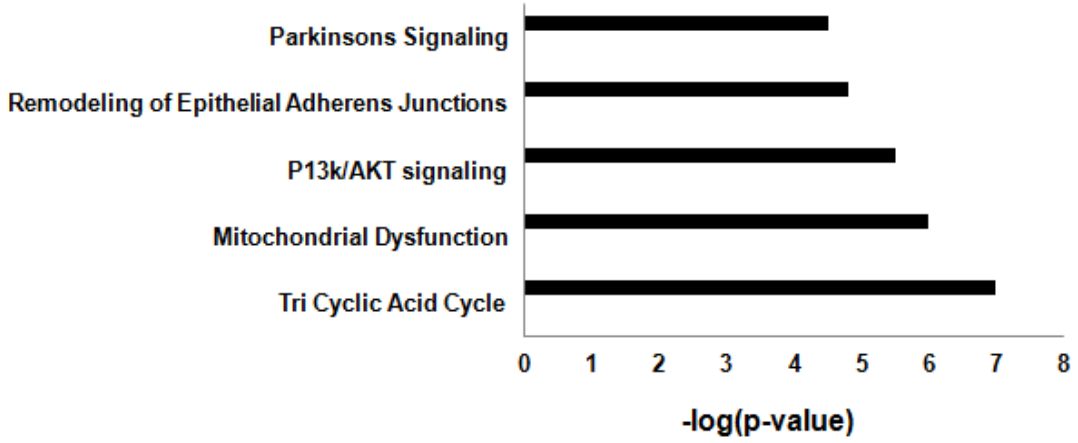
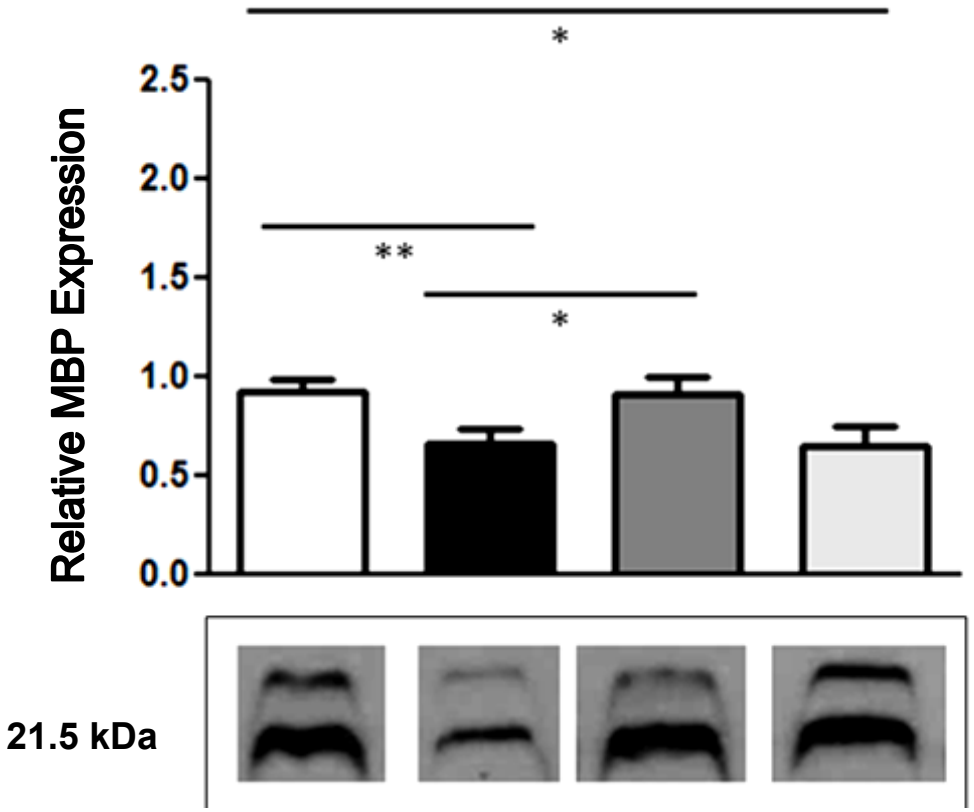


Figure 3 Western blotting confirmation of reduced MBP expression following iron deficiency. Image shows a representative protein expression and protein stain for each group for MBP. Expression was measured by densitometry in ImageJ software. 10 μg of protein was electrophoresed on a 12 % bisacrylamide gel. Data were normalized to Con-sal and the Memcode staining of the same membrane. The mean of three independent experiments +SEM is presented, n= 4-5 per group. *p< 0.05, **p< 0.005 unpaired Students t-test. ****p< 0.001 unpaired Students t-test.



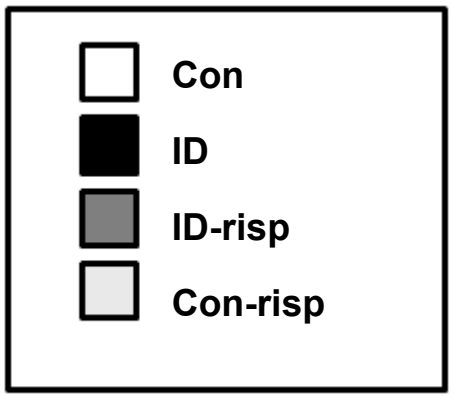


Table 1. Blood parameters measured in ID and C animals at PND90 demonstrated that ID systemic features were reversed to control values upon normal diet reinstatement. HCT: Hematocrit. HGB: Hemoglobin. RBC: red blood cells. Data are expressed as mean values ± SEM

	HCT (%)	**HGB** (g/dl)	**RBC** ($1*10^6$cell/ml)
ID	45.67±0.78	15.95±0.19	8.305±0.145
C	45.14±0.33	14.15±0.36	7.95±0.13

Table 2: List of proteins relating to the tri-cyclic acid cycle (**A**), mitochondrial dysfunction (**B**) and AKt signaling (**C**) as identified by Ingenuity Pathway Analysis from main effect of prenatal iron deficiency. *= p< 0.05, **= p< 0.005, ***=p< 0.001. Fold Changes in Bold are significant after FDR correction.

A: TCA cycle

Gene Name	ID and Ris Interaction FDR (p-value)	ID vs Con FDR (p-value)	ID vs Con *Fold Change*	ID-risp vs ID-sal *Fold Change*	Con-risp vs Con-sal *Fold Change*	ID-risp vs Con-risp *Fold Change*
DLD	0.414	0.027	**1.3***	-1.2	-1.4*	1.4*
FH	0.607	0.005	**-1.3****	1.2	1.1	-1.2
OGDH	0.120	0.028	**-1.2***	-1.1	1.2	-1.4
SDHA	0.363	0.041	**1.1***	1.0	-1.1	1.2
SDHA	0.749	0.008	**1.3****	-1.2	-1.1	1.2

B Mitochondrial dysfunction

Gene Name	ID and Ris Interaction FDR (p -value)	ID vs Con FDR (p-value)	ID vs Con *Fold Change*	ID-risp vs ID-sal *Fold Change*	Con-risp vs Con-sal *Fold Change*	ID-risp vs Con-risp *Fold Change*
ATP5B	0.398	0.037	**-1.0***	1.0	-1.0	-1.0
ATP5J	<0.0001	<0.0001	**2.5*****	1.3	**-2.5*****	**4.6*****
NDUFA9	0.096	<0.0001	**-2.0*****	-1.1	1.4*	**-2.4*****
PARK	0.012	<0.0001	**2.0*****	1.0	-2.0**	**2.9*****
SNCA	0.426	0.019	**1.2***	1.1	-1.0	1.3

C P13K/Akt pathway

Gene Name	ID and Ris Interaction FDR (p -value)	ID vs Con FDR (p-value)	ID vs Con *Fold Change*	ID-risp vs ID-sal *Fold Change*	Con-risp vs Con-sal *Fold Change*	ID-risp vs Con-risp *Fold Change*
HSP90aa1	0.007	<0.001	**-1.2****	-1.2**	1.0	**-1.3*****
HSP90ab1	0.839	0.001	**-1.1****	-1.0	1.0	-1.1*
HSP90b1	0.780	0.037	**-1.2***	1.0	-1.0	-1.2
MAPK1	0.266	<0.001	**-1.9****	1.0	1.5	-2.2**
PPP2r1a	0.119	0.007	**1.1***	1.0	-1.1	1.2*
YWHAE	0.146	<0.0001	**-1.3*****	1.0	1.2*	**-1.4*****

YWHAQ	0.230	0.034	**-1.5***	1.5	2.5**	-2.0*

Table 3: Myelin specific proteins significantly differentially expressed by prenatal iron deficiency. Bold marks changes that remained significant after FDR testing for multiple comparisons. (*= p< 0.05, **= p< 0.005, ***=p< 0.001)

Gene Name	ID and Ris Interaction FDR (p-value)	ID vs Con FDR (p-value)	ID vs Con *Fold Change*	ID-risp vs ID-sal *Fold Change*	Con-risp vs Con-sal *Fold Change*	ID-risp vs Con-risp *Fold Change*
CNPase	0.006	**<0.001**	**-1.1*** **	-1.5**	1.1	**-1.6*** **
PLP	0.687	**0.001**	**-1.2** **	-1.1	-1.1	-1.2*
MAG	0.513	**0.002**	**-1.3** **	-1.4*	-1.2	-1.5*
MOG	0.015	**0.003**	**-1.1** **	-1.4**	1.1	**-1.6*** **
MBP	0.302	0.019	-1.2*	-1.2	-1.0	-1.3*
OMG	0.433	0.161	-1.2	1.1	-1.1	-1.1