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Diabetic db/db Mice Exhibit Central Nervous System and Peripheral Molecular Alterations as Seen in Neurological Disorders.

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Diabetic *db/db* mice exhibit central nervous system and peripheral molecular alterations as seen in neurological disorders

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The *db/db* mouse is a widely used preclinical model in diabetes research. Recent studies have shown that these mice also display aspects of psychosis and depression-like behaviors as seen in some psychiatric disorders. Here, we have performed multiplex immunoassay and liquid chromatography mass spectrometry profiling of the plasma and brain samples from *db/db* and control mice to identify altered pathways, which could be related to these behavioral abnormalities. This is the first study to carry out profiling of the brain proteome in this model. Plasma from the *db/db* mice had increased levels of leptin and insulin, decreased levels of peptide YY, glucagon and prolactin and alterations in inflammation-related proteins, compared with control mice. Frontal cortex tissue from the *db/db* mice showed changes in proteins involved in energy metabolism, cellular structure and neural functioning, and the hippocampus had changes in proteins involved in the same pathways, with additional effects on cellular signalling proteins. The overlap of these findings with effects seen in type 2 diabetes, schizophrenia, major depressive disorder and Alzheimer's disease might contribute to a common endophenotype seen in metabolic and neurological disorders. *Translational Psychiatry* (2013) 3, e263; doi:10.1038/tp.2013.42; published online 28 May 2013

Introduction

The link between metabolic conditions such as diabetes and psychiatric disorders has been reported widely.¹ In most cases, metabolic abnormalities have been identified in association with psychiatric disorders as a side effect induced by commonly prescribed anti-psychotic medications.² However, recent studies have shown that such changes can occur at disease onset even before medications have been applied. For example, hyperinsulinemia and insulin resistance have been reported in first onset, antipsychotic naive schizophrenia patients in several studies over the past decade.^{3,4} This indicates that there may be an etiological link between metabolic and psychiatric conditions, which could be an important consideration in downstream treatment approaches.

The *db/db* mouse has been used as a model of type 2 diabetes mellitus and other metabolic conditions, such as obesity and dyslipidemia.⁵ This model was first described in 1965 by Hummel *et al.*,⁶ who identified random mutations in mice associated with obesity and excessive hunger. The models were later segregated as *ob/ob* mice with gene mutations for leptin, and *db/db* mice that have mutations for the gene encoding the long isoform of the leptin receptor, localized mainly in the hypothalamic region of the brain.⁷ The *db/db* mouse model mirrors the clinical picture of type 2 diabetes mellitus.

A recent study found that hippocampal inflammation in *db/db* mice is associated with increased anxiety-like behavior.⁸ It has been reported that patients with diabetes have an increased probability of developing major depressive

disorder⁹ and Alzheimer's disease or cognitive impairments¹⁰ compared with non-diabetic people. It has also been suggested that type 2 diabetes and Alzheimer's disease share similar biochemical and molecular features.¹¹ Consistent with the link between psychiatric and metabolic disorders, recent studies have shown that *db/db* mice exhibit behavioral abnormalities, such as impairments in memory function and long-term potentiation, as well as depression and psychosis-like behaviors.^{12,13} Furthermore, treatment of *db/db* mice with anti-diabetic medications such as rosiglitazone has led to improved insulin signalling along with a decrease in measures of depression but not in psychosis-like behaviors.¹⁴

In this study, we have carried out molecular profiling of plasma samples from *db/db* mice in order to identify peripheral molecular pathways, which are altered in this model. In addition, we have carried out the first proteomic profiling analysis of brain tissues from these mice in order to gain insight into the altered molecular pathways associated with the observed schizophrenia- and depression-like behaviors.

Materials and methods

Animals. Male *db/db* mice (10-week old; background strain C57BL/KsJ (BKS-Cg-Dock7m +/+ Leprdb/J)) and age-matched non-diabetic lean control C57BL/KsJ mice (Jackson Laboratories; Bar Harbor, ME, USA) were housed singly in plastic cages with wooden shavings in a temperature controlled room (22–23 °C) on a 12-h/12-h light/dark

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cycle (lights on at 0500 hours) (Table 1). Single housing of *db/db* mice is routinely practiced in our lab and by most of the labs worldwide. Housing at this temperature range results in no change in diurnal rhythm of *db/db* mice compared with control mice.¹⁵ To assess the metabolic status of the mice, body weight, food consumption and water intake were monitored weekly (Table 2). Blood samples were taken from a cut made on the tip of the tail from each mouse and glucose concentrations were determined using an Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, Indianapolis, IN, USA) to confirm development of diabetes in *db/db* mice compared with that in lean controls. Body fat composition was measured as described previously.¹³ The EchoMRI whole-body composition analyzer (Houston, TX, USA) was used to determine fat and lean body mass.¹⁶ Fat and lean mass were calculated as percentage of total mass. A total of 20 mice (10 *db/db* and 10 lean control mice) were killed according to schedule 1, decapitated and trunk blood was collected in ice-chilled tubes containing EDTA and centrifuged at 1100 *g*, 4 °C for 15 min. Plasma was immediately separated and stored frozen at –80 °C for later use. Frontal cortex and hippocampal regions of the brain were dissected on ice and immediately frozen at –80 °C. All experiments were carried out in full compliance with the principles of laboratory animal care (NIH publication no. 85–23, revised 1985), and the protocols were approved by the Wright State University Animal Care and Use Committee.

Plasma insulin measurements. Plasma insulin levels were determined at Mouse Metabolic Phenotyping Centre (Cincinnati, OH, USA) by using the commercial immunoassay kits (Millipore, St Charles, MO, USA) according to the manufacturer's specifications. Briefly, standards, quality controls and samples were incubated for 2 h in microplate wells pre-coated with mouse anti-rat insulin antibodies (80 µl per well). After washing, biotin-labeled polyclonal antibodies were added (100 µl per well) followed by incubation for 30 min. After washing, horse radish peroxidase conjugate and the substrate 3,3',5,5'-tetramethylbenzidine solution (100 µl per well) were added for a 15-min incubation. The

reactions were halted by addition of 100 µl per well 0.3 M HCl, and the absorbance of the resulting color product was measured at $\lambda = 450$ nm. The concentrations of the experimental samples were determined by comparison of the absorbance values with those of a standard curve, constructed using 0.2, 0.5, 1, 2, 5 and 10 ng ml^{–1} insulin.

Multiplex immunoassay profiling. Plasma samples were analyzed using the RodentMAP, Rat MetabolicMAP and Rat KidneyMAP platforms comprising multiplexed immunoassays for a total of 89 analytes (86 proteins and 3 steroid hormones) (Supplementary Table S1) in a Clinical Laboratory Improved Amendments (CLIA)-certified laboratory at Myriad-RBM (Austin, TX, USA) as described recently.¹⁷ Immunoassays were calibrated using duplicate standard curves for each analyte and raw intensity measurements converted to protein concentrations using proprietary software. Multiplexed calibrators (eight levels per analyte) and controls (three levels per analyte) are developed to monitor key performance parameters, such as lower limit of quantification, precision, cross-reactivity, linearity, spike-recovery, dynamic range, matrix interference, freeze–thaw stability, and short-term sample stability are established for every assay as described by the manufacturer (<http://www.myriadrbm.com/technology/data-quality/>). Data analyses were performed using the statistical software package R (<http://www.r-project.org>) and the levels of analytes were determined. Analyses were conducted under blinded conditions with respect to sample identities, and samples were analyzed in random order to avoid any sequential biases.

Liquid chromatography mass spectrometry (LC-MS^E) profiling. Frontal cortex and hippocampus tissues from *db/db* ($n = 10$) and control ($n = 10$) mice were sonicated in a buffer comprised of 7 M urea, 2 M thiourea, 4% chaps, 2% ASB14, 70 mM dithiothreitol and protease inhibitors (5:1 (v/w) ratio) at a 5:1 (v/w) ratio and mixed for 30 min. Samples were centrifuged for 3 min at 17 000 *g* at 4 °C and the supernatants recovered. Protein concentrations of the lysates were determined using a Bradford assay (Bio-Rad; Hemel Hempstead, UK). Proteins (approximately 100 µg) were precipitated using acetone, centrifuged for 3 min at 17 000 *g* at 4 °C and the pellets suspended in 50 mM ammonium bicarbonate. Disulfide bonds were reduced in 5 mM dithiothreitol at 60 °C for 30 min, and free sulfhydryl groups were alkylated by incubation in 10 mM iodoacetamide in the dark at 37 °C for 30 min. Protein digestion was conducted using porcine trypsin at a 1:50 (w/w) ratio for 17 h at 37 °C, and reactions were stopped by addition of 8.8 M HCl at a 1:60 (w/w) ratio. Quality control samples consisting of frontal cortex and hippocampus protein extracts from a wild-type mouse were prepared as above and underwent all experimental steps in parallel with the test samples to monitor machine performance.

LC-MS^E analyses were carried out in triplicate of individual samples as described previously.¹⁸ Samples were diluted in 0.1% formic acid (Sigma-Aldrich; Poole, UK) to a final peptide concentration of 0.12 µg µl^{–1} and reverse phase LC separation was performed using a nanoACQUITY system (Waters Corporation; Milford, MA, USA) comprised of a C18 trapping

Table 1 Physical and metabolic characteristics of *db/db* and control mice

Parameter	Control	<i>db/db</i>
Age (weeks)	10	10
Body weight (g)	25.2 ± 0.6	36.0 ± 1.2*
Blood glucose (mg dl ^{–1})	140.3 ± 8.8	583.2 ± 35.9*
Plasma insulin (ng ml ^{–1})	1.3 ± 0.2	4.6 ± 1.5*
Percentage of fat mass	17.9 ± 1.0	58.9 ± 0.8*
Percentage of lean mass	70.4 ± 1.0	42.7 ± 1.4*

* $P < 0.05$ (unpaired *t*-test).

Table 2 Food and water consumption of *db/db* and control mice

Mouse strain	N	Food intake (g per day per mouse)	Water intake (g per day per mouse)
Lean control	10	3.85 ± 0.11	9.76 ± 0.89
<i>db/db</i>	10	6.74 ± 0.37*	20.15 ± 2.39*

* $P < 0.05$ versus lean control mice, unpaired *t*-test.

column (180 $\mu\text{m} \times 20\text{ mm}$; 5 μm particle size) and a C18 BEH nanocolumn (75 $\mu\text{m} \times 200\text{ mm}$; 1.7 μm particle size). The buffers were as follows: (A) $\text{H}_2\text{O} + 0.1\%$ formic acid and (B) acetonitrile (Sigma-Aldrich) + 0.1% formic acid. Initial buffer concentrations were 3% B (97% A) followed by 3–30% B over 90 min, 30–90% B over 25 min, 90–97% B over 5 min, constant 97% B for 10 min and 97–3% B over 1 min. The column was coupled online to a nanoESI emitter on a quadrupole time-of-flight (Q-TOF Premier) mass spectrometer (Waters Corporation). Approximately 500 fmol μL^{-1} of Glu Fibrinopeptide B (Waters Corporation) was infused every 30 s using a lock spray for mass calibration. The mass spectrometer was operated in V mode and analyses were performed using positive nanoESI ion mode. The collision energy was 5 eV for low-energy scans and ramped from 17 to 40 eV for high-energy scans with a cycle time of 1.3 s. The low collision energy generates intact precursor ion data, and the high collision energy provides information about the peptide fragments.

Data analysis. Analysis of multiplex immunoassay profiling data was conducted using nonparametric Wilcoxon rank-sum tests to account for any uneven distribution of the data. $P < 0.05$ was considered as statistically significant. LC-MS^E data were processed using ProteinLynx Global Server (PLGS) v.2.4 (Waters Corporation) and Rosetta Elucidator v.3.3 (Rosetta Biosoftware; Seattle, WA, USA) for time and mass/charge alignment of ion fragments as described previously.¹⁸ The *Mus musculus* complete proteome FASTA (sourced from www.uniprot.org) sequence Integr8 database was used for the appropriate protein searches. Acceptable modifications were set to include oxidation of methionine and phosphorylation of serine, threonine or tyrosine residues. The criteria for valid protein identifications were set at a minimum of three fragment ions/peptide ion and seven fragment ions/protein and detection of peptides in at least two out of three replicates. In addition, proteins had to be identified in 60% of samples to ensure biological reproducibility. The results were imported into Elucidator for alignment and annotation of the features. The end result was a matrix that included peptide intensities for each sample. Proteins identified by ≥ 2 peptides were considered for further analyses (Supplementary Table S2).

Statistical analyses. Significant differences in protein expression generated by the LC-MS^E analyses, between *db/db* and control mice, were determined using Wilcoxon signed-rank tests ($P < 0.05$). The false discovery rate was calculated, although no adjustments were made for multiple comparisons in order to not exclude possible true positives. Nevertheless, only those molecules with a false discovery rate < 0.4 and a fold change cut off of 0.10 for the LC-MS^E analyses were applied in order that only those molecules with the most robust changes were considered.

Classification of differentially expressed proteins. The UniProt accession codes of significantly altered proteins found in brain tissues from *db/db* and control mice were uploaded to the Ingenuity Pathway Analysis Knowledge Base (IPKB; Ingenuity Systems; Mountain View, CA, USA).

Networks and pathways that were most significant to the data set were determined by overlaying the uploaded proteins onto predefined pathway maps in the IPKB. A right-tailed Fisher's exact test was used to calculate the *P*-values associated with the identified canonical pathways.

Results

Plasma analysis. Multiplex immunoassay profiling of plasma samples showed that 31 molecules were present at different levels between *db/db* and control mice (Table 3). As expected, leptin levels were significantly higher (approximately 18-fold) in *db/db* compared with the control mice. Several other molecules were also present at higher levels in the *db/db* mice, including insulin, C-reactive protein, serum amyloid P component, immunoglobulin A, interleukin 18, monocyte chemotactic proteins 1 and 3 and fibrinogen. In addition, other proteins showed decreased levels in the

Table 3 Multiplexed immunoassay of plasma from *db/db* and control mice

Molecule	P-value	Ratio	Function
▲Leptin	0.001	18.68	Hormone
▲C-Reactive protein	0.001	2.59	Immune response
▲Serum amyloid P component	0.001	2.07	Immune response
▲Myeloperoxidase	0.001	1.90	Enzyme
▲Plasminogen activator inhibitor 1	0.002	1.82	Proteinase inhibitor
▲Immunoglobulin A	0.001	1.80	Immune response
▲Interleukin-18	0.012	1.64	Immune response
▲Insulin	0.002	1.63	Hormone
▲Monocyte chemotactic protein 1	0.001	1.51	Immune response
▲Monocyte chemotactic protein 3	0.005	1.49	Immune Response
▲Matrix Metalloproteinase 9	0.037	1.33	Enzyme
▲Fibrinogen	0.042	1.23	Immune response
▲Fibroblast growth factor basic	0.032	1.19	Growth factor
▲Serum glutamic oxaloacetic transaminase	0.019	1.19	Enzyme
▼Endothelin-1	0.002	0.79	Vascular homeostasis
▼Peptide YY	0.010	0.79	Hormone
▼Glucagon	0.003	0.79	Hormone
▼Prolactin	0.016	0.76	Hormone
▼Monocyte chemotactic protein 5	0.003	0.73	Immune response
▼Apolipoprotein A1	0.001	0.73	Lipid metabolism
▼Osteopontin	0.004	0.70	Immune response
▼Haptoglobin	0.014	0.70	Immune response
▼Cystatin-C	0.005	0.65	Protein Metabolism
▼Stem cell factor	0.004	0.63	Signal transduction
▼Factor VII	0.001	0.62	Enzyme
▼Macrophage colony stimulating factor 1	0.001	0.59	Cell differentiation
▼Interleukin-10	0.049	0.53	Immune response
▼Epidermal growth factor	0.002	0.51	Growth factor
▼Vascular cellular adhesion molecule 1	0.001	0.48	Signal transduction
▼Beta 2 microglobulin	0.031	0.48	Immune response
▼Lymphotoxin	0.001	0.41	Immune response

The ratio change was calculated as *db/db*/control. Functions were assigned using the Ingenuity Pathway Analysis Knowledge Base software as described in the Research Design and Methods section. Increased and decreased levels of analytes are represented by green and red arrows respectively.

Table 4 LC-MS^E analysis of frontal cortex from *db/db* and control mice

UniProt	Gene	Protein	Function	P-value	Ratio
▲Q88477	<i>IF2B1</i>	Insulin-like growth factor 2 mRNA-binding protein 1	Translation	0.016	1.29
▲P11798	<i>KCC2A</i>	Ca ²⁺ calmodulin dep. kinase type II α chain	Kinase	0.037	1.25
▲Q9Z2Q6	<i>SEPT5</i>	Septin 5	Enzyme	0.035	1.24
▲Q89053	<i>COR1A</i>	Coronin 1A	Other	0.030	1.16
▼Q08709	<i>PRDX6</i>	Peroxiredoxin 6	Enzyme	0.035	-1.10
▼Q8K183	<i>PDXK</i>	Pyridoxal kinase	Kinase	0.019	-1.11
▼P00920	<i>CAH2</i>	Carbonic anhydrase 2	Enzyme	0.038	-1.13
▼P63085	<i>MK01</i>	Mitogen-activated protein kinase 1	Kinase	0.007	-1.14
▼Q02053	<i>UBA1</i>	Ubiquitin-like modifier activating enzyme 1	Enzyme	0.036	-1.14
▼Q61171	<i>PRDX2</i>	Peroxiredoxin 2	Enzyme	0.026	-1.16
▼P55821	<i>STMN2</i>	Stathmin 2	Other	0.043	-1.19
▼Q61207	<i>SAP</i>	Sulfated glycoprotein 1	Other	0.046	-1.14
▼P06837	<i>NEUM</i>	Neuromodulin	Other	0.009	-1.18

Abbreviations: LC-MS^E, liquid chromatography mass spectrometry; UniProt, UniProt accession code.

Cellular locations and molecular functions were assigned using Ingenuity Pathway Analysis Knowledge Base. The ratio was calculated as in the legend for Table 3.

Increased and decreased levels of analytes are represented by green and red arrows respectively.

db/db mice peptide YY, glucagon, prolactin, apolipoprotein A, monocyte chemoattractant protein 5, osteopontin, haptoglobin, interleukin 10, beta 2 microglobulin and lymphotactin.

Frontal cortex analysis. Brain proteome analyses of the *db/db* and control mice were performed using LC-MS^E to determine whether changes occurred as described in some post-mortem studies of neuropsychiatric and neurodegenerative diseases. LC-MS^E analysis of whole frontal cortex extracts led to the identification of 478 proteins. Of these proteins, 13 were found to be significantly altered in *db/db* compared with control mice (Table 4). Four of these proteins were increased and nine decreased. The highest increase was observed for the insulin-like growth factor 2 mRNA binding protein 1 and the calcium calmodulin-dependent protein kinase type II α chain (CAMK2A). The most robust decreases were observed for stathmin 2 and neuromodulin. *In silico* functional analysis using the IPKB software analysis showed that 12 of the 13 altered proteins could be incorporated into a single interaction network (Figure 1). The top disease identified with the uploaded proteins was neurological disorder ($P=7.1\text{E-}5$ – $4.4\text{E-}2$) and Parkinson's disease-related signalling was the top canonical pathway ($P=7.4\text{E-}5$; 2/16 proteins).

Hippocampus analysis. Proteome analysis of the hippocampus from *db/db* and control mice led to identification of 441 proteins. Differential analysis showed that more proteins were altered in this tissue compared with the frontal cortex, with 56 proteins found to be present at significantly different levels between *db/db* and control mice (Table 5). The highest increases were observed for tubulin beta 5 chain and Thy 1 membrane glycoprotein. The largest decreases were found for groups of proteins involved in metabolic processes (malate dehydrogenase mitochondrial, cytochrome b c1 complex subunit 2, malate dehydrogenase cytoplasmic) or intracellular transport (alpha internexin, heat shock 70 kDa

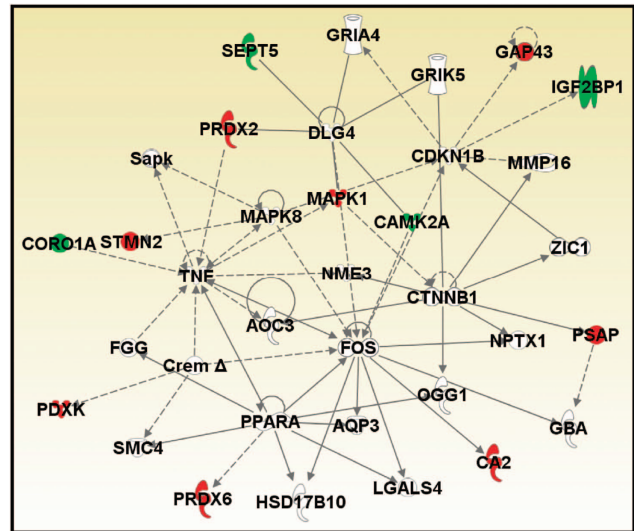


Figure 1 *In silico* functional analysis of proteomic changes in the frontal cortex of *db/db* mice. UniProt accession codes were uploaded into the Ingenuity Pathway Analysis Knowledge Base to determine the most over-represented networks and canonical pathways associated with the data. Straight lines = direct connections; dotted lines = indirect connections; green colour = increased; red colour = decreased.

protein 1L, dihydropyrimidinase related protein 1, ras-related protein rab 39B). IPKB analysis of the altered proteins identified a network comprising 14 of the target proteins (Figure 2). Most of the altered proteins were involved in cellular signalling, cell structure and maintenance, neural functioning and energy metabolism. Three members of the 14-3-3 protein family, conserved regulatory molecules expressed in all eukaryotes, were among the group of altered proteins. Furthermore, several molecules linked to regulation of calcium signalling were also altered. The top disease associated with the altered hippocampal proteins was hematological disease ($P=8.4\text{E-}5$ – $1.7\text{E-}2$) and the top canonical pathway was clathrin-mediated endocytosis signalling ($P=3.0\text{E-}6$; 7/181 proteins).

Discussion

This is the first proteomic profiling study investigating plasma and brain tissues from the *db/db* mouse model. The advantage of using the multiplex profiling approach is the potential to identify differences in hundreds of molecules simultaneously, which can lead to the identification of changes in functional pathways. Profiling of plasma from *db/db* mice showed the expected robust increase in leptin levels, along with significantly higher levels of insulin compared with the levels in control mice. In line with these findings, we also found decreased levels of other hormones such as peptide YY, glucagon and prolactin, which suggested that lack of leptin receptors in the *db/db* mice can lead to either altered biosynthesis or secretion of other major hormones.

The use of the multiplex immunoassay profiling analysis resulted in identification of several novel serum molecules, which have not been described previously in association with this model. The finding of lower peptide YY levels is consistent

Table 5 LC-MS^E analysis of hippocampus from *db/db* and control mice

UniProt	Gene	Protein	Function	P-value	Ratio
▲P99024	TBB5	Tubulin beta 5 chain	Other	0.031	1.31
▲P01831	THY1	Thy 1 membrane glycoprotein	Other	0.032	1.29
▲P51881	ADT2	ADP ATP translocase 2	Transporter	0.007	1.26
▲P68033	ACTC	Actin alpha cardiac muscle 1	Enzyme	0.046	1.26
▲Q9R0K7	AT2B2	Plasma membrane calcium transporting ATPase 2	Transporter	0.005	1.25
▲P11499	HS90B	Heat-shock protein HSP 90	Other	0.006	1.21
▲Q80W21	GSTM7	Glutathione S transferase Mu 7	Enzyme	0.041	1.18
▲Q9D0J8	PTMS	Parathymosin	Other	0.030	1.15
▲Q6GSS7	H2A2A	Histone H2A type 2 A	Other	0.031	1.14
▲P02088	HBB1	Hemoglobin subunit beta 1	Transporter	0.002	1.14
▲P68254	1433T	14 3 3 protein theta	Other	0.002	1.14
▲Q9D6F9	TBB4A	Tubulin beta 4 chain	Other	0.019	1.13
▲Q99LX0	PARK	Protein DJ 1	Enzyme	0.026	1.12
▲P20029	GRP78	78 kDa glucose-regulated protein	Other	0.002	1.11
▲P00920	CAH2	Carbonic anhydrase 2	Enzyme	0.030	1.10
▲Q6PHZ2	KCC2D	Ca2 + calmodulin dep protein kinase type II Δ chain	Kinase	0.019	1.10
▲P63268	ACTH	Actin gamma enteric smooth muscle	Other	0.026	1.10
▲P17183	ENOG	Gamma enolase	Enzyme	0.040	1.10
▼O35660	GSTM6	Glutathione S transferase Mu 6	Enzyme	0.001	– 1.10
▼P70696	H2B1A	Histone H2B type 1 A	Other	0.042	– 1.10
▼Q61990	PCBP2	Poly rC binding protein 2	Other	0.007	– 1.10
▼P15532	NDKA	Nucleoside diphosphate kinase A	Kinase	0.030	– 1.11
▼P28184	MT3	Metallothionein 3	Other	0.038	– 1.12
▼Q9D1G1	RAB1B	Ras related protein Rab 1B	Enzyme	0.001	– 1.12
▼P09041	PGK2	Phosphoglycerate kinase 2	Kinase	0.026	– 1.13
▼O70456	1433S	14 3 3 protein sigma	Other	0.024	– 1.13
▼P21550	ENOB	Beta enolase	Enzyme	0.009	– 1.13
▼Q62420	SH3G2	Endophilin A1	Enzyme	0.045	– 1.13
▼P08553	NFM	Neurofilament medium polypeptide	Other	0.012	– 1.14
▼P62827	RAN	GTP-binding nuclear protein Ran	Enzyme	0.019	– 1.14
▼Q63810	CANB1	Calcineurin subunit B type 1	Phosphatase	0.035	– 1.15
▼Q9WV27	AT1A4	Na + /K + transporting ATPase subunit alpha 4	Transporter	0.035	– 1.17
▼P06837	NEUM	Neuromodulin	Other	0.012	– 1.18
▼Q61598	GDIB	Rab GDP dissociation inhibitor beta	Other	0.012	– 1.18
▼Q9CQV8	1433B	14 3 3 protein alpha	Other	0.019	– 1.18
▼P31001	DESM	Desmin	Other	0.019	– 1.19
▼P31786	ACBP	Acyl CoA-binding protein	Other	0.046	– 1.19
▼P26443	DHE3	Glutamate dehydrogenase 1 mitochondrial	Enzyme	0.023	– 1.19
▼P28652	KCC2B	Ca2 + calmodulin dep protein kinase type II β chain	Kinase	0.046	– 1.20
▼Q9R0P9	UCHL1	Ubiquitin carboxyl terminal hydrolase isozyme L1	Peptidase	0.017	– 1.21
▼P05202	AATM	Aspartate aminotransferase mitochondrial	Enzyme	0.001	– 1.21
▼P63328	PP2BA	Ser/Thr protein phosphatase 2B catalytic subunit α	Phosphatase	0.004	– 1.22
▼Q01768	NDKB	Nucleoside diphosphate kinase B	Kinase	0.047	– 1.22
▼O35737	HNRH1	Heterogeneous nuclear ribonucleoprotein H	Other	0.014	– 1.23
▼P50516	VATA	V-type proton ATPase catalytic subunit A	Transporter	0.030	– 1.23
▼Q8BHC1	RB39B	Ras related protein Rab 39B	Enzyme	0.004	– 1.24
▼P09671	SODM	Superoxide dismutase Mn mitochondrial	Enzyme	0.017	– 1.26
▼O08709	PRDX6	Peroxiredoxin 6	Enzyme	0.035	– 1.28
▼P02104	HBE	Hemoglobin subunit epsilon Y2	Transporter	0.030	– 1.29
▼P61750	ARF4	ADP ribosylation factor 4	Enzyme	0.012	– 1.30
▼P46660	AINX	Alpha internexin	Other	0.004	– 1.32
▼P16627	HS71L	Heat shock 70 kDa protein 1 l	Other	0.023	– 1.38
▼P97427	DPYL1	Dihydropyrimidinase related protein 1	Enzyme	0.009	– 1.45
▼P08249	MDHM	Malate dehydrogenase mitochondrial	Enzyme	0.005	– 1.45
▼Q9DB77	QCR2	Cytochrome b c1 complex subunit 2 mitochondrial	Enzyme	0.030	– 1.47
▼P14152	MDHC	Malate dehydrogenase cytoplasmic	Enzyme	0.003	– 1.47

Abbreviations: LC-MS^E, liquid chromatography mass spectrometry; UniProt, UniProt accession code. Cellular locations and molecular functions were assigned using Ingenuity Pathway Analysis Knowledge Base. The ratio was calculated as in the legend for Table 3. Increased and decreased levels of analytes are represented by green and red arrows respectively.

with the increased appetite and body weight of the *db/db* mice and with previous studies which have shown that obese humans secrete lower levels of this hormone.¹⁹ Lower levels of peptide YY have also been found in drug-free schizophrenia patients in a biomarker study of cerebrospinal fluid, suggesting another link between metabolic regulation and psychiatric disorders.²⁰ The finding of decreased circulating prolactin levels in *db/db* mice has also not been reported previously. Prolactin is secreted by the anterior pituitary and this process can be

inhibited by release of dopamine from the arcuate nucleus of the hypothalamus.²¹ This is interesting as one of the major theories of how schizophrenia develops involves increased dopaminergic activity in the brain.²² Therefore, further studies are warranted to determine whether hypothalamic–pituitary dopamine signalling is altered in this model.

In addition to the effects on hormonal pathways, we also identified alterations in immune and inflammation-related molecules in plasma from *db/db* mice. Several studies

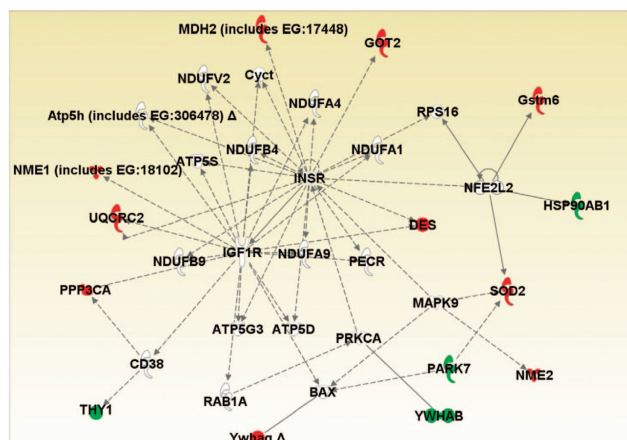


Figure 2 *In silico* functional analysis of protein changes in the hippocampus of *db/db* mice. UniProt accession codes were uploaded into the Ingenuity Pathway Analysis Knowledge Base to determine the most over-represented networks and canonical pathways associated with the data. Straight lines = direct connections; dotted lines = indirect connections; green colour = increased; red colour = decreased.

have shown that immune system perturbations occur in type 2 diabetes mellitus,²³ as well as in psychiatric and neurodegenerative disorders, including schizophrenia,¹⁷ depression²⁴ and Alzheimer's disease.²⁵ However, the overlap of immune-related molecules across these conditions with the *db/db* mice model is not surprising given that these pathways are affected in a variety of diseases. In the case of the *db/db* mouse, the observed perturbations in immune system molecules could be due to the high circulating levels of leptin, as this hormone can also act as pro-inflammatory adipokine.²⁶ Leptin has been tested as a possible treatment for autoimmune and/or inflammatory diseases in a recent study²⁷ and preclinical studies have shown that the depression-like symptoms in the chronic stress and social defeat rat models was associated with lower serum leptin concentrations, and the behavioral effects could be reversed by systemic leptin administration.²⁸

Comparison of circulating molecules changing in the *db/db* mouse model to those identified in our previous study of first onset schizophrenia patients shows that 12 proteins were altered in common and 6 of these showed the same directional changes (insulin, serum amyloid P, interleukin 18, serum glutamic-oxaloacetic transaminase, apolipoprotein A1 and epidermal growth factor).¹⁷ In addition, seven circulating proteins (insulin, serum glutamic-oxaloacetic transaminase, prolactin, cystatin C, stem cell factor, macrophage colony-stimulating factor and beta-2 microglobulin) were changed in common between the *db/db* mouse and the acute PCP rat model of schizophrenia, using the same molecular profiling platform.²⁹ Taken together, these findings suggest that the *db/db* model shows an overlap of molecular profiles with schizophrenia and other psychiatric or neurodegenerative disorders.

The most novel findings of the current study involved the identification of changes in brain proteins as these have not been described previously in association with this model. Consistent with the effects on behavior seen in the *db/db*

mice,^{13,14} we identified multiple proteins that were altered in brain tissues extracted from *db/db* mice compared with control mice. The finding that more proteins were altered in the hippocampus of the *db/db* mouse is consistent with the reported effects on cognition and learning in this model. In line with this, we found changes in three members of the 14-3-3 protein family in *db/db* mouse hippocampi. These proteins are highly conserved and have an important role in neurodevelopment due to their involvement in cellular processes, such as cell signalling, growth and apoptosis.³⁰ Also, perturbations in these proteins have been found in schizophrenia, autism, bipolar disorder and in subjects with impaired sensory motor gating and memory.^{30,31} The latter finding is consistent with the impairments seen in paired-pulse inhibition in the *db/db* mice. Furthermore, our findings of altered expression of Ca^{2+} -regulatory proteins, such as CAMK2B, CAMK2D and calcineurin subunit B type 1, are in line with studies that identified impaired cardiac function in *db/db* mice through suppression of Ca^{2+} permeability.³² This also supports the possibility that the defect found in the *db/db* mice is systemic, with effects seen in both the brain and periphery.

Fewer proteomic changes were found in the frontal cortex region of the *db/db* mice using the LC-MS^E proteomic profiling approach. However, *in silico* analysis of the 13 significantly altered proteins resulted in identification of 'neurological disorder' as the top associated disease and 'Parkinson's signalling' as the top canonical pathway. Again, this indicated potential effects on dopaminergic pathways as seen most notably in Parkinson's disease and schizophrenia. In addition, one of the brain proteins found to be altered in both the *db/db* and the acute PCP rodent model described above,²⁹ was protein DJ-1 (also known as PARK7). DJ-1 is an oxidative stress response protein that defends cells against reactive oxygen species. Furthermore, CAMK2A was found to be changing in the *db/db* mice as found in our previous study of the acute PCP model of schizophrenia.²⁹ Previous studies have also found perturbed expression of these proteins in post-mortem frontal cortex tissues from schizophrenia patients^{33,34} as well as in disorders associated with effects on learning and memory.^{35–37}

One limitation of this study was the small numbers of animals used to generate the data. However, we have attempted to compensate for this through comparison of the findings with those in the literature on the same mouse model, the acute PCP model, drug-naïve schizophrenia subjects and on protein pathways altered in schizophrenia post-mortem brain tissues. Nevertheless, these studies require repetition in independent cohorts of *db/db* mice, and future studies should attempt to further explore the specificity of the effects through comparison with other animal models and other human psychiatric and neurodegenerative diseases. Also, the *db/db* mouse has been mainly used in type 2 diabetes research, and it should be noted that the alterations in central nervous system could result from the change of leptin receptor signalling in the brain or through complications of diabetes itself.

In conclusion, this study resulted in the identification of novel central nervous system proteomic alterations that link the metabolic abnormalities found in the *db/db* mouse model to neurodegenerative and neuropsychiatric diseases.

This has laid the ground work for further studies of the *db/db* mouse model in testing whether treatment with drugs such as antidepressants, antipsychotics or anti-diabetic compounds has a positive effect on the behaviors and brain proteome changes observed. Sharma *et al.*¹⁴ have already carried out a study which showed that treatment of *db/db* mice with rosiglitazone could affect depression measures, but this had no effect on readouts for psychosis-like behaviours. Taken together with previous investigations on the behavioural effects in this model, the current study supports the use of the *db/db* mouse as a potential preclinical model for schizophrenia and potentially other psychiatric or neurodegenerative disorders. Also, the combined use of this model with incorporation of high throughput assays for the plasma and brain molecular candidates identified here would potentially offer a useful novel tool for drug discovery, with a focus on the metabolic aspects of mental disorders and/or the psychopathology associated with diabetes.

Conflict of interest

SB, HR and PCG are consultants for Myriad-RBM, although this does not alter adherence to Translational Psychiatry policies on sharing data and materials. The other authors declare no conflict of interest.

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