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Adolescent Risperidone treatment alters protein expression associated with protein trafficking and cellular metabolism in the adult rat prefrontal cortex.

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DATASET BRIEF

Adolescent Risperidone treatment alters protein expression associated with protein trafficking and cellular metabolism in the adult rat prefrontal cortex.

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Abbreviations: PFC, prefrontal cortex; DAVID, Database for Annotation, Visualization and Integrated Discovery; PND, postnatal day; FDR, False discovery rate; LFQ, Label free quantitation.

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The prefrontal cortex is associated with mental health illnesses including schizophrenia, depression, bipolar disorder and autism spectrum disorders. It richly expresses neuroreceptors which are the target for antipsychotics. However as the precise mechanism of action of antipsychotic medications are not known, proteomic studies of the effects of antipsychotic drugs on the brain are warranted. In the current study we aimed to characterise protein expression in the adult rodent prefrontal cortex (n=5 per group) following low dose treatment with Risperidone or saline in adolescence (postnatal-days 34-47). The prefrontal cortex was examined by triplicate one hour runs of label-free LC-MS/MS. The raw mass spectral data were analyzed with the MaxQuant™ software. Statistical analysis was carried out using SAS ® Version 9.1. Pathway and functional analysis was performed with IngenuityPathway Analysis and in the Database for Annotation, Visualization and Integrated Discovery (DAVID) respectively, the most implicated pathways were found to be related to mitochondrial function, protein-trafficking and the cytoskeleton. This report adds to the current repertoire of data available concerning the effects of antipsychotic drugs on the brain and sheds light on their biological mechanisms. The mass spectrometry data has been deposited with the ProteomeXchange Consortium with dataset identifier PXD000480.

Antipsychotics are the main pharmacological agents used to treat patients with psychotic disorders and while their use is critical to the management of psychosis they are often incompletely effective. Furthermore, their mechanism of action is not clear, with evidence for effects on numerous signalling and receptor functions, and little clarity regarding predictors of drug treatment response and resistance [1-3]. Although clinical studies have revealed potential metabolomic and proteomic changes in patients undergoing antipsychotic treatment [4-7], few proteomic studies have specifically studied the effects of antipsychotic drugs on the mammalian brain. Proteomic studies of antipsychotic drug exposure to the rodent brain have revealed altered protein expression associated with presynaptic function and cellular assembly/organization in the frontal cortex [8] and differentially regulated proteins relating to the cytoskeleton, calcium regulation, metabolism, signal transduction and oxidative stress in rat neural stem cells [9].

The prefrontal cortex (PFC) is implicated in the pathophysiology of psychotic disorders such as schizophrenia, bipolar disorder, depression, anxiety and addiction

[10-14]. Neuropsychological studies showing executive and working memory deficits in psychotic disorders also implicate this region [15-18]. The executive functions of the PFC develop to their peak throughout the adolescent period. During this time, maturation of the PFC is vulnerable to environmental insults and susceptible to the development of neuropsychiatric disorders [19].

It is appreciated that the timing of antipsychotic drug exposure is important. Recent research have shown that early identification and treatment of psychosis improves outcome [20] and a great debate has arisen concerning the risks and benefits of early treatment of patients during the 'at risk mental state' for psychosis [21]. The goal of the present study was to comprehensively evaluate the PFC proteome of the rat following administration of a low dose of the atypical antipsychotic Risperidone, during adolescence.

Adolescent male Wistar rats were housed 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 (animal welfare assurance number A5010-01). Ethical approval was granted by Royal College of Surgeons in Ireland Research Ethics Committee (REC-585bb). Work flow is depicted in Figure 1. Animals were injected daily, intraperitoneally, with Risperidone (0.045 mg/kg) or saline (n= 5 per group) on postnatal days (PND) 34-47. This dose of Risperidone was utilised as it is well established in the model of Weiner and Colleagues [22, 23] and earlier work by Richtand found this lose dose to be effective in a neurodevelopmental model, more effective at a lower dose, more selective for serotonin receptors, and also found it to be effective in preventing amphetamine-induced hyperactivity caused by neonatal ventral hippocampal lesions [24]. Additionally, it is also in the range of the average clinical dosage for adolescents; 2 mg/day to a 50 kg adolescent. The duration of treatment (postnatal days 34-47) is again described well by Weiner and colleagues [22, 23]. This period represents peri-adolescence or adolescence, a timeframe crucial to brain development [25] and secondly, animals were sacrificed on PND 120, mirroring adulthood, meaning we can assess long-term brain changes from antipsychotic intervention at adolescence.

sacrificed during adulthood at postnatal day 120 under pentobarbital anaesthesia, where brains were quickly removed, dissected, and frozen in liquid nitrogen. The prefrontal cortex was sonicated (Sonics© Newtown, CT, USA) in tri-ethyl-ammonium-bicarbonate buffer (Sigma Aldrich, Ireland) containing protease inhibitors (Roche, Ireland) and protein concentrations were determined using the Bradford assay [26]. Fifty micrograms of protein from each homogenate was denatured in 10 µl 2% *Rapi*Gest™ solution (Waters, United Kingdom) at 80ºC for 10 min. Samples were subsequently reduced in the presence of 50mM TCEP (*tris*2-carboxyethylphosphine) (Sigma Aldrich, Ireland) at 60ºC for 60 minutes and alkylated in the dark with 200mM iodoacetic acid (Sigma Aldrich, Ireland). Protein was digested with 5 µg of sequence grade modified trypsin (Promega, United Kingdom), overnight in a 37°C shaking incubator. Digestion was stopped and the *Rapi*Gest precipitated with formic acid (0.1% v/v). After digestion, peptides were resuspended in 0.5% trifluoroacetic acid, dried in an Eppendorf Vacufuge™ (Eppendorf©, USA) and desalted using 5 µg capacity, C_{18} resin, ZipTips (Millipore, USA). Label free liquid chromatography-mass spectrometry was performed on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Each sample was loaded onto Biobasic Picotip Emitter (120 mm length, 75 µm ID) packed with Reprocil Pur C18 (1.9 µm) reverse phase media column, and was separated by an increasing acetonitrile gradient, using a 60 min reverse phase gradient at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 200°C, a capillary voltage of 45V, a tube lens voltage of 100V and with a potential of 1800V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the seven most intense ions prior to MS/MS analysis using the ion-trap. Three technical replicates were performed for every biological sample. The data analysis was carried out with MaxQuant software [27]. Label free quantitation (LFQ) was performed as previously described [28]. As described by Luber and colleagues, LFQ algorithms were added to MaxQuant by extracting isotope patterns for each peptide in each run. 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 set at 1%. In brief, for every peptide, corresponding total signals from the multiple runs were compared to determine peptide ratios. Pair-wise peptide

ratios were only determined when the corresponding peak is detected in all LC-MS runs. The median values of all peptide ratios of one protein then represent a powerful estimate of the protein ratio [29].

The dataset is composed of 1259 proteins through the identification of 5551 unique peptides. Because of the stringent FDR settings (both FDR for peptide and protein identification set at 1%) we used all proteins identified for further statistical analysis. This approach ensures that we get a maximum number of identifications for future studies, maximum coverage of the prefrontal cortex proteome and potentially interesting candidates for further quantitative investigations. LFQ intensity values were used for protein quantification across the groups in subsequent statistical analysis with SAS ® Version 9.1. The LFQ scores for each protein were $\log_2$ transformed to remove the possible influence of skew in the data. Regression normalisation was performed to remove technical error across the samples. A Students t-test was performed between the two treatment groups. Comparisons were performed at a 5% level of significance. From a total of 1,259 identified proteins, 492 were significantly differentially expressed between the two groups at a 5% level of FDR. From these 492 proteins, 28% were originally identified with one unique peptide, 232 were increased and 260 were decreased. All significant proteins were converted to UNIPROT gene identifications and submitted to the Ingenuity Pathway Analysis software. The top significant molecular and cellular functions identified to be enriched within the proteins were mapped to cellular assembly and organisation, cellular function and maintenance, cell-to-cell signalling and interaction, cell morphology and cellular development (Table 1a). The biological pathways of the significantly expressed proteins were mapped to clathrin-mediated endocytosis, the tricyclic acid cycle, remodelling of epithelial adherens junctions, rho GTPase signalling and mitochondrial dysfunction (Table 1b). The GO-term functional annotation was performed with DAVID [30] (Figure 2). The GO terms of cellular component, molecular function, and biological process were explored for all significantly differentially expressed proteins. The identified pathways regarding clathrin-mediated endocytosis and metabolic function are in agreement with neuroproteomic investigations implicating these to be altered in the pathology of schizophrenia [31, 32]. Significant proteins that were identified with high scores of unique peptides (< 20 unique peptides) were predominantly involved in core metabolic pathways, such as aconitate hydratase, fructose-bisphosphate and

hexokinase and relating to the cytoskeleton such as synapsin, glial fibrillary acidic protein and spectrin beta. These results reflect the known metabolic and brain remodelling effects of atypical antipsychotics [33, 34]. Overall, these findings are particularly interesting as they provide insights into the mechanistic action of antipsychotic medication. The results have relevance for drug discovery where such pathways mentioned could be specifically targeted. Additionally, metabolomic studies could be beneficial in dissecting the effect of the large amount of metabolic proteins implicated in the data.

The results pertaining to this data set should be interpreted with care as there are limitations which should be addressed. Firstly, it is likely that there will be differences in the protein expression changes identified in the brain following drug administration in rodents compared with that observed in humans with neuropsychiatric disorders following similar drug administration. Secondly, drug metabolism can differ significantly between species. For example, the half life for Risperidone ranges from 3-20 hours in human where it is just under 3 hours in the rat [35-37]. Thirdly, drug exposure in our study was of relatively short duration and may not equate, in terms of effects on brain proteome, with chronic administration. Nonetheless, our study has the strength that by focussing on drug treatment during adolescence, we have targeted a time period of particular importance in psychosis and of PFC maturation. In sum, we have used a high throughput proteomic platform to achieve in-depth coverage of the proteome of the adult rodent PFC following a short-term administration of Risperidone. These data contribute to the understanding of the mechanistic action of Risperidone in the rodent, and possibly point to its' role in disease. The mass spectrometry proteomics data are deposited in ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) [38] via the PRIDE partner repository with the data set identifier PXD000480. The authors express no conflict of interest.

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294 Table Captions

295 Table 1: (a) Top molecular and cellular function associated with proteins significantly
296 differentially expressed between adult rats who either received Risperidone (0.045
297 mg/kg) or saline during adolescence (PND 34-47). #molecules denotes number of
298 molecules associated with each function (b) Top canonical pathways associated with
299 proteins significantly differentially expressed between adult rats who either received
300 Risperidone (0.045 mg/kg) or saline during adolescence (PND 34-47).Ratio indicates
301 number of molecules identified in data set from total number of pathway associated
302 molecules listed within the database.

303

Figure Captions

Figure 1: Proteomic workflow for the study described. In brief, male adolescent rodents were treated with Risperidone (0.045 mg/kg) or saline for two weeks (PND 34-47) (*n= 5 per group*) and sacrificed in adulthood at postnatal PND120. The prefrontal cortex was dissected, prepared for mass spectrometry and each sample was run in triplicate. Bioinformatics was then carried out.

Figure 2: The distribution of differentially expressed proteins in the prefrontal cortex between adult rats which were either treated with Risperidone (0.045 mg/kg) or saline in adolescence. Annotations of biological process (a) cellular component (b) and molecular function (c) were according to GO analysis. Percentages indicate the proportion of proteins within that category with respect to the total number of significantly expressed proteins (FDR; $p < 0.05$).