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Maternal immune activation induces changes in myelin and metabolic proteins, some of which can be prevented with risperidone in adolescence.

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Maternal immune activation induces changes in myelin and metabolic proteins, some of which can be prevented with Risperidone in adolescence

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Abstract

BACKGROUND:Maternal infection is a risk factor for schizophrenia but the molecular and cellular mechanisms are not fully known.Myelin abnormalities are amongst the most robust neuropathological changes observed in schizophrenia and preliminary evidence suggests that prenatal inflammation may play a role.

METHODS:Label-free liquid chromatography-mass spectrometry was performed on the prefrontal cortex of adult rat offspring born to dams that were exposed on gestational day15 to the viral mimic polyinosinic:polycytidylic acid (Poly(I:C)[4 mg/kg]) or saline and treated with the atypical antipsychotic drug Risperidone(0.045 mg/kg) or saline in adolescence.Western blotting was employed to validate protein changes.

RESULTS:Over 1000 proteins were quanitifed in the prefrontal cortex with pathway analyses implicating changes in core metabolic pathways, following prenatal Poly(I:C) exposure.Some of these protein changes were absent in the prefrontal cortex of Poly(I:C) treated offspring that subsequently received Risperidone treatment in adolescence.Particularly interesting reductions in the expression of the myelin related proteins, myelin basic protein isoform 3(MBP1) and rhombex29 were observed, which were reversed by risperidone treatment.Validation by western blotting confirmed changes in myelin basic protein isoform 3(MBP1), and mitogen activated kinase 1(MAPK1).Western blotting was extended to assess the MAPK signalling proteins due to their roles in inflammation, namely phosphorylated mitogen activated kinase 1(pMAPK1) and phosphorylayed MAPK-activated protein kinase 2(pMAPKAPK2).Both were upregulated by Poly(I:C) treatment and reversed by risperidone treatment.

CONCLUSIONS:Overall, our data suggests that maternal inflammation may contribute to an increased risk for schizophrenia through mechanisms involving

metabolic function and myelin formation and that Risperidone in adolescence may prevent or reverse such changes.

Introduction

Exposure to prenatal infection has been linked to an increased risk of schizophrenia in keeping with increasing recent evidence implicating inflammation in psychosis[1-5]. However, the pathological mechanisms which confer vulnerability to the disease are not known. Proteomics, the high-throughput large scale study of proteins, holds potential to reveal pathological pathways at the molecular level and new insights into disease mechanisms.Proteomic investigations of schizophrenia are steadily increasing and are providing unique insights to unravel the complexities of this disorder[6]. The expression of proteins involved in cell communication, signal transduction, cellular metabolism, synaptic plasticity, cell growth and oxidation have been reported to be altered in postmortem schizophrenia[6-8].

The prefrontal cortex (PFC) is involved in the higher brain functions such as memory, perception and cognitive processes and is therefore strongly implicated in schizophrenia[9]. Using proteomic methods we have previously shown protein expression changes in the PFC of schizophrenic patients relating to myelin, metabolic and cytoskeletal function[7, 8, 10].Studies involving post-mortem tissue can be confounded by factors such as post-mortem delay, tissue pH and drug treatment. However, animal models based on known risk factors can aid considerably in elucidating putative cellular and molecular pathways, test specific mechanistic hypotheses and increase our insight into schizophrenia pathophysiology.

In recent years, an extensive body of research has validated the maternal immune activation (MIA) model involving gestational exposure to infection or immune stimulation as a means to simulate schizophrenia-like neuroanatomical, neurochemical, behavioural and gene expression abnormalities[11-14]. The MIA model is in line with the epidemiological association between prenatal infection and

increased risk for schizophrenia[4, 15-18]. In the present study, we studied the effect of MIA by injecting pregnant dams with the viral mimic Poly(I:C) on protein expression in the PFC. Poly(I:C) is a synthetic analogue of virus-specific double-stranded RNA, which induces a cytokine-associated acute phase response typically seen following viral infections[11, 18]. As reviewed in[18], prenatal Poly(I:C) treatment in rodents induces a variety of behavioural and brain dysfunctions implicated in schizophrenia. Furthermore, a dramatic preventive effect of the atypical antipsychotic drugs clozapine and Risperidone in the offspring of rat dams exposed to Poly(I:C) has previously been demonstrated in adolescence[19-21]. Risperidone, within this timeforame, was successful in preventing the emergence of brain structural pathology and behavioural abnormalities induced by prenatal Poly(I:C) treatment.

Specifically, administration of these drugs during an asymptomatic period of adolescence prevented the emergence of schizophrenia-like brain structural and behavioural abnormalities in adulthood[19-21]. Therefore the prenatal Poly(I:C) model is suitable not only for studying the molecular effects of prenatal immune challenge but can also shed light on molecular mechanisms of preventing such effects.

Myelin and glial abnormalities are amongst the most robust neuropathological changes observed in schizophrenia[6, 22-24] however the precise mechanisms are not fully known and warrants proteomic investigation. As oligodendroglial function, including myelin production, are highly energy dependent [25], it is is possible that the myelin abnormalities described in schizophrenia relate to alterations in metabolic function such as that involving oxidative phosphorylation and glycolysis/gluconeogenesis as described previously [6-8, 26-28]. While it is not clear whether these abnormalities have their origins relating to prenatal risk factors such as maternal infection, preliminary evidence suggests that it may play a role.

Investigations of animal models of prenatal infection have demonstrated white matter atrophy, reductions in myelin specific genes, reduced fractional anisotropy in white matter and alterations in mitochondrial function and metabolism [29-32]. Inflammation may influence myelin formation by causing a dysregulation of iron homeostasis which is also central to this process[33, 34]. It has also been proposed that one effect of atypical antipsychotics may be to enhance or normalise myelination changes in schizophrenia[35, 36].

Another mechanism by which inflamation may act is through activation of the MAPK signalling pathway which increases the transcription of pro-inflammatory cytokines[37]. Recent research has also implicated MAPK signalling in the proposed antiinflammatory properties of Risperidone in a model of lipopolysaccharide induced inflammation[38], and also in the growth and maintenance of myelin[39-41].

In the current investigation we specifically hypothesized that myelin protein expression changes would be induced in the PFC by prenatal Poly(I:C) and that these changes could be reversed in adulthood by adolescent treatment with Risperidone. Risperidone was utilised due to its preventative effect on behaviour and brain pathology following prenatal Poly(I:C) exposure, and its previously described antiinflammatory properties[20, 21, 38].

By using discovery proteomic methods, and combining this with specific hypothesis testing, this study will provide more insight into the possible mechanisms underlying myelin deficiencies in schizophrenia and to the knowledge of mechanisms by which atypical antipsychotics may act.

Methods

Animals

Adult (350–400 g) male Wistar rats were housed 3–4 per cage under reversed cycle lighting (lights on: 1900–0700 h) with unlimited access to food and water.

All protocols conformed to the guidelines of the Institutional Animal Care and Use Committee of Tel-Aviv University, Israel, and to the guidelines of the National Institutes of Health (NIH) (animal welfare assurance number A5010-01). Ethical approval was granted by Royal College of Surgeons in Ireland Research Ethics Committee (REC-585bb).

Prenatal Poly(I:C) Treatment

At 3 months of age, rats were mated and the first day after copulation was defined as day 1. On gestational day 15, pregnant dams were anesthetized with 3% isoflurane (Minrad, Bethlehem, PA) in 98% O_2 and given a single intravenous injection at the tail vein of 4 mg/kg Poly(I:C) (Sigma, Rehovot, Israel) dissolved in saline, or saline alone. The volume of injection was 1 ml/kg. At birth, pups were grouped to 10, composed of 5 females and 5 males when possible. On postnatal day 21, the pups were weaned and housed 3–4 per cage by sex and litter and maintained undisturbed until drug injections that commenced on PND 34. Only male offspring were used in the following experiments and each experimental group consisted of subjects derived from multiple independent litters (6 Poly(I:C) and 6 saline litters), with no more than one rat from the same litter.

153 Risperidone Treatment

154 Preventative treatment was given on PND 34–47 representing adolescence. This
155 period was chosen because Poly(I:C) offspring are behaviourally and
156 neuroanatomically asymptomatic during this period, and a previous study showed that
157 antipsychotic administration at this time window prevented the emergence of
158 behavioral and brain structural abnormalities in adulthood. Offspring of Poly(I:C) or
159 saline dams were injected daily intraperitoneally with 0.045 mg/kg risperidone or
160 vehicle (saline). This particular low dose of Risperidone was chosen as it was
161 previously found to be effective in preventing amphetamine-induced hyperactivity
162 caused by neonatal ventral hippocampal lesions [42]. The volume of injection was 1
163 ml/kg. Risperidone (Janssen, Beerse, Belgium) was dissolved in 0.1M tartaric acid
164 (7.5 µl/1 mg) and diluted with saline. A graphical represenatation of the study design
165 can be found in Figure 1A. In total, four experimental groups were prepared (see
166 Figure 1B) with a total of five pups per group.

167 Sample preparation

168 Male offspring of Poly(I:C) or saline treated dams that were injected with Risperidone
169 or saline on PND34-47, were sacrificed at PND120 under pentobarbital anaesthesia
170 ,brains were quickly removed, dissected, and frozen in liquid nitrogen. The location of
171 the PFC was chosen on the basis of the stereotaxic atlas of Paxinos and Watson
172 (1998). The PFC was hand-dissected from the slice ~2.5 - 4.5 mm anterior to bregma
173 and included prelimbic cortex, infralimbic and cingulate cortices.

174 The PFC was sonicated (Sonics© Newtown, CT, USA) in tri-ethyl-ammonium-
175 bicarbonate buffer (Sigma Aldrich, Ireland) containing protease inhibitors (Roche,
176 Ireland). Protein concentrations were determined using the Bradford assay[43] and
177 fifty micrograms of protein from each homogenate was processed for mass

spectrometry as previously described[7]. Additional details can also be found in the Supplementary methods.

Mass Spectrometry Analysis

Label free liquid chromatography-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 can be found in the Supplementary methods.

Data Processing with MaxQuant™

The data analysis was carried out with MaxQuant™ software, supported by Andromeda as a database search engine for peptide identification[44]. Label free quantitation was performed as previously described[45]. Carbamidomethylation was defined as a fixed modification, while oxidation and acetylation of the protein N-terminus were defined as variable modifications. Only peptides with seven or more amino acid residues were allowed for identification. Additionally, at least one unique peptide was required to identify a protein. The cut off for the false discovery rate (FDR) for peptide and protein identification was 1%[46]. The FDR of 1% is inbuilt, and standard for users of MaxQuant, as it allows maximum stringency for the analysis of thousands of identified peptides. Additonally, it has been described that peptide intensity based on an FDR of 1% correlates well with western blotting analyses as shown in the yeast proteome [47]. The label free algorithm takes the maximum number of identified peptides between any two samples and compares the intensity of these peptides to determine peptide ratios. Label free quantitation (LFQ) intensity values were used for protein quantification across the groups.

Statistical and Biostatistical Analysis

The LFQ scores for each protein 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 (66%) LFQ intensities were available. A total 23,739 LFQ values were subsequently used in the statistical analyses. Regression normalisation was performed to remove technical error across the samples[48]. An FDR of 5% as advocated by Benjamini and Hochberg[49] was used to determine the filtered protein set of interest. Group comparisons were performed using the traditional 5% level of significance. Group differences of log-LFQ scores were exponentiated, giving fold changes as measures of treatment effects. The overall study had 4 potential comparison groups, Poly(I:C)/Sal (Prenatal treatment), Poly(I:C)/Ris (Preventative treatment), Sal/Ris (Risperidone alone) and Sal/Sal (Saline controls), reflecting the 2x2 factorial study design involving treatment type (Poly(I:C)/Ris) and timing (prenatal/adolescent). However, as the primary objective of the study was to determine the differential protein expression in offspring following Poly(I:C) administration in dams, a *planned*, *discovery based* comparison was first performed involving prenatal treatment compared to control offspring. See Supplementary methods for more details regarding the statistical design.

Effect of Prenatal treatment - *pathway and functional analyses of proteins*

This analysis was used to provide a list of proteins differentially expressed in adult offspring by prenatal Poly(I:C) compared to saline controls. In order to identify which signalling pathways were most affected by the prenatal treatment, the differentially expressed proteins were inputted to DAVID bioinformatics software according to KEGG™ (http://david.abcc.ncifcrf.gov/). Proteins related to *these* pathways were then assessed across the other treatment groups using a 2x2 ANOVA with prenatal

Poly(I:C) and Risperidone as factors, plus the treatment interaction term, was used to model the data and obtain particular comparisons of interest.

Prenatal treatment compared to Preventative treatment.

Among the proteins linked to pathways identified from our discovery based approach (above), we then assessed these proteins in offspring from prenatal treatment and in offspring following preventative treatment. This latter assessment could determine potential reversible effects of Risperidone on the affected, filtered protein set.

The effect of Risperidone in control offspring

We also undertook an analysis of Risperidone treatment alone on offspring from saline-treated dams.

Preventative treatment compared to Risperidone alone

Although not of primary interest, this comparison illustrated the effects of Poly(I:C) under Risperidone conditions. Relative to the effect of prenatal Poly(I:C), this illustrated possible drug interaction effects.

Hypothesis based approach

Finally, we applied a hypothesis based approach to the analysis of i). 'myelin and myelin-related proteins' ii). 'postmortem candidate proteins' involving proteins previously shown to be dysregulated in reviews of proteomic studies of postmortem schizophrenia[6, 50] and iii). 'MAPK signalling related kinases'.

Data management and analysis was done using SAS ® Version 9.1.

Western Blot analysis

Western blots were used to confirm the mass spectrometry derived differential expressions of transferrin (TF), myelin basic protein isoform 3 (MBP1) and mitogen activated kinase 1 (MAPK1) in addition to MAPK signalling related phosphorylated mitogen activated kinase 1 (pMAPK1) and phosphorylated MAP kinase-activated protein kinase 2 (pMAPKAPK2)[51]. For additional western blotting details such as antibodies, please refer to Supplementary methods.

Results

Mass spectrometry

A total of 1259 proteins, from 80,628 unique peptides and 125,173 MS/MS counts were identified with 1% FDR, after data input to the MaxQuant bioinformatics software.

Prenatal Poly(I:C) treatment induced changes in core metabolic pathways

130 proteins were dysregulated following prenatal treatment compared to saline controls (Students t-test; FDR, $p < 0.05$) and KEGG^{TM} (http://david.abcc.ncifcrf.gov) was used to identify the key biological pathways within all FDR-significant differentially expressed proteins. The top five significant pathways are listed in Table 1 (oxidative phosphorylation, tricyclic acid (TCA) cycle, glycolysis, ribosome, cysteine and methionine metabolism) along with genes identified from each pathway.

Proteins identified from the *top three* implicated pathways were assessed across the groups using a 2X2 ANOVA (Table 2). The results of our *pair wise comparisons* within the context of the 2X2 ANOVA provide insight into the effects of Poly(I:C) and Risperidone. Offspring from Poly(I:C) dams exhibited significant alterations in proteins involved in metabolic function compared to saline controls. Particularly prominent changes in proteins relating to oxidative phosphorylation, the TCA cycle and glycolysis/gluconeogenesis were observed (Table 2). Prenatal treatment upregulated

six out of eight proteins, and downregulated one protein, relating to oxidative phosphorylation. Additonally, the prenatal exposure also upregulated four proteins and downregulated one protein relating to the TCA cycle. (Table 2).Preventative treatment reversed the change observed in malate dehydrogenase 2 (MDH2), cytochrome c oxidase (COX) (subunit VIc) and triosephosphate isomerise (TPI) in Poly(I:C) treated offspring. Overall, prenatal Poly(I:C) exposure had an upregulating effect on proteins relating to metabolic function, with some of these effects being reversed by preventative treatment (see Table 2).

Risperidone treatment of control offspring

Risperidone alone exhibited an overall pattern of protein changes which were highly similar to that observed within offspring prenatally treated with Poly(I:C) and Risperidone in adolesence. Thus, of 15 proteins differentially expressed within the latter treatment group, 13 were also significantly differentially expressed in the same direction in the offspring treated with Risperidone alone, (see Table 2). A similar pattern was seen for myelin and iron proteins with the exception of oligodendrocyte myelin glycoprotein (see Figure 2 and Figure 3). In terms of schizophrenia candidate proteins assessed, Risperidone alone once again influenced the majority of proteins in a similar way to that of offspring prenatally treated with Poly(I:C) and Risperidone in adolesence (Table 3). The most implicated pathways with adolescent Risperidone treatment were found to be related to mitochondrial function, protein-trafficking and the cytoskeleton. A more comprehensive analysis of this dataset can be found in Farrelly et al (2014) [52].

Preventative treatment compared to Risperidone alone

In comparison to how Poly(I:C) acts in a saline setting, (Poly(I:C)/Sal versus Sal/Sal) our analysis and study design also demonstrates how Poly(I:C) acts in a Risperidone setting, (Poly(I:C)/Ris versus Sal/Ris). For the majority of proteins assessed, the direction of fold changes were similar in both comparisons (Poly(I:C)/Sal versus Sal/Sal and Poly(I:C)/Ris versus Sal/Ris) (see Table 2 and 3, Figure 1 and 2).

Hypothesis based approach

i). Myelin and myelin-related Protein Changes

Seven myelin specific proteins were identified in our MS analysis: isoform 1 of myelin basic protein (MBP3), isoform 3 of myelin basic protein (MBP1), myelin associated glycoprotein (MAG), myelin oligodendrocyte protein (MOG), oligodendrocyte myelin glycoprotein (OMG), proteolipid protein (PLP) and a structural analogue of PLP, rhombex29 (Figure 2 (a)).

Prenatal treatment was associated with significantly decreased expression of MBP1 ($p<0.05$; -2 fold), and rhombex29 ($p<0.005$; -2.5 fold) while preventative treatment was associated with an increased expression of these proteins; MBP1 ($p<0.05$; +2.1 fold), and rhombex29 ($p<0.005$; +3 fold) suggesting a reversing effect of Risperidone. Preventative treatment was also associated with a significant increased expression of MAG ($p<0.005$; +8.2 fold).

Iron is an essential cofactor in myelination, and with this in mind, we assessed the expression of any core iron homeostasis proteins that we identified in our proteomic analysis. These were ferritin heavy chain (FTH), transferrin (TF) and hemopexin (HPX). FTH was decreased in adulthood following prenatal Poly(I:C) treatment ($p<0.05$; -1.4 fold) but this effect was reversed for preventative treatment ($p<0.001$; +4.4 fold,Figure 2 (b)).

TF, which controls the level of free iron in the blood was unchanged following prenatal Poly(I:C) treatment. However there was a trend present to increase the expression ($p = 0.1$; +1.1 fold).

HPX, an iron binding protein, was also unchanged following prenatal treatment, however, risperidone treatment increased its' expression ($p < 0.05$; +2.6 fold).

ii). Postmortem candidate proteins

We next asked whether our observations in the animal model agreed with specific protein changes previously observed in postmortem schizophrenia by comparing our findings to recently published postmortem proteomic reviews[6, 50]. Remarkably, while a majority of the candidate proteins were found to be differentially expressed following Poly(I:C) treatment, the direction of change was dissimilar for the majority of these from that observed in schizophrenia (see Table 3). Specifically, of the 28 candidate proteins listed in these reviews, 15 were found to be differentially expressed by prenatal Poly(I:C) treatment, and of these only three were altered in the same direction as observed in the postmortem schizophrenia brain. These were MBP, Spectrin beta and Septin 3. Preventative treatment reversed the expression changes of four candidate proteins compared to prenatal Poly(I:C) exposure alone (CKB, MBP, PRDX2 and TPI1 Table 3).

<u>Western Blot Validation of Selected Proteins</u>

Western blotting was undertaken to confirm/investigate the differential/possible differential expression of 5 proteins (TF, MBP1, MAPK1, pMAPK1 and pMAPKAPK2).

TF

In our proteomic study we had observed increased TF expression in offspring following Risperidone alone (+2.1 fold change) (Figure 2b). This finding was not confirmed by western blotting ($p=0.59$). However, we were able to achieve a significant increase in TF expression in poly(I:C) treated offspring compared to saline controls ($p<0.05$) and in offspring who received preventative treatment compared to those treated with poly(I:C) ($p<0.05$) (Figure 3a). These latter two results, did not reach significance via mass spectrometry although protein expression was in the same direction and a trend was evident for the effect of prenatal poly(I:C) treatment ($p= 0.1$)

MBP1

In our proteomic study we had observed increased MBP1 (21.5 kDa) expression following preventative treatment and decreased expression following prenatal treatment (Figure 2a). Both of these changes were confirmed by western blotting ($p=0.01$; +1.5 fold and $p=0.01$; -1.5 fold) (Figure 3b).

MAPK1

In our proteomic study we had observed increased expression of MAPK1, although, following our data filtering with DAVID, there were not enough MAPK related proteins to implicate the pathway for our further group wise comparisons. However, we found increased expression of MAPK1 following prenatal treatment ($p=0.04$), preventative treatment ($p=0.004$) and Risperidone treatment alone ($p<0.001$) (Data not shown, see Supplementary data). In keeping with these findings, our western blotting studies confirmed a significant increase in MAPK1 expression following prenatal treatment (Student unpaired t-test; $p=0.02$; +1.2 fold), and following preventative treatment (Student unpaired t-test; $p=0.04$; +1.3 fold) but increased MAPK1 expression following

Risperidone alone could not be confirmed by western blotting (Student's unpaired t-test; p= 0.49) (Figure 3c).

pMAPK1

The activated form of MAPK1, known as phosphorylated pMAPK1, was significantly upregulated in offspring following prenatal treatment compared to controls (Student's unpaired t-test; p=0.03; +1.3 fold). However, pMAPK1 expression was significantly downregulated in offspring with preventative treatment compared to Poly(I:C) alone (p=0.002; -1.5 fold; Figure 3d). This downregulation was close to the effect of Risperidone treatment alone of control offspring. Overall, Poly(I:C) had an upregulating effect whereas risperidone acted to decrease expression.

pMAPKAPK2

Phosphorylation of MAPKAPK2 by MAPK1 induces its activaton and the consequential transcription of proinflammatory cytokines [51]. We discovered a significant upregulation in offspring from prenatal treatment compared to controls (p=0.001; +1.5 fold). Interestingly, in offspring who received preventative treatment, pMAPKAPK2 expression was downregulated compared to those who were prenatally treated (p=<0.0001; -3.0 fold). Similarly, this decrease was comparable to the effect of Risperidone treatment alone. Risperidone treated offspring exhibited a 2 fold decrease in pMAPKAPK2 expression compared to controls (p=0.005; -2.0 fold; Figure 3e). Overall, Poly(I:C) had an upregulating effect whereas risperidone acted to decrease expression.

Discussion

Our study is the first to characterise global differential protein expression in adult offspring following MIA using mass spectrometry based discovery proteomic methods. Pathway and functional analysis implicates metabolic dysfunction, particularly

oxidative phosphorylation, the TCA cycle and glycolysis/gluconeogenesis (see Table 2). The findings are thus in keeping with previous investigations of the Poly(I:C) model which implicated metabolic protein changes[53], and with evidence implicating mitochondrial function in schizophrenia[6, 8, 54]. Intruigingly, we also observed that some but not all of the protein changes induced in the MIA model were normalised or reversed by adolescent risperidone treatment, this providing preliminary evidence that that antipsychotic medications may act through normalising inflammation related protein changes.

In our data, metabolic dysfunction was highlighted by an upregulation in COX, ATP synthase delta subunit and in all identified isoforms of NADH dehydrogenase. As COX is regulated by oxidative stress[55] which is influenced by infective processes[56] our findings suggest that altered COX expression, may be a consequence of Poly(I:C) induced oxidative stress. In keeping with our findings of altered NADH dehydrogenase expression, postmortem studies have also observed dysregulated NADH dehydrogenase protein[57] and gene expression in schizophrenia[58]. Dysregulation of glycolytic enzymes such as TPI, has also been observed in schizophrenia[6, 59] and our finding of altered oxoglutarate dehydrogenase (OGDH) following MIA mirrors a previous investigation which reports its decrease in the plasma/urine of first-episode neuroleptic-naïve adult schizophrenia patients[60].

There was pattern of altered protein expression following Poly(I:C) being reversed by preventative treatment (Table 2) for several metabolic proteins such as COX, MDH2, and TPI. We identified seven myelin specific proteins in our proteomic data set and two of these, MBP1 and rhombex29, a structural analogue of PLP, were decreased with prenatal Poly(I:C). This is consistent with previous investigations in showing MBP,

PLP and MAG mRNA reduced in an animal model of prenatal viral infection and inflammation[31].

For the most part we saw little evidence of Risperidone reversing changes following prenatal exposure.This is explained by a general absence of prenatal Poly(I:C) X Risperidone treatment interaction. This is suggestive of different physiological mechanisms by which the two agents are eliciting their effects.

Blood iron availability is reduced by inflammation and its prenatal deficiency is associated with myelin reductions in animal models[34, 61], behavioural deficits in infants [62] and also schizophrenia [63, 64]. We found ferritin heavy chain (FTH), which is expressed abundantly in oligodendrocytes [65], to be downregulated following Poly(I:C). These findings are consistent with the possibility that prenatal inflammation may act to reduce iron availability and so contribute to myelin dysregulation. Interestingly, FHC was also reversed by Risperidone treatment.

We also specifically tested differentially proteins implicated in recent proteomic reviews of schizophrenia studies [6, 50, 66] (Table 3). Of the 28 candidate proteins identified in these reviews, over 55% (15 individual proteins) were found to be differentially expressed by prenatal Poly(I:C). However, only three were altered in the same direction as observed in the postmortem schizophrenia brain, namely MBP, spectrin and septin 3. This is in keeping with a previous study where changes in protein expression were also reported to differ between a Poly(I:C) model and the postmortem situation[14]. These differences may be attributed to environmental factors such as lifelong medication in the clinical samples, duration of psychosis, technical differences such as postmortem interval, and species differences. It may also be related to the fact that the offspring were sacrified closer to the emergence of ‘symptoms’ in comparison to the clinical situation. In addition our current study only

used male offspring and therefore gender specific changes may need to be considered in future studies.

We attempted validatation by western blotting in three proteins, TF MBP1 and MAPK1, and were successful for MBP1 and MAPK1 for both the effect of prenatal Poly(I:C) and preventive treatment. While changes in TF following Risperidone treated offspring compared to controls were not confirmed, trend changes observed in TF in the proteomic analysis following preventative treatment compared to prenatal Poly(I:C) ($p=0.1$) were significantly different in the western blotting experiments. In addition, based on our finding of upregulated MAPK1, which we validated, we also quantified its related kinases pMAPK1 and pMAPKAPK2[51] by western blotting. We found these proteins to upregulated following prenatal inflammation, in agreement with the current literature[67-69] and decreased following preventative treatment. The MAPK pathway, is known to be highly implicated in the cellular response to inflammation[70] and it has been linked to myelin formation where gain of MAPK1 function promotes CNS developmental myelination[40]. To our knowledge this is the first time that early intervention with an atypical antipsychotic in a Poly(I:C) model of prenatal inflammation has been shown to decrease activation of core MAPK kinases.

Our study has several limitations. Firstly, the interpretation of the effects of Risperidone on the Poly(I:C) model is not straightforward because we don't know when expression changes occurred. Thus we are unable to state whether changes are prevented or reversed. Additionally, it is possible that we were not able to define many Poly(I:C) X Risperidone interactions due to the strong main effect of Risperidone treatment. Future preclinical work examining expression changes following different Poly(I:C) and Risperidone dosing regimes (including different exposure times), will

help address this issue. Secondly, although we did not validate changes in proteins implicating prenatal Poly(I:C) exposure in mitochondrial functions such oxidative phosphorylation, TCA cycle and glycolysis/gluconeogenesis these pathways were very strongly implicated by the level of significance of the individual protein changes in pathway analysis. Nonetheless future studies are planned to confirm and extend these findings.

Additionally, offspring were injected with Risperidone between PND 34-47 and protein expression was analysed at PND120. This is a large time span between drug administration and analyses , and this allowed us to examine relatively long-term drug effects. However, more short-term drug effects were not examined. Future studies are planned which will address the time course of drug effects. It must also be noted that we found risperidone treatment to have a similar effect to that of Poly(I:C). However, they may be acting through distinct mechanisms as to the best of our knowledge risperidone does not signal through toll like receptors. Additionally, it must be recognised that the effect of risperidone in control offspring does not completely represent its clinical mechanisms as here we are dealing with a ‘non diseased’ sample. Lastly we must also take into account the handling of the animals as this has been reported to contribute to changes we can not measure or control[71]. Our study also has several strengths. Firstly, we believe our proteomics methods are sensitive and reliable as they are based on triplicate MS data. Reliability is demonstrated by consistency in the identification of trypsin autolysis peaks, and stable retention times as identified by the MS (data not shown). Furthermore, the consistency of the effects of Risperidone on protein expression both with and without Poly(I:C) treatment is very remarkable (Table 2 and Table 3) and points to a consistent biological effect identified by a reliable quantitative assay. Finally, it should be noted that the effects of low dose

Risperidone, as observed in our study, suggest that selective $5HT_{2A}$ antagonism, which is more prominent at a low dose, may contribute to the observed effects[20]. However, as markers of serotonin activity were not specifically addressed in this study, future studies employing quantitative receptor autoradiography could provide more confident insights in to the action of Risperidone on 5HT antagonism in this study [72].

In sum, our data extends previous data by demonstrating that prenatal Poly(I:C) exposure leads particularly to dysregulation of core metabolic and myelin associated proteins. Furthermore, adolescent treatment with low dose Risperidone, which has previously been shown to prevent Poly(I:C) induced brain structural, cellular/molecular and behavioural abnormalities[20, 73] was shown to upregulate proteins relating to metabolic pathways and myelin function in the PFC. These findings indicate that molecular abnormalities produced by prenatal insults are not always permanent and that they are modifiable by early drug treatment. This may have implications to treatment studies of subjects considered to be at risk of psychosis[74].

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523

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Figure 1: (a) Experimental design. Pregnant wistar dams were injected with Poly(I:C) (4 mg/kg) or saline (Sal) on gestational day 15. The resulting Sal and Poly(I:C) offspring were then divided into two cohorts, those that received Risperidone (Ris) (0.045 mg/kg) in adolescence (PND 34–47) and those that received Sal. Four groups of offspring were created in total (see Figure 1b). **(b)** Four groups of offspring were produced in total for further proteomic analysis; offspring from Poly(I:C) treated dams which received Sal or Ris at adolescence (group 1 and group 2) and offspring from Sal treated dams which received Ris or Sal at adolescence (group 3 and group 4).

Figure 2: (a) Myelin and (b) iron protein expression changes observed in offspring treated with prenatal Poly(I:C)/Sal compared to Sal/Sal(black), Poly(I:C)/Ris compared to Poly(I:C)/Sal (white), Sal/Ris in adolescence compared to Sal/Sal(light grey) and prenatal Poly(I:C)/Ris compared to Sal/Ris (dark grey).

Abbreviations: MBP1, myelin basic protein isoform 3; MBP3, myelin basic protein isoform 1; MAG, myelin associated glycoprotein; MOG, myelin oligodendrocyte glycoprotein; OMG, oligodendrocyte myelin glycoprotein; PLP, proteolipid protein; FTH, ferritin heavy chain; TF, transferrin; HPX, hemopexin.

(Pairwise comparisons * $p<0.05$; ** $p<0.005$)

Figure 3: Protein validation with western blotting of (a) TF, (b) MBP1, (c) MAPK1, (d) pMAPK1, (e) pMAPKAPK2. The images show a representative blot and the graphs represent the relative protein level as measured by densitometry. (Data normalised to saline controls and the Memcode staining of the same membrane). The mean of three independent experiments +SEM is presented. * $P < 0.05$, ** $P < 0.005$, ***$P < 0.001$, ****$P < 0.0001$

Table 1: Top 5 significant pathways identified amongst the proteins differentially expressed (t-test; $p<0.05$) between Poly(I:C)/Sal and Sal/Sal controls. Pathways were identified according to KEGG^{TM} (Kyoto Encyclopaedia of Genes and Genomes) and significance of each pathway identified is denoted by; * $p<0.001$, ** $p<0.0001$.

Table 2: Top 3 significant pathways identified by KEGG^{TM} of the significantly altered proteins between Poly(I:C)/Sal and Sal/Sal controls (t-test $p<0.05$).

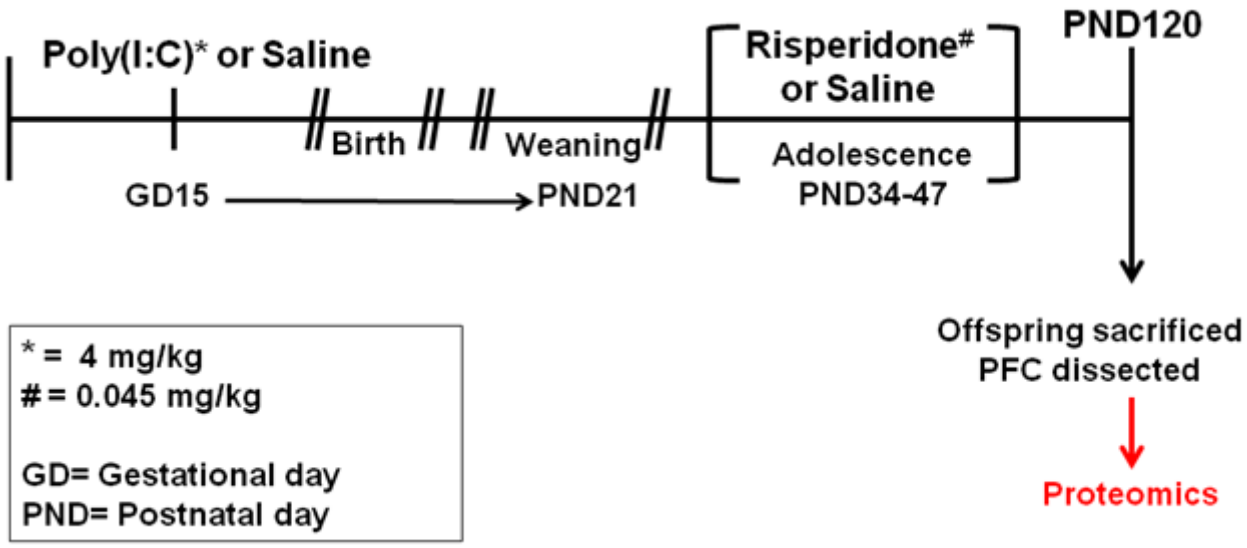
Fisher exact value is reported for each pathway and altered proteins within the data relating to these altered pathways are also shown. Significance of protein fold changes within the data is also reported (2X2 way ANOVA; * $p<0.05$; ** $p<0.01$). Proteins in **bold** text are those which were reversed$^{\#}$ in Poly(I:C)/Ris vs Poly(I:C)/Sal compared to Poly(I:C)/Sal vs Sal/Sal.

$^{\#}$ = indicated by a change of sign providing the fold change is significant in Poly(I:C)/Sal vs Sal/Sal and in Poly(I:C)/Ris vs Poly(I:C)/Sal.

Table 3: Candidate proteins taken from proteomic reviews by English et al. (2011), Martins-De-Souza et al. (2009). Candidate proteins were analysed across the data with a two way ANOVA and fold changes are expressed with related p values (2X2 way ANOVA; * $p<0.05$; ** $p<0.01$). Proteins underlined are those which share the

same direction of expression in post-mortem reviews with Poly(I:C)/Sal compared to Sal/Sal controls.Proteins in **bold** text are those which were reversed in Poly(I:C)/Ris vs Poly(I:C)/Sal compared to Poly(I:C)/Sal vs Sal/Sal controls.

780 **Figure 1: (a)**

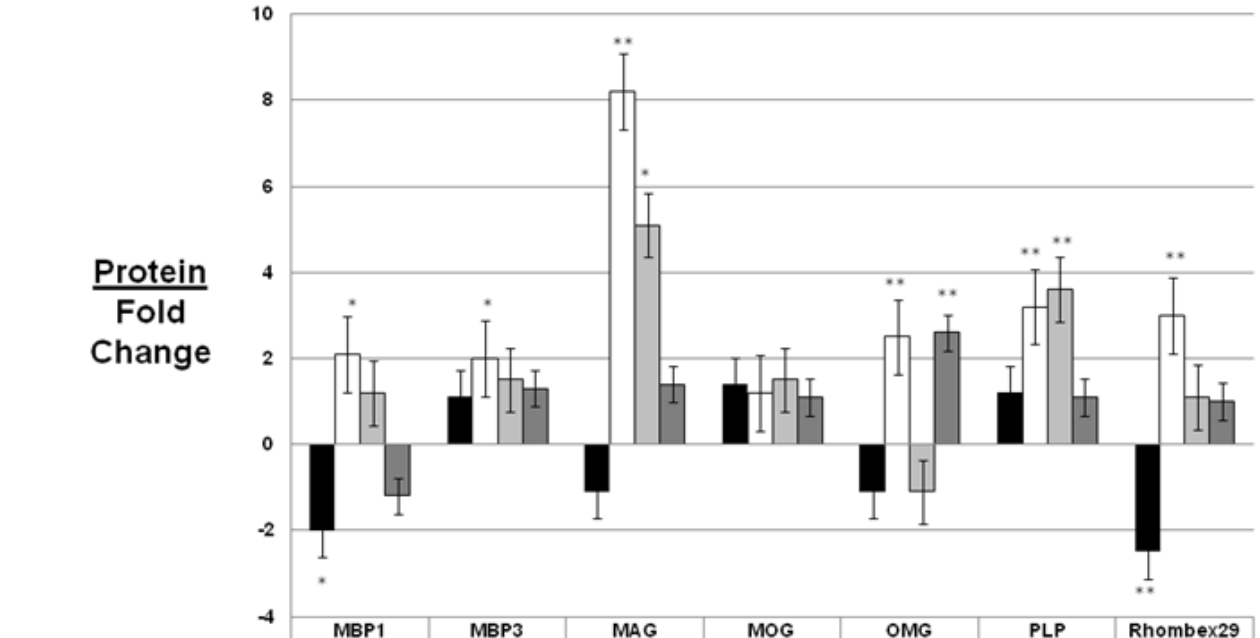


781

Figure 1 (b):

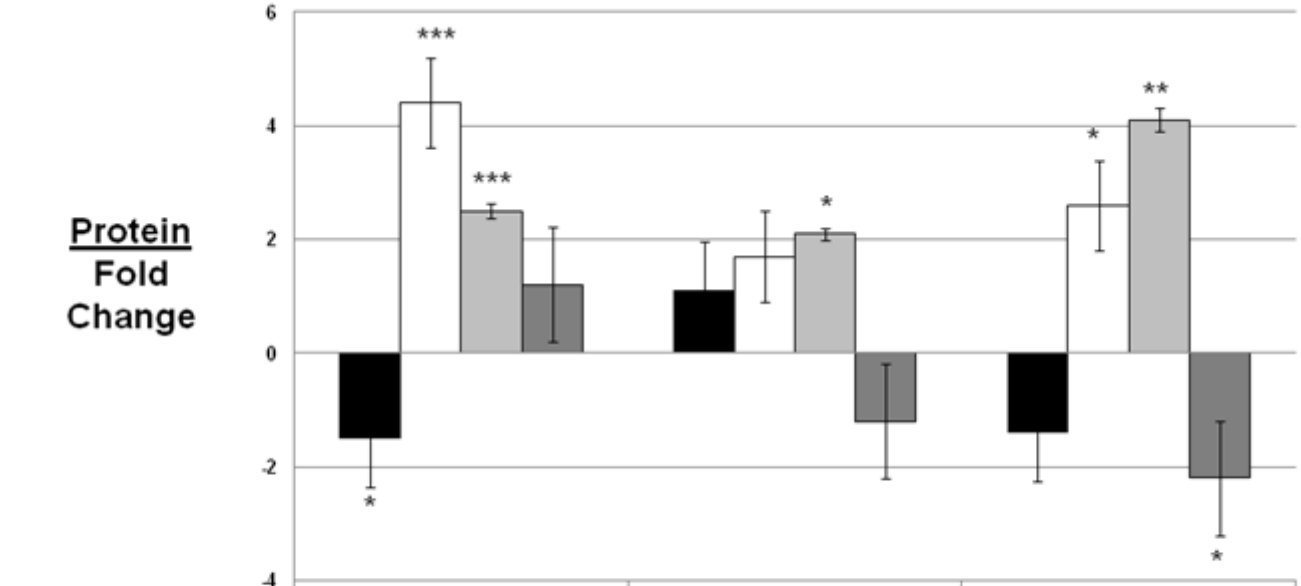
Group	Pregnant Dams	Adolescent Offspring
1	Poly (I:C)	Saline
2	Poly(I:C)	Risperidone
3	Saline	Risperidone
4	Saline	Saline

Figure 2(a)



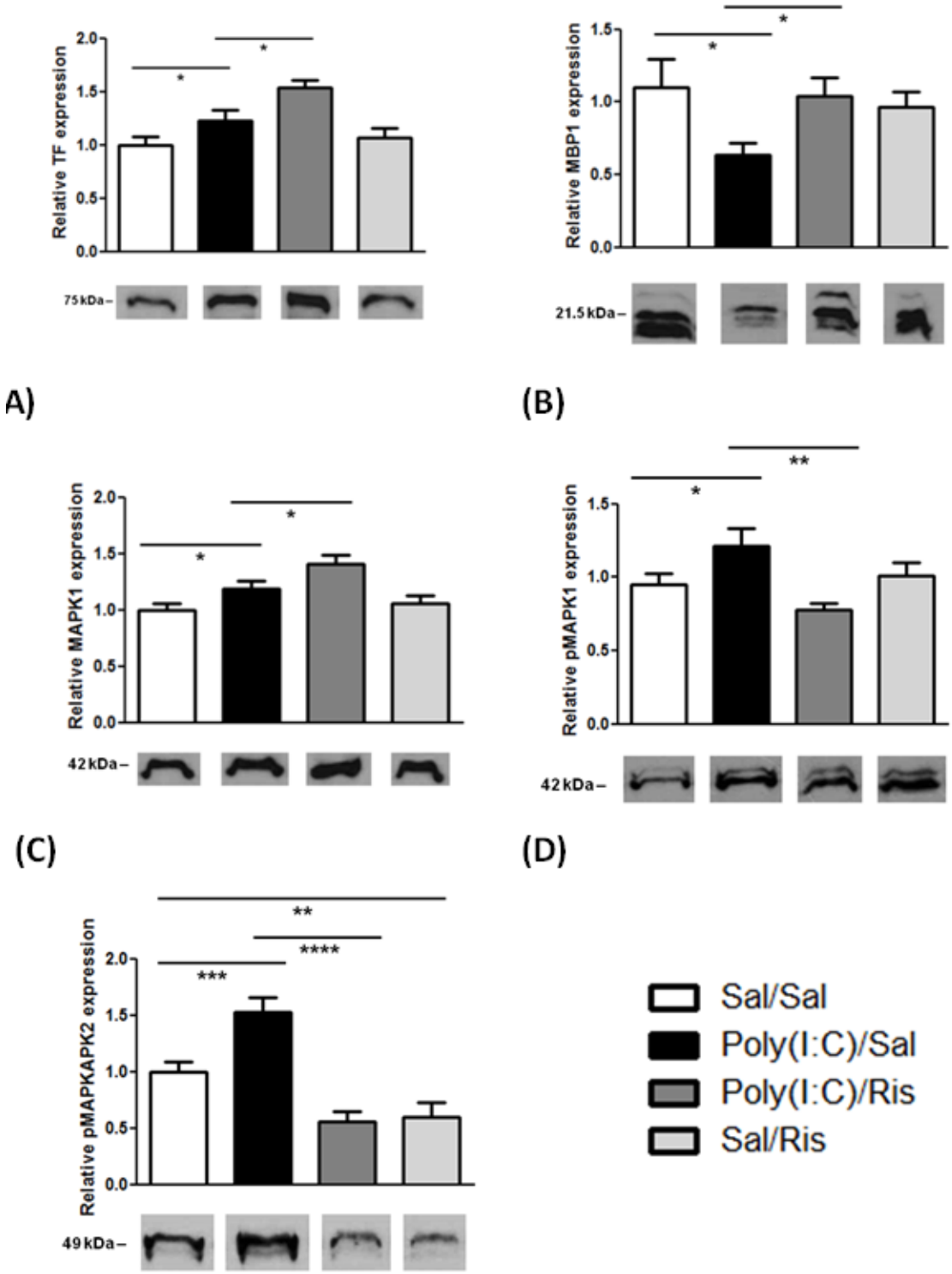
	MBP1	MBP3	MAG	MOG	OMG	PLP	Rhombex29
■Poly(I:C)/Sal vs Sal/Sal	-2	1.1	-1.1	1.4	-1.1	1.2	-2.5
□Poly(I:C)/Ris vs Poly(I:C)/Sal	2.1	2	8.2	1.2	2.5	3.2	3
▫Sal/Ris vs Sal/Sal	1.2	1.5	5.1	1.5	-1.1	3.6	1.1
▪Poly(I:C)/Ris vs Sal/Ris	-1.2	1.3	1.4	1.1	2.6	1.1	1

Figure 2(b)



	FTH	TF	HPX
■Poly(I:C)/Sal vs Sal/Sal	-1.5	1.1	-1.4
□Poly(I:C)/Ris vs Poly(I:C)/Sal	4.4	1.7	2.6
▫Sal/Ris vs Sal/Sal	2.5	2.1	4.1
▪Poly(I:C)/Ris vs Sal/Ris	1.2	-1.2	-2.2

Figure 3


Relative TF expression
2.0
1.5
1.0
0.5
0.0
75kDa–
A)
Relative MBP1 expression
1.5
1.0
0.5
0.0
21.5kDa–
(B)
Relative MAPK1 expression
2.0
1.5
1.0
0.5
0.0
42kDa–
(C)
Relative pMAPK1 expression
1.5
1.0
0.5
0.0
42kDa–
(D)
Relative pMAPKAPK2 expression
2.0
1.5
1.0
0.5
0.0
49kDa–
Sal/Sal
Poly(I:C)/Sal
Poly(I:C)/Ris
Sal/Ris

Top 5 significant pathways Poly (I:C)/Sal vs Sal/Sal	Gene names
Oxidative Phosphorylation**	ATP5F1;ATP5O;ATP5A1:ATP5B;ATP5D;ATP6V1D;ATP6V1E1;NDUFB9 NDUFC2;NDUFS7;NDUFV1;COX6C;COX5A;NDUFV3;SDHB;UQCRC2;UQCRFS1
Tricyclic acid cycle**	ACO2;CS;DLST;IDH3B;MDH1;MDH2;OGDH;PDHA1;SDHB
Glycolysis/Gluconeogenesis**	AKR1A1;ALDOA;ALDOC;ENO2;GAPDH;HK1;LDHA;LDHB;PGAM1:PDHA1:TPI1
Ribosome**	ARBP;RPS19;RPS29;RPS4X;RPS16;RPS7;RSL34;RPL3;RPLP1;SPSA
Cysteine and Methionine metabolism*	GOT1;GOT2;LDHA;LDHB;MAT2A;MPST

Table 1:

KEGG™ Pathway (Fischer Exact test P-value)	Gene name	Prenatal X preventive interaction (P-value)	Prenatal main effect (P-value)	Preventive main effect (P-value)	Poly(I:C)/sal vs sal/sal	Poly(I:C)/ris Vs poly (I:C)/sal	sal/ris vs sal/sal	Poly(I:C)/ris Vs sal/ris
Oxidative Phosphorylation (3.1E10)	ATP5A1	**0.040**	<0.001	0.491	1.6**	-1.5	1.8*	1.1
	ATP6V1H	0.860	0.594	<0.0001	1.0	-2.6**	-2.7**	1.1
	COX6C	0.236	0.034	<0.0001	**-1.4***	**2.2****	1.8**	-1.1
	COX5A	0.106	0.001	<0.0001	2.0**	2.5**	3.8**	1.3
	NDUFC2	0.531	0.019	<0.001	2.4**	2.0**	3.5**	-1.1
	NDUFS7	0.251	<0.001	0.002	2.4**	1.4	2.1**	1.6*
	NDUFV1	0.801	<0.001	<0.001	1.5**	1.4**	1.4*	1.6**
	NDUFV3	0.333	0.045	0.149	1.5*	-1.3	-1.1	1.1
Citrate Cycle (TCA cycle) (9.4E-9)	ACO2	0.249	0.043	0.007	**1.2***	**1.1**	1.3**	1.0
	CS	0.481	0.003	<0.0001	1.4*	1.8**	2.0**	1.2
	IDH	0.074	0.530	0.003	-1.2	1.5**	1.1	1.1
	MDH1	0.004	<0.001	<0.0001	1.7**	1.2	2.0**	1.1
	MDH2	0.369	<0.001	<0.0001	**1.5****	**-1.8****	-1.6**	1.3*
	OGDH	0.004	0.017	<0.001	-2**	1.1	-2.3**	1.0
	PDHB	0.033	0.002	0.086	-1.0	1.0	-1.2**	1.3**
	SDHA	0.387	0.004	<0.0001	1.1	-1.3**	-1.5**	1.3**
Glycolysis/ Gluconeogenesis (2.6E-6)	ADH	0.078	0.022	0.052	-1.2	-1.2	-1.2	-1.3
	ALDOA	0.016	<0.0001	<0.0001	1.8**	1.8**	2.4**	1.3**
	ALDOC	0.365	0.001	<0.0001	1.2**	1.4**	1.6**	1.1
	ENO2	0.060	<0.001	0.731	1.6**	-1.1	1.2	1.2
	GAPDH	<0.001	<0.0001	0.001	2.0	-1.7**	1.0	1.1
	HK1	0.058	<0.0001	<0.0001	1.6**	1.6**	2.0**	1.3**
	LDHA	0.940	0.003	0.072	1.4*	1.2	1.2	1.4*
	LDHB	0.022	0.034	<0.0001	2**	2.1**	4.4**	-1.0
	PGAM1	0.570	0.001	0.012	1.3**	1.3*	1.2	1.4**
	TPI1	0.316	0.028	<0.0001	1.2*	-2.0**	-1.8**	1.0

Table 2

KEGG™ Pathway	Candidate Proteins from previous Postmortem schizophrenia human studies	Direction of altered expression in human studies	Prenatal X preventive interaction (P-value)	Prenatal main effect (P-value)	Preventive main effect (P-value)	Poly(I:C)/sal vs sal/sal	Poly(I:C)/ris Vs poly (I:C)/sal	sal/ris vs sal/sal	Poly(I:C)/ris Vs sal/ris
Metabolic changes	**CKB**	↓	0.538	0.837	<0.0001	**2****	**-1.4****	1.5**	-1.0
	DPYSL2	↓	0.122	0.070	<0.0001	1.1	-1.6**	-1.5*	1.0
	ENO1	↓	0.751	0.005	<0.0001	1.2	-1.5**	-1.6**	1.2*
	ENO2	↓	0.060	<0.001	0.7313	1.6**	-1.1	1.2	1.2
	ALDOC	↓	0.365	0.001	<0.0001	1.2**	1.4**	1.6**	1.1
	IMPA1	↕	0.126	0.350	<0.001	-1.3	2.0**	1.4*	1.1
	LDHB	↕	0.022	0.034	<0.0001	2.0**	2.1**	4.4**	-1.0
Cytoskeletal changes	PPP3CA	↓	0.682	0.015	<0.0001	2.4	2.4	4.3	1.1
	CA2	↓	0.976	0.467	0.901	1.2	-1.0	-1.0	1.2
	CLTA	↓	0.507	<0.001	<0.0001	1.4*	2.1**	1.9**	1.6**
	DNM1	↕	0.366	0.578	<0.0001	-1.0	1.4**	1.5**	1.3
	EZR	↕	0.902	0.541	0.510	1.1	-1.1	-1.2	1.1
	GFAP	↕	0.351	0.008	<0.0001	1.2	1.9**	1.6**	1.5*
	GLUL	↓	0.085	0.01	<0.001	1.5**	1.3*	1.8**	1.1
	INA	↓	0.973	0.061	0.011	1.3	1.5	1.5	1.3
	MBP	↓	**0.631**	0.054	<0.05	**-2.0***	**2.0***	1.2	-1.2
	NFL	↓	0.981	0.647	<0.001	1.1	2.6**	2.7**	1.1
	PRDX2	↓	0.494	0.027	0.026	**1.2***	**-1.2***	-1.1	1.1
	PGAM1	↓	0.570	0.001	0.012	1.3*	1.3*	1.2	1.4**
	PHB	↕	0.660	0.372	0.052	1.1	-1.2	-1.1	1.0
	SEPT3	↕	0.093	0.006	0.002	1.6**	1.2	1.7**	1.1
	SEPT11	↕	0.004	0.094	0.192	1.8**	-1.2	1.5	-1.2
	SPTB	↓	0.040	0.045	0.487	-2.4**	1.8	-1.3	1.0
	STMN1	↑	0.974	0.107	0.249	-1.2	-1.1	-1.1	-1.2

	TPI1	↓	0.316	0.028	<0.0001	**1.2***	**-2.0****	1.8**	1.0
	YWHAZ	↓	0.247	0.002	0.003	1.4	1.1**	1.4**	1.1
	YWHAH	↓	0.005	<0.0001	<0.0001	2.2**	2.1**	3.8**	1.3
	YWHAG	↓	0.413	0.039	0.424	2.0*	-1.0	1.4	1.3

Table 3