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<td><strong>Author(s)</strong></td>
<td>Deng, MY; Lam, S; Meyer, U; Feldon, J; Li, Q; Wei, R; Luk, L; Chua, SE; Sham, P; Wang, Y; McAlonan, GM</td>
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Frontal-Subcortical Protein Expression following Prenatal Exposure to Maternal Inflammation

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Abstract

Background: Maternal immune activation (MIA) during prenatal life is a risk factor for neurodevelopmental disorders including schizophrenia and autism. Such conditions are associated with alterations in fronto-subcortical circuits, but their molecular basis is far from clear.

Methodology/Principal Findings: Using two-dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometry, with targeted western blot analyses for confirmation, we investigated the impact of MIA on the prefrontal and striatal proteome from an established MIA mouse model generated in C57B6 mice, by administering the viral analogue PolyI:C or saline vehicle (control) intravenously on gestation day (GD) 9. In striatum, 11 proteins were up-regulated and 4 proteins were down-regulated in the PolyI:C mouse, while 10 proteins were up-regulated and 7 proteins down-regulated in prefrontal cortex (PFC). These were proteins involved in the mitogen-activated protein kinase (MAPK) signaling pathway, oxidation and auto-immune targets, including dual specificity mitogen-activated protein kinase kinase 1 (MEK), eukaryotic initiation factor (eIF) 4A-II, creatine kinase (CK)-B, L-lactate dehydrogenase (LDH)-B, WD repeat-containing protein and NADH dehydrogenase in the striatum; and guanine nucleotide-binding protein (G-protein), 14-3-3 protein, alpha- enolase, olfactory maker protein and heat shock proteins (HSP) 60, and 90-beta in the PFC.

Conclusions/Significance: This data fits with emerging evidence for disruption of critical converging intracellular pathways involving MAPK pathways in neurodevelopmental conditions and it shows considerable overlap with protein pathways identified by genetic modeling and clinical post-mortem studies. This has implications for understanding causality and may offer potential biomarkers and novel treatment targets for neurodevelopmental conditions.

Introduction

Dysfunction of fronto-subcortical circuits is implicated in psychiatric conditions with developmental origin, including schizophrenia [1] and autism [2]. The prefrontal cortex (PFC) is intimately linked to striatum, pallidum and thalamus via a serial of fronto-subcortical circuits [3]. The PFC is responsible for executive function and is heavily involved in social-emotional processes [4]. Dysfunction of this region results in disorders of thoughts and actions, part and parcel of the psychopathology of schizophrenia. The dopamine rich striatal region interfaces between the PFC and other brain regions. Therefore, disruption to prefrontal cortical networks incorporating the striatum are thought crucial to schizophrenia [5] and also implicated in autism [2,6]. Consistent with this, fronto-striatal prefrontal circuit anomalies have been detected in autism [2,6,7] and first episode schizophrenia prior to medication [1,8,9]; and functional imaging and sensorimotor gating abnormalities in both conditions are consistent with fronto-striatal perturbation [6,10,11,12]. However, the molecular basis of fronto-subcortical dysfunction is uncertain, therefore, novel treatment targets remain under-exploited.

Proteomics holds potential to uncover pathological mechanisms at the molecular level. Compared to conventional analysis, two-dimensional differential in-gel electrophoresis (2D-DIGE) has higher reproducibility and sensitivity because of its internal standard design which minimizes gel-to-gel variation, improves spot-matching, reduces number of gels needed, and permits quantitative analysis of small sample amounts [13,14]. Recently, post-mortem proteomic profiles of different brain regions from patients with schizophrenia [15,16,17,18,19] have been investigated. The expression of proteins involved in cell communication, signal transduction, cellular metabolism, synaptic plasticity, cell growth...
Brain Proteome Changes after Prenatal Inflammation

and oxidation proteins differs in samples from patients who had schizophrenia. However drug treatment, chronicity and cause of death, may confound interpretation and such studies cannot easily probe causative mechanisms. In contrast, animal models allow direct examination of aetiological risk factors on brain proteome. For example, rats with acute methamphetamine-induced behavioral sensitization have changes in mitochondrial dysfunction, oxidative, cytoskeletal, and synaptic proteins, which may hold clues to the nature of drug-induced psychosis [20,21]. In a schizophrenia model simulated by neonatal hippocampal lesions in rats, reduced protein expression in the PFC has been reported, with alterations in proteins involved in neurotransmitter signaling systems [22].

Maternal immune activation (MIA) during prenatal life increases risk of development of schizophrenia and related illnesses such as autism [23,24,25,26]. A substantial body of work has validated MIA in rodents as a means to simulate neuroanatomical [27,28], neurochemical [29,30], behavioural [31] and gene expression [32] abnormalities similar to those in schizophrenia, autism and related disorders. In this study, we adopted an established mouse model of MIA to study the effects of prenatal immune challenge on protein expression in striatum and PFC using 2D-DIGE, coupled with western blot analysis of selected proteins. The MIA model is based on prenatal maternal treatment with polyriboinosinic-polyribocytidilic acid (PolyI:C). PolyI:C is a synthetic analogue of virus-specific double-stranded RNA, which induces a cytokine-associated acute phase response typically seen following viral infections. As reviewed elsewhere [33,34], prenatal PolyI:C treatment in rodents induces a variety of behavioral, cognitive and pharmacological dysfunctions primarily implicated in schizophrenia and autism. Hence, the prenatal PolyI:C model is highly suitable for proteomic analyses because of its solid face validity to schizophrenia and related disorders.

Results

2D-DIGE Processing and Protein Identification

Total soluble brain proteins from CPu and PFC labeled with Cy3 or Cy5 fluorescent dyes, mixed to form random pairs on each 2D-DIGE gel, were evenly distributed across the pH 4–7 range and separated between 10 to 250 kDa on the 12% SDS-PAGE. For CPu, an average 1500 spots were detected in each image by DeCyder DIA module, and around 1300 spots included for further BVA module analysis after protein exclusion filters applied (Area<250; Max Peak<100; Max Volume<10,000; Max Slope>1.75). For PFC, around 1800 spots were detected and 1300 spots were included for BVA module with similar protein exclusion filters (Area<250; Max Peak<200; Max Volume<25,000; Max Slope>1.5). In BVA module, protein spots detected from each image were automatically matched to the master image which contained the most protein spots in an experiment and each protein’s standardized abundance volume was calculated against the internal standard. The protein fold changes were of a similar order of magnitude to other studies which have adopted 2D-DIGE techniques [35]. In addition, protein spots were manually checked to exclude dust particles and other artifact before further statistical analysis. Independent Student t-tests compared protein expression between PolyI:C- treated mice and controls. According to the significance criteria above, 15 spots in CPu showed significant alteration in expression (p<0.05; presented >75% images) in PolyI:C-treated mice compared to the control group. Of these 15 spots, 11 spots showed up-regulation and 4 spots showed down-regulation. Table 1 lists the 9 proteins identified by mass spectrometry. In PFC, compared to control group, 18 spots with significant alteration in expression were detected (p<0.05; presented in >75% images) in PolyI:C-treated mice, of which 10 spots were detected as up-regulated and 8 spots down-regulated. Nine protein spots were identified using mass spectrometry (Table 2), including olfactory marker protein; alpha- enolase protein; Guanine nucleotide-binding protein (G-protein); protein kinase C inhibitor protein1 (KCIP-1) and heat shock proteins. Figure 1 shows representative expression patterns.

Multivariate Statistical Analysis of Full Proteome Profile

In CPu (Figure 2, A) and PFC (Figure 2, B) respectively, 790 and 524 protein spots present in at least 11 out of 14 (>75%) images were included in partial least squares-discriminant analyses

<table>
<thead>
<tr>
<th>Classification</th>
<th>Spot No.</th>
<th>Fold change</th>
<th>p value</th>
<th>Protein name</th>
<th>Protein MW (kDa)</th>
<th>Protein PI</th>
<th>Protein score</th>
<th>C.I.%</th>
<th>Pep. count</th>
<th>NCBInr accession number</th>
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<tr>
<td>Isomerase</td>
<td>CPu356</td>
<td>1.06 down</td>
<td>0.02</td>
<td>WD repeat-containing protein 1 (Actin-interacting protein 1) (AIP1)</td>
<td>67.0492</td>
<td>6.11</td>
<td>103</td>
<td>99.998</td>
<td>11</td>
<td>gi</td>
</tr>
<tr>
<td>CPu357</td>
<td>1.09 down</td>
<td>0.023</td>
<td></td>
<td>WD repeat-containing protein 1 (Actin-interacting protein 1) (AIP1)</td>
<td>67.0492</td>
<td>6.11</td>
<td>82</td>
<td>99.979</td>
<td>10</td>
<td>gi</td>
</tr>
<tr>
<td>Transferase</td>
<td>CPu717</td>
<td>1.05 down</td>
<td>0.049</td>
<td>Dual specificity mitogen-activated protein kinase kinase 1 (MAP kinase kinase 1) (MAPK1 or MEK1)</td>
<td>43.7885</td>
<td>6.24</td>
<td>186</td>
<td>100</td>
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<td>CPu664</td>
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<td>Creatine kinase B-type</td>
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<td>5.4</td>
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<td>100</td>
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<tr>
<td>CPu650</td>
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<td>Creatine kinase B-type</td>
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<td>142</td>
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<tr>
<td>CPu659</td>
<td>1.09 up</td>
<td>0.05</td>
<td></td>
<td>Creatine kinase B-type</td>
<td>42.9714</td>
<td>5.4</td>
<td>768</td>
<td>100</td>
<td>21</td>
<td>gi</td>
</tr>
<tr>
<td>Hydrolase/RNA binding protein</td>
<td>CPu706</td>
<td>1.16 up</td>
<td>0.036</td>
<td>Eukaryotic initiation factor 4A-II</td>
<td>46.6009</td>
<td>5.33</td>
<td>194</td>
<td>100</td>
<td>10</td>
<td>gi</td>
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<tr>
<td>Oxido-reductase</td>
<td>CPu907</td>
<td>1.07 up</td>
<td>0.0045</td>
<td>L-lactate dehydrogenase B chain (LDH-B) (LDH-H)</td>
<td>36.8341</td>
<td>5.7</td>
<td>82</td>
<td>99.773</td>
<td>7</td>
<td>gi</td>
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<td>CPu1070</td>
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<td>0.0024</td>
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<td>NADH dehydrogenase (ubiquinone) iron-sulfur protein 3</td>
<td>30.3016</td>
<td>6.67</td>
<td>279</td>
<td>100</td>
<td>15</td>
<td>gi</td>
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Table 1. Differential protein expression in striatum of PolyI:C-treated mice compared to controls.

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Discussion

Using an epidemiologically motivated neurodevelopmental animal model of schizophrenia and related disorders, we show for the first time that prenatal immune challenge in the form of a viral-like acute phase response triggered in early/middle gestation (GD9) in mice leads to long-lasting changes in protein expression at the proteome level. Our quantitative 2D-DIGE techniques revealed differential expression of proteins in the striatum and PFC of adult mice prenatally exposed to the viral mimic PolyI:C relative to adult control mice. More specifically, proteins involved in the MEK/ERK pathway, oxidation and auto-immune targets were found to have altered expression in PolyI:C exposed mice, including dual specificity mitogen-activated protein kinase kinase 1 (MEK1), eukaryotic initiation factor (eIF) 4AII, creatine kinase (CK)-B, L-lactate dehydrogenase (LDH)-B, WD repeat-containing protein and NADH dehydrogenase in the CPu (Table 1); guanine nucleotide-binding protein (G-protein), 14-3-3 protein zeta/delta (KCIP-1), alpha-enolase, olfactory maker protein and heat shock nucleotide-binding protein (G-protein), 14-3-3 protein zeta were significantly up-regulated while HSP-60 and HSP-90-beta in the PFC (Table 1). These proteome wide results were generally confirmed using western blot analysis of the full proteome profile clearly separated PolyI:C and control groups (Figure 2).

In the maternal infection model, cytokine elevation rapidly triggers cellular defense programs [36]. Smith and Patterson concluded several cytokine signaling pathways, such as NF-κB and/or JAK/STAT, are involved in the modulation of cell proliferation, differentiation and migration during prenatal brain development. Activation of JAK/STAT pathway is particularly important in neuronal regeneration, embryonic development [37], neurogenesis and gliogenesis [38]. Pro-inflammatory cytokines have been shown to inhibit cortical neuron dendrite development in embryonic day 18 rat cortical cultures [39]. In our study, when triggered by pro-inflammatory cytokines, the intracellular signal transduction networks modified downstream of JAK/STAT, included the mitogen-activated protein kinase (MAPK) cascade which contains Ras/Raf, MEK, ERK. Confirmatory western blot analysis indicated lower level of phosphorylated ERK (pERK) was indeed a consequence of prenatal cytokine exposure, probably due to down-regulation of MEK. This finding is consistent with cell-culture study showing viral infection interferes with ERK1/2 phosphorylation in hippocampal neurons [40].

Post-mortem studies in patients of schizophrenia suggest the MAPK pathway is disrupted [41] with decreased MAPK1 gene expression reported in schizophrenia post-mortem samples [42]. In addition, preclinical investigation suggests the MAPK pathway is modified by anti-psychotic drug treatment [43,44]. Chronic treatment with olanzapine activates the MAPK pathway leading to phosphorylation of MEK1/2 and ERK1/2 [43], suggesting that the lower MEK1 observed in our study may constitute a potential pharmacological target. Consistent with this, epigenetic regulation of MEK1 via methylation of an upstream CpG island has been reported to correlate with antipsychotic use in schizophrenia [44]. In addition, in both the knock-out mouse model of Fragile X and peripheral blood lymphocytes from individuals with fragile X, a condition associated with autism, Berry-Kravis et al [45] showed delayed phosphorylation of ERK, partly overlapping our observation that PolyI:C mice have lower MEK and pERK.

Activation of STAT3 trigged by cytokines may be rapidly inhibited by MEK-ERK negative regulation [46]. Our finding that prenatal inflammation down-regulates expression of MEK and pERK in the CPu region suggests the prenatal PolyI:C

### Table 2. Differential protein expression in prefrontal cortex of PolyI:C-treated mice compared to controls.

| Chaperone | PFC324 | 1.1 up | 0.028 | Heat shock protein HSP 90-beta | 83.5712 | 4.97 | 466 | 100 | 29 | gi|123681 |
|-----------|--------|--------|-------|-------------------------------|---------|-----|-----|-----|----|----------|
| PFC522    | 1.15 down | 0.003 | Heat shock protein HSP60 | 60.8849 | 5.38 | 163 | 100 | 8 | gi|51452 |
| Lyase     | PFC731 | 1.11 down | 0.013 | Alpha-enolase (2-phospho-D-glycerate hydro-lyase) | 47.6345 | 6.63 | 288 | 100 | 17 | gi|6679651 |
|           | PFC739 | 1.15 down | 0.013 | Alpha-enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) | 47.4533 | 6.37 | 118 | 100 | 11 | gi|70794816 |
| Hydrolyase | PFC1061 | 1.07 down | 0.029 | Platelet-activating factor acetylhydrolase IB subunit | 25.7242 | 5.57 | 196 | 100 | 4 | gi|33440467 |
| Signaling protein | PFC942 | 1.07 up | 0.023 | Guanine nucleotide-binding protein G0/G13/G15/G17 subunit beta-1 | 38.1513 | 5.6 | 470 | 100 | 13 | gi|6680045 |
|           | PFC1069 | 1.29 up | 0.028 | 14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1) (KCIP-1) | 27.9284 | 4.73 | 104 | 99.999 | 8 | gi|6756041 |
|           | PFC1070 | 1.2 up | 0.059 | 14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1) (KCIP-1) | 28.1829 | 4.77 | 183 | 100 | 12 | gi|3065925 |
|           | PFC1176 | 2.95 up | 0.038 | Olfactory marker protein | 18.8547 | 5 | 128 | 100 | 7 | gi|6754936 |

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exposure might disrupt this feedback circuit, and have potentially widespread impact upon multiple cellular processes. The MAPK cascade is a vital cellular pathway for neuronal differentiation and function [47]. The 14-3-3 protein is key intracellular binding protein and one of its actions is to facilitate RAF activation of MEK [48]. Heat shock protein (HSP)-90 is also required to activate the RAF signaling cascade [49]. Our results showing both 14-3-3 and HSP-90 proteins were up-regulated in PolyI:C exposed offspring might indicate a facilitation of RAF to compensate for lower expression of MEK. Moreover, activation of HSP-90 appears to be coupled to the activation of IL6/STAT3 [50], and is over expressed in patients with schizophrenia [51], while 14-3-3 proteins were have been reported to be elevated in nucleus accumbens of isolation reared rats [52], and in the posterior hippocampus of post-mortem tissue from patients with schizophrenia [17].

Figure 1. Differential expression patterns of proteins-of-interest and their abundances relative to the internal standard in the BVA module.
doi:10.1371/journal.pone.0016638.g001
(A) 

![Graph 1](image1)

(B) 

![Graph 2](image2)
Another important function of HSP90 is to ‘restrain’ activation of ErbB receptor network by ligands such as neuregulin 1 (nrg1). ERK phosphorylation is an end result of ErbB activation. In addition to possible disruption of MAPK signaling in schizophrenia and related conditions discussed above, nrg1 is a susceptibility gene for schizophrenia and expression of ErbB3 protein is defective in schizophrenia [53]. Our finding that a key inhibitory regulator of neuregulin-ErbB is up-regulated in the MIA model along with a down-regulation of MAPK signaling is therefore compelling, suggesting the same pathways are targeted by genetic and environmental perturbations relevant to neurodevelopmental conditions.

There is considerable cross-talk between MAPK pathway and others such as PKC/PTEN, mTOR and fragile X mental retardation protein (FMRP) pathways [54], PTEN mutations are associated with autism [55,56], as are tuberose sclerosis and fragile X, which disrupt mTOR signaling. These pathways modulate protein translation via the eIF4 family proteins. In PolyI:C exposed offspring the eukaryotic initiation factor (eIF) 4A-II protein was up-regulated in the CPu. Recently novel association of the eIF4E protein component of this family with autism was reported [57]. Thus disruption of a number of critical converging pathways including MAPK, PKC, mTOR and FMRP appears important in neurodevelopmental conditions, either as a consequence of genetic or environmental risk factors during prenatal life [58], GSK3b interfaces between these pathways [59,60], inhibited by MEK and mGLUR signaling [61] and increased by PTEN inhibition of PI3K/AKT. Lastly, in addition to facilitating Raf, protein 14-3-3, up-regulated in PolyI:C exposed mice regulates GSK3b interactions including phosphorylation of Tau [62]. These multiple clues pointing to GSK3b may be important to follow up in future studies because GSK3b inhibitors, such as Lithium, offer potential therapeuthic options [61,63].

Down-regulation of heat shock protein (HSP)-60 detected in PFC of PolyI:C exposed offspring, agrees with other schizophrenia-like models [52,64]. In Roncada’s study [52], isolation-reared rats showed significant pre-pulse inhibition (PPI) deficits correlated with the level of HSP-60 decrease. The MIA model has also reliably been shown to precipitate PPI impairment [28,31]. HSP-60 is a proposed auto-immune target in schizophrenia [65,66]. Similarly, alpha-enolase (NNE), significantly down-regulated in PolyI:C exposed offspring, is considered an auto-antigen [67]. In the MK801 rat model, NNE increased in the thalamus of MK801 short-term treated rats, but decreased with long-term treatment [64]. Combined with the evidence of lower HSP-60, which echoes reduction in liver proteome of patients with schizophrenia [65], our results raise the issue of whether the immune system is chronically affected following activation in fetal life. Certainly immune mechanisms have been suggested to explain glutameric and dopaminergic disregulation characteristic of schizophrenia [68], and autism is accompanied by persistent immunological anomalies [69]. Proteomic and transcriptome studies in schizophrenia are also consistent with dysregulation of immune pathways [42,70].

Other proteins altered in PolyI:C mice included creatine kinase (CK), actin-interacting proteins, L-lactate dehydrogenase proteins, NADH dehydrogenase iron-sulfur (Fe-S) proteins and WD repeat protein 1. The direction of change identified here is often, but not always, similar to that reported in analyses of schizophrenia post-mortem proteome. For example, NADH dehydrogenase was up-regulated here and has previously been reported as upregulated in schizophrenia posterior hippocampal post-mortem samples but reduced in anterior hippocampus [17]. CK type B was up-regulated here similar to findings in schizophrenia post-mortem tissue [16]. Moreover, CK isoforms are associated with the 14-3-3 protein complex [71,72] so, that both are up regulated within this model is internally consistent. In contrast, WD repeat protein 1 has been reported as up-regulated in schizophrenia post-mortem tissue but was down-regulated in our model [70]. Possible reasons for divergent findings are many-fold, but may include contribution from medication exposure and illness progression in the clinical samples.

Our study has a number of limitations. A standard global scale proteomic analysis was applied to brain homogenate lysates. However, this approach does not fully sample different intracellular organelles in the brain. A further limitation was the modest rate of successful protein identification in mass spectrometric analysis, particularly in PFC. This false negative error may be because the regional homogenate was insensitive to low protein abundance. Given that the gels are loaded with a fixed protein quantity, more proteins separated, theoretically means lower concentrations of individual proteins. One solution is the use of subcellular fractions such as mitochondria, nuclear and plasma membrane which can further separate from brain lysates with chemical or physical fractionation techniques. Fractionation techniques, though challenging, will form good future partnership with proteomics [22].

In conclusion, prenatal immune challenge causes changes across the fronto-striatal proteome echoing findings from diverse studies on post-mortem tissue, genetic analyses and animal model systems. Specifically, our data fits with evidence for disruption of critical proteins within converging intracellular pathways involving MAPK in neurodevelopmental conditions. Further investigation of protein pathways in this and other related models may hold promise for further identification of disease biomarkers and novel treatment targets.

Figure 3. Western blot analysis of the differentially expressed target proteins in PolyI:C and control group. (A) Gel image of protein products in the control and PolyI:C group. (B) The relative expression of the target proteins/β-actin protein. Columns show mean ± SEM (n = 8 for PFC and n = 6 for CPu). *p<0.05 and **p<0.005 vs. corresponding control group.

doi:10.1371/journal.pone.0016638.g003
Materials and Methods

Animals and Sample Purification
C57BL6/J mice were used for the experiment. All procedures described in the present study had been previously approved by the Cantonal Veterinarian’s Office of Zurich (permit number 28-2009) and are in agreement with the principles of laboratory animal care in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 86-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering. After local ethic review board approval, and following previously published protocol [27,28,31], 5mg/kg PolyLC (potassium salt dissolved in saline, Sigma Aldrich) was administered in an injection volume of 5ml/kg to pregnant C57BL6/J mice on gestation day (GD) 9 via the tail vein. Control animals received 5ml/kg saline via tail vein on GD9. The resulting offspring were weaned and sexed at postnatal day (PND) 21. Littermates of the same sex were caged separately, three to four per cage. At adult age (PND 70), 7 mice (4 male and 3 female offspring) from each prenatal treatment group (saline and PolyLC) were randomly selected from different litters. Hence, a total of 14 mice (7 control, 7 PolyLC) were included in the 2D-DIGE analyses. Animals were sacrificed by decapitation and brains dissected, immediately frozen and stored at −80°C. Brain tissue from striatum (CPu) and PFC of saline- and PolyLC-treated mice were individually thawed and homogenized in 2D-DIGE lysis buffer (30mM Tris, 7M Urea, 2M Thiourea, 4% CHAPS, pH 8.5; at 4°C) for 30 seconds, then centrifuged at 15,000×g for 15 min to remove cell debris. Supernatant was collected and quantified with 2-D Quant Kit (Amersham Biosciences) before loading gels for 2D-DIGE separation.

2D-DIGE Analysis and Protein Identification by Mass Spectrometry

2D-DIGE analyses of CPu and PFC samples were conducted independently. The procedure has been described previously [35]. Briefly, for each region, 50µg of protein was randomly labeled with 400 pmol of CyDye DIGE Fluor minimal dye (GE Healthcare), Cy3 or Cy5, and the pooled internal standard with Cy2. Labeled samples were randomly mixed and assigned to seven gels, each of which contained Cy2-, Cy3- and Cy5-labeled samples, respectively [73]. Mixed pre-labeled samples were loaded to the 18cm, pH 4–7, IPG DryStrips (Bio-Rad) for first dimensional isoelectric focusing (IEF) separation (Bio-Rad IEF system), and secondary dimensional separation by 12% SDS-PAGE (Bio-Rad vertical system). Fluorescent images were obtained by Typhoon™ 9410 scanner (GE Healthcare). For each region, a total of 14 images from 7 gels with good separation quality were analyzed using DeCyder software version 6.5 containing Differential In-gel Analysis (DIA) module and Biological Variation Analysis (BVA) module (Amersham Biosciences). The abundance of protein spots was intra- and inter- gel normalized according to the internal standard. Statistical analysis of different protein spot intensity between groups was performed using independent Student’s t-tests run on the normalized protein abundance in DeCyder software. Protein spots with p-value less than 0.05 and present in at least 11 out of 14 (>75%) images [65] were considered significant. Spots of interest were picked from the silver-stained 2D-DIGE gels and followed by in-gel trypsin digestion and peptide extraction [35] for protein identification by tandem mass spectrometry analysis using ABI 4800 MALDI TOF/TOF™ MS Analyzer (Applied Biosystems, Foster City, CA, USA). The combined peptide mass fingerprinting (PMF) and MS/MS peptide fragmentation data were submitted to the NCBIhr database and SwissProt database using the software GPS Explorer, version 3.6 (Applied Biosystems) and in-house MASCOT version 2.2 (Matrix Science) with parameters: one missed cleavage allowed; initial mass tolerance of 3ppm; fixed modification, carbamidomethyl (Cys); variable modification, oxidation (Met); precursor tolerance of ±0.2 Da. For all significant protein identifications, both protein and total ion scores were above or equal to C.I. 95% [35].

Multivariate Statistical Analysis of Full Proteome Profile

Multivariate analysis using partial least squares-discriminant analysis (PLS-DA) was performed to examine whether PolyLC groups could be separated from control groups on the basis of the full proteome profile [63,74]. Data was imported into SIMCA 9.0 software (Umetrics, Umea, Sweden) as previously described [39]. Only protein spots present in at least 11 out of 14 (>75%) images were included in the analyses [63,74].

Western Blot Validation

Brain tissues from CPu (n = 8) and PFC (n = 6) of equal numbers of male and female saline and PolyLC-treated mice were homogenized at 4°C in lysis buffer (1:5, wt/vol) containing 1 mM EDTA and 20 mM phenylmethylsulphonyl fluoride. The proteins from resultant supernatant were determined by (Bio-Rad Protein Assay) for Sodium Dodecyl-sulphate Polyacrylamide gel electrophoresis (SDS-PAGE). Proteins (20 µg/lane) were subjected to electrophoresis on a 12% (wt/vol) polyacrylamide gel in SDS, and gels subsequently processed for electroblotting to polyvinylidine difluoride (PVDF) membranes. The blotted PVDF membranes were saturated with 5% (wt/vol) of skimmed milk in Tris Buffer Saline, pH 7.4 and 0.1% (vol/vol) of Tween 20 for 1 h at room temperature. The membranes were sequentially incubated with primary antibodies to the following proteins: HSP90 (rabbit polyclonal IgG antibody, 1:2000 dilution, Cat # ab13495, Abcam, MA, USA), HSP60 (rabbit polyclonal IgG antibody, 1:2000 dilution, Cat # ab68416, Abcam, MA, USA), α-ENOLASE (rabbit polyclonal IgG antibody, 1:5000 dilution, Cat # ab49343, Abcam, MA, USA), 14-3-5 ZETA (rabbit polyclonal IgG antibody, 1:1000 dilution, Cat # ab51129, Abcam, MA, USA), eIF4A2 (rabbit polyclonal IgG antibody, 1:1000 dilution, Cat # ab32128, Abcam, MA, USA), MEK1 (rabbit polyclonal IgG antibody, 1:500 dilution, Cat # ab59294, Abcam, MA, USA), ERK (rabbit polyclonal IgG antibody, 1:1000 dilution, Cat # sc-51, Santa Cruz Biotechnology, CA, USA) and pERK (mouse monoclonal IgG antibody, 1:500 dilution, Cat # sc-7333, Santa Cruz Biotechnology, CA, USA), overnight at 4°C following by a peroxidase-labeled anti-mouse/rabbit IgG (1:2000 dilution, Boehringer Mannheim, Germany) for 1 h at room temperature. After thorough washing, the positive bands were revealed using ECL western blotting detection reagents and autoradiography film (Amersham, Biosciences, UK). The intensities of the bands were quantified using IMAGE QUANT software (Molecular Dynamics, USA).

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Author Contributions
Conceived and designed the experiments: MYD UM JF SEC PS YW GMM. Performed the experiments: MYD SL QL RW LL. Analyzed the data: MYD SL GMM. Contributed reagents/materials/analysis tools: SEC PS YW GMM. Wrote the paper: MYD UM GMM.
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