

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

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

3

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26 *Abstract*

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

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

36 **RESULTS:**Over 1000 proteins were quantified in the prefrontal cortex with pathway
37 analyses implicating changes in core metabolic pathways, following prenatal Poly(I:C)
38 exposure.Some of these protein changes were absent in the prefrontal cortex of
39 Poly(I:C) treated offspring that subsequently received Risperidone treatment in
40 adolescence.Particularly interesting reductions in the expression of the myelin related
41 proteins, myelin basic protein isoform 3(MBP1) and rhombex29 were observed, which
42 were reversed by risperidone treatment.Validation by western blotting confirmed
43 changes in myelin basic protein isoform 3(MBP1), and mitogen activated kinase
44 1(MAPK1).Western blotting was extended to assess the MAPK signalling proteins due
45 to their roles in inflammation, namely phosphorylated mitogen activated kinase
46 1(pMAPK1) and phosphorylated MAPK-activated protein kinase 2(pMAPKAPK2).Both
47 were upregulated by Poly(I:C) treatment and reversed by risperidone treatment.

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

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

52

53

54 **Introduction**

55 Exposure to prenatal infection has been linked to an increased risk of schizophrenia in
56 keeping with increasing recent evidence implicating inflammation in psychosis[1-5].

57 However, the pathological mechanisms which confer vulnerability to the disease are
58 not known. Proteomics, the high-throughput large scale study of proteins, holds
59 potential to reveal pathological pathways at the molecular level and new insights into
60 disease mechanisms. Proteomic investigations of schizophrenia are steadily increasing
61 and are providing unique insights to unravel the complexities of this disorder[6]. The
62 expression of proteins involved in cell communication, signal transduction, cellular
63 metabolism, synaptic plasticity, cell growth and oxidation have been reported to be
64 altered in postmortem schizophrenia[6-8].

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

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

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

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

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

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

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

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

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

126

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128

129 **Methods**

130 Animals

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

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

138 Prenatal Poly(I:C) Treatment

139 At 3 months of age, rats were mated and the first day after copulation was defined as
140 day 1. On gestational day 15, pregnant dams were anesthetized with 3% isoflurane
141 (Minrad, Bethlehem, PA) in 98% O₂ and given a single intravenous injection at the tail
142 vein of 4 mg/kg Poly(I:C) (Sigma, Rehovot, Israel) dissolved in saline, or saline alone.
143 The volume of injection was 1 ml/kg. At birth, pups were grouped to 10, composed of
144 5 females and 5 males when possible. On postnatal day 21, the pups were weaned
145 and housed 3–4 per cage by sex and litter and maintained undisturbed until drug
146 injections that commenced on PND 34. Only male offspring were used in the following
147 experiments and each experimental group consisted of subjects derived from multiple
148 independent litters (6 Poly(I:C) and 6 saline litters), with no more than one rat from the
149 same litter.

150

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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 representation 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

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

180 Mass Spectrometry Analysis

181 Label free liquid chromatography-mass spectrometry (LC-MS) was performed on a
182 Thermo Scientific LTQ ORBITRAP XL mass spectrometer connected to a Dionex
183 Ultimate 3000 (RSLCnano) chromatography system. Further information regarding
184 LC-MS settings can be found in the Supplementary methods.

185 Data Processing with MaxQuant™

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

201 Statistical and Biostatistical Analysis

202 The LFQ scores for each protein were \log_2 transformed to remove the possible
203 influence of skew in the data. Under-represented proteins in a treatment-sequence
204 group were excluded in cases where less than three (66%) LFQ intensities were
205 available. A total 23,739 LFQ values were subsequently used in the statistical
206 analyses. Regression normalisation was performed to remove technical error across
207 the samples[48]. An FDR of 5% as advocated by Benjamini and Hochberg[49] was
208 used to determine the filtered protein set of interest. Group comparisons were
209 performed using the traditional 5% level of significance. Group differences of log-LFQ
210 scores were exponentiated, giving fold changes as measures of treatment effects.
211 The overall study had 4 potential comparison groups, Poly(I:C)/Sal (Prenatal
212 treatment), Poly(I:C)/Ris (Preventative treatment), Sal/Ris (Risperidone alone) and
213 Sal/Sal (Saline controls), reflecting the 2x2 factorial study design involving treatment
214 type (Poly(I:C)/Ris) and timing (prenatal/adolescent). However, as the primary
215 objective of the study was to determine the differential protein expression in offspring
216 following Poly(I:C) administration in dams, a *planned, discovery based* comparison
217 was first performed involving prenatal treatment compared to control offspring. See
218 Supplementary methods for more details regarding the statistical design.

219

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

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

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

229 *Prenatal treatment compared to Preventative treatment.*

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

234 *The effect of Risperidone in control offspring*

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

237 *Preventative treatment compared to Risperidone alone*

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

241

242

243 *Hypothesis based approach*

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

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

249

250 Western Blot analysis

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

257 **Results**

258 Mass spectrometry

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

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

262 130 proteins were dysregulated following prenatal treatment compared to saline
263 controls (Students t-test; FDR, $p < 0.05$) and KEGGTM (<http://david.abcc.ncifcrf.gov>)
264 was used to identify the key biological pathways within all FDR-significant differentially
265 expressed proteins. The top five significant pathways are listed in Table 1 (oxidative
266 phosphorylation, tricyclic acid (TCA) cycle, glycolysis, ribosome, cysteine and
267 methionine metabolism) along with genes identified from each pathway.

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

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

283 *Risperidone treatment of control offspring*

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

297

298 *Preventative treatment compared to Risperidone alone*

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

304 *Hypothesis based approach*

305 *i). Myelin and myelin-related Protein Changes*

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

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

315 Preventative treatment was also associated with a significant increased expression of
316 MAG ($p < 0.005$; +8.2 fold).

317 Iron is an essential cofactor in myelination, and with this in mind, we assessed the
318 expression of any core iron homeostasis proteins that we identified in our proteomic
319 analysis. These were ferritin heavy chain (FTH), transferrin (TF) and hemopexin
320 (HPX). FTH was decreased in adulthood following prenatal Poly(I:C) treatment
321 ($p < 0.05$; -1.4 fold) but this effect was reversed for preventative treatment ($p < 0.001$;
322 +4.4 fold, Figure 2 (b)).

323 TF, which controls the level of free iron in the blood was unchanged following prenatal
324 Poly(I:C) treatment. However there was a trend present to increase the expression ($p=$
325 0.1; +1.1 fold).

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

328 *ii). Postmortem candidate proteins*

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

341

342

343

344 Western Blot Validation of Selected Proteins

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

347 **TF**

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

357 **MBP1**

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

362 **MAPK1**

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

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

374 **pMAPK1**

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

382 **pMAPKAPK2**

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

393 **Discussion**

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

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

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

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

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

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

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

433 Interestingly, FHC was also reversed by Risperidone treatment.

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

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

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

463

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

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

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

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

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

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523

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731

732

733

734 **Figure 1: (a)** Experimental design. Pregnant wistar dams were injected with Poly(I:C)
735 (4 mg/kg) or saline (Sal) on gestational day 15. The resulting Sal and Poly(I:C)
736 offspring were then divided into two cohorts, those that received Risperidone (Ris)
737 (0.045 mg/kg) in adolescence (PND 34–47) and those that received Sal. Four groups
738 of offspring were created in total (see Figure 1b). **(b)** Four groups of offspring were
739 produced in total for further proteomic analysis; offspring from Poly(I:C) treated dams
740 which received Sal or Ris at adolescence (group 1 and group 2) and offspring from Sal
741 treated dams which received Ris or Sal at adolescence (group 3 and group 4).

742
743 **Figure 2:** (a) Myelin and (b) iron protein expression changes observed in offspring
744 treated with prenatal Poly(I:C)/Sal compared to Sal/Sal(black), Poly(I:C)/Ris compared
745 to Poly(I:C)/Sal (white), Sal/Ris in adolescence compared to Sal/Sal(light grey) and
746 prenatal Poly(I:C)/Ris compared to Sal/Ris (dark grey).

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

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

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

758
759 **Table 1:** Top 5 significant pathways identified amongst the proteins differentially
760 expressed (t-test; $p < 0.05$) between Poly(I:C)/Sal and Sal/Sal controls. Pathways were
761 identified according to KEGGTM (Kyoto Encyclopaedia of Genes and Genomes) and
762 significance of each pathway identified is denoted by; * $p < 0.001$, ** $p < 0.0001$.

763 **Table 2:** Top 3 significant pathways identified by KEGGTM of the significantly altered
764 proteins between Poly(I:C)/Sal and Sal/Sal controls (t-test $p < 0.05$).

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

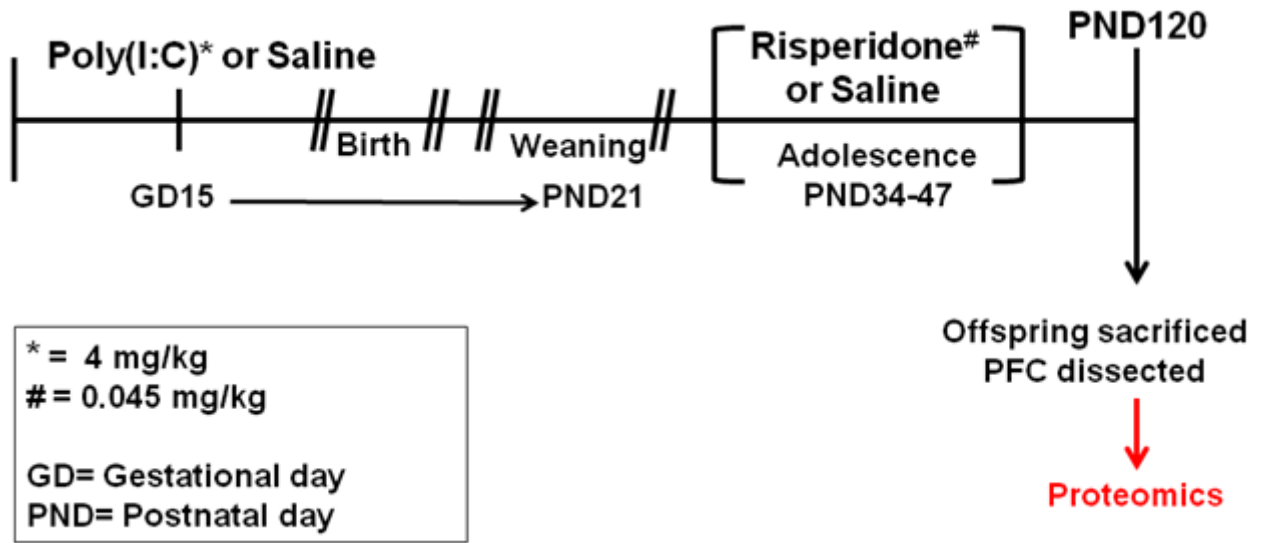
770 [#] = indicated by a change of sign providing the fold change is significant in
771 Poly(I:C)/Sal vs Sal/Sal and in Poly(I:C)/Ris vs Poly(I:C)/Sal.

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

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

779

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)

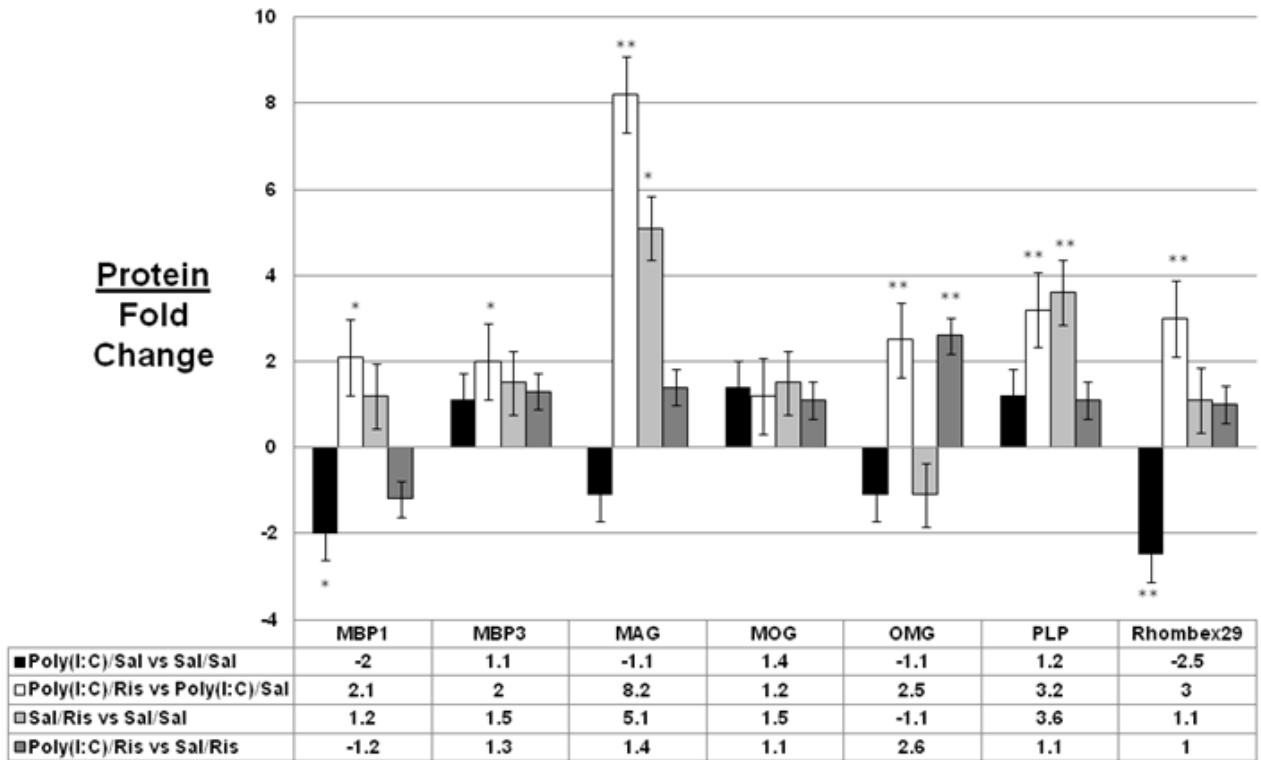


Figure 2(b)

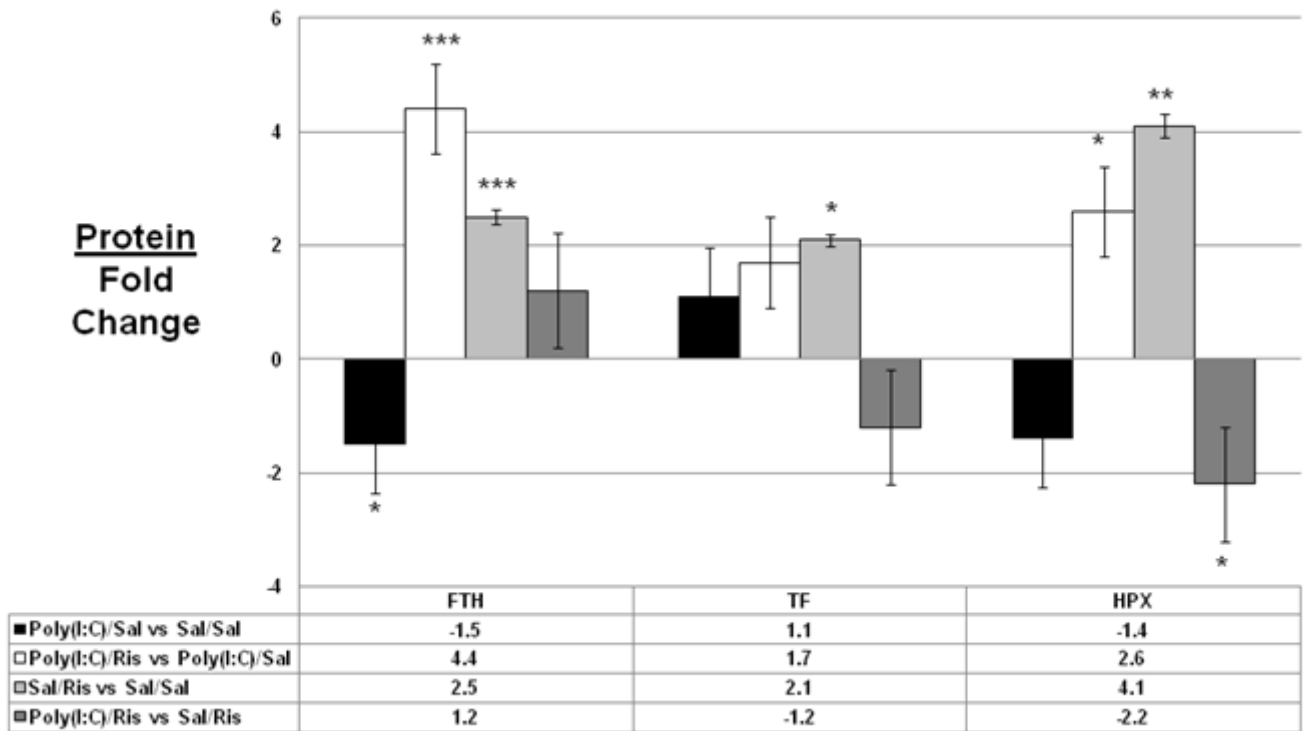
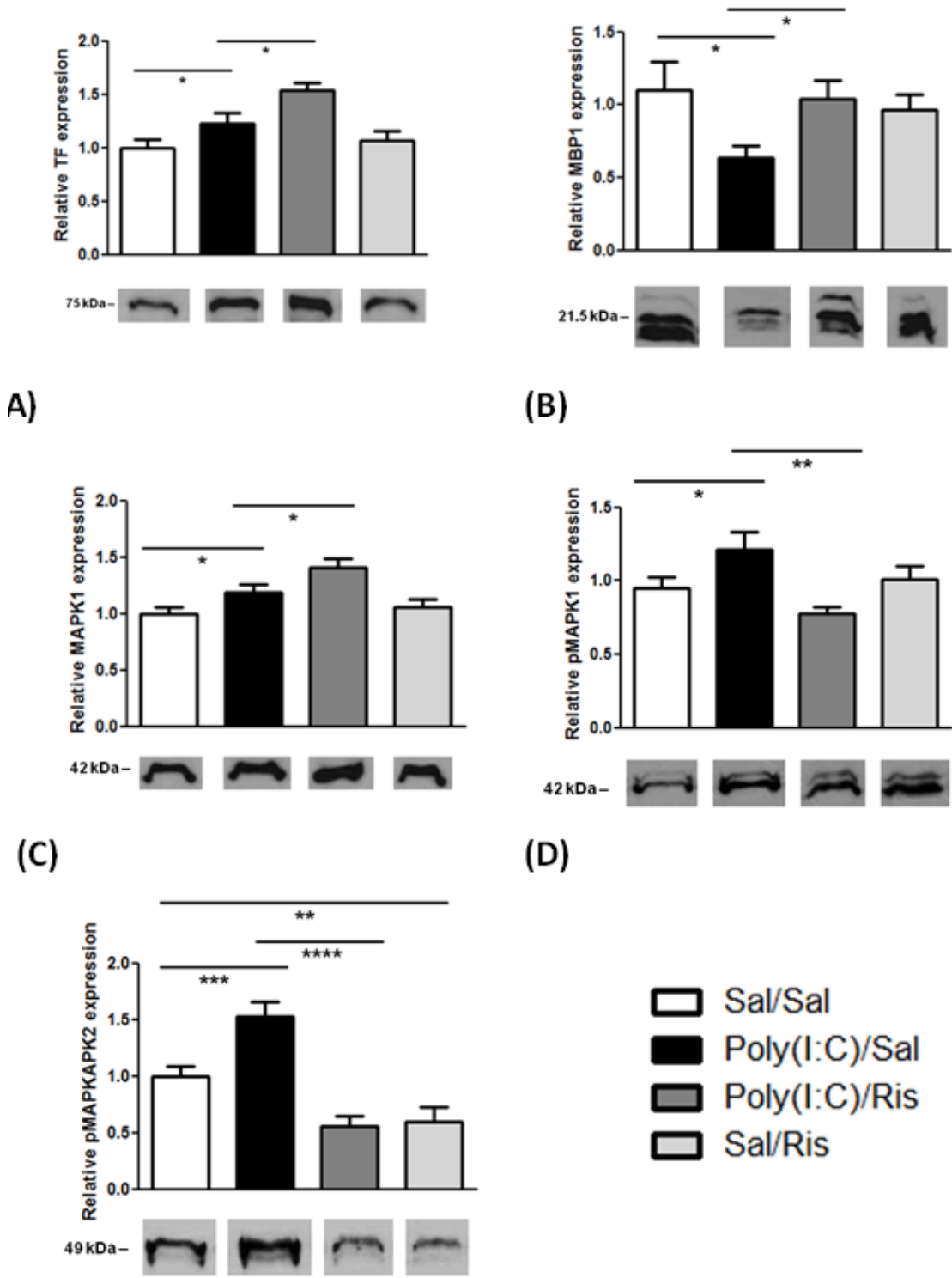


Figure 3



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

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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
	<u>SEPT3</u>	↑	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
	<u>SPTB</u>	↓	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