RESEARCH ARTICLE

Developmental vitamin D deficiency alters brain protein expression in the adult rat: Implications for neuropsychiatric disorders

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An increased risk for multiple sclerosis and schizophrenia is observed at increasing latitude and in patients born in winter or spring. To explore a possible link between maternal vitamin D deficiency and these brain disorders, we examined the impact of prenatal hypovitaminosis D on protein expression in the adult rat brain. Vitamin D-deficient female rats were mated with vitamin D normal males. Pregnant females were kept vitamin D-deficient until birth whereupon they were returned to a control diet. At week 10, protein expression in the progeny's prefrontal cortex and hippocampus was compared with control animals using silver staining 2-D gels associated with MS and newly devised data mining software. Developmental vitamin D (DVD) deficiency caused a dysregulation of 36 brain proteins involved in several biological pathways including oxidative phosphorylation, redox balance, cytoskeleton maintenance, calcium homeostasis, chaperoning, PTMs, synaptic plasticity and neurotransmission. A computational analysis of these data revealed that (i) nearly half of the molecules dysregulated in our animal model have also been shown to be misexpressed in either schizophrenia and/or multiple sclerosis and (ii) an impaired synaptic network may be a consequence of mitochondrial dysfunction.

Keywords:
Mitochondria / Multiple sclerosis / Schizophrenia / Synapse / Vitamin D

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Abbreviations: DVD, developmental vitamin D; **NGF,** nerve growth factor; **VDR,** vitamin D receptor

1 Introduction

Vitamin D, the seco-steroid hormone (1,25-dihydroxyvitamin D_3, calcitriol), is associated with a wide range of physiological functions (for recent reviews, see [1–4]). Vitamin D acts *via* a nuclear receptor (the vitamin D receptor,

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VDR), the smallest member of the phylogenetically conserved superfamily of nuclear receptors, and induces transcription of a large number of genes [5] affecting calcium/phosphorus homeostasis. It also influences key cellular functions such as cell proliferation, differentiation and survival of various cell types in almost every tissue, including the nervous system [6, 7].

There is accumulating evidence that vitamin D deficiency may contribute to the risk of developing a wide range of common chronic diseases. Vitamin D deficiency is associated with increased risks of cancer (colon, breast and prostate), chronic inflammatory disease, autoimmune diseases (including multiple sclerosis, type I diabetes, inflammatory bowel disease) and metabolic disorders (metabolic syndrome, hypertension) in addition to the increased risks of skeletal disorders such as osteoporosis [8]. Vitamin D deficiency confined to prenatal or early life development may also increase the risk of schizophrenia [9–11] and multiple sclerosis [12–14]. The incidence of vitamin D deficiency is more prominent at higher latitudes [15]. Indeed a strong correlation between increasing latitude and increased risk for MS [16] and to a lesser extent schizophrenia [17] exists. Collectively these observations suggest vitamin D deficiency may affect many organ systems, including the brain. Indeed, receptors for vitamin D are distributed widely in the nervous system [18, 19] and the VDR and 1 α-hydroxylase (the key enzyme for the synthesis of the active moiety) are present in human brain [20].

Our laboratory is exploring the consequences of prenatal and early life vitamin D deficiency on the adult brain structure and function. Clearly, prenatal vitamin D deficiency has significant effects on the brain at birth [21]. The brain was shown to be longer, lateral ventricles were enlarged, cortical thickness was reduced, cell proliferation was increased, expression of certain growth factors was reduced (nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF)) and the expression of the low-affinity neurotrophin receptor ($p75^{NTR}$) was also reduced [21]. When offspring of vitamin D-deficient mothers were restored to a normal diet at birth and examined in adulthood, their brain still had larger lateral ventricles and reduced NGF protein and altered expression of genes involved in cytoskeleton maintenance (MAP2, NF-L) and neurotransmission (GABA-Aα4) [22]. The behaviour of these developmental vitamin D (DVD) deficient adult rats was also altered, compared to controls, showing increased locomotion in response to novelty [23, 24] and disrupted latent inhibition of learning [25]. The aim of the present study was to further explore the molecular alterations in the adult brain after DVD deficiency using proteomic and computational analyses. This systems approach provides evidence that prenatal vitamin D deficiency results in altered mitochondrial, cytoskeletal and neuronal function in the adult brain.

2 Materials and methods

2.1 Animals, diet, housing

Female Sprague-Dawley rats (Herston Medical Research Centre, Queensland, Australia) were housed as previously described [21, 22]. All procedures were performed with approval from the Griffith University and University of Queensland Animal Ethics Committees, under the guidelines of the National Health and Medical Research Council (NHMRC) of Australia.

Vitamin D-depleted female rats were supplied with a diet deficient in vitamin D (Dyets, CA, USA) and lighting (200–500 Lux) was provided by incandescent bulbs that do not emit UV radiation in the vitamin D_3 action spectrum (290–315 nm). Control females were housed under a UVB-emitting light cycle and were fed a normal diet. After 6 wk, serum vitamin D_3 depletion was confirmed prior to mating using a commercial RIA (Diasorin, MN, USA) for calcidiol (25-OH D_3). Animals were mated at 10 wk of age and successful mating was established *via* the presence of a vaginal sperm plug. Dams were kept under their respective premating diet until birth.

At birth, depleted dams were given a normal vitamin D containing diet (control diet) and housed under UVB-emitting light. Offspring were weaned at 3 wk of age and housed in same sex groups of 3. At 10 wk of age, the offspring were killed by an overdose of pentobarbital (Nembutal, IL, USA) and brains rapidly obtained. Brains were cut into 1 mm coronal sections on ice and from these the entire hippocampus (Bregma −1.72, −7.92 mm) and frontal cortex (Bregma 5.64, 2.76 mm) were dissected according to the criteria of Paxinos and Watson [26]. Serum concentrations of calcium, measured at birth and death with an AutoAnalyser (Hitachi Instruments, Tokyo, Japan), were shown to be normal.

2.2 Protein profiling

2.2.1 2-DE

In this study, we focused on two brain regions of particular interest to schizophrenia and multiple sclerosis (*i.e.* frontal cortex, hippocampus). Tissue was sampled from four control and six DVD-deficient female adult offspring. These animals were all from separate litters. Frontal cortices were sampled from all animals and hippocampi were available from only six of these animals (two controls and four DVD deficient). Resected tissue was homogenised in a lysis buffer (7 M urea/2 M thiourea, Sigma, St. Louis, MO, USA), 50 mM *N*-octyl glucoside (Sigma), antiprotease cocktail (Sigma), 1% w/v DTT (Sigma) at a final concentration of 10 mg tissue/mL and was stored frozen at −80°C. An aliquot (10 μL) of each sample was used to determine protein concentration. All aliquots were diluted in water (90 μL) before protein quantitation to minimise detergent interferences. Protein quantitation was measured using BioRad Lowry-based DC assay (BioRad,

Hercules, CA, USA) according to the manufacturer's instructions. Prior to the first-dimension of electrophoresis, 17 cm linear IPG strips pH 3–10 (Immobilines, BioRad) were rehydrated overnight with 150 μg of brain protein *per* IPG strip added to the reswelling solution (9 M urea, 1% w/v DTT, Triton 0.5X (Sigma) and 1% v/v pharmalytes pH 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden)). Samples were isoelectrically focused for a total of 80 kV using the MultiPhor II (Amersham Pharmacia Biotech) and then stored at −70°C until further use.

Following IEF, strips were equilibrated with 6 mL of a solution containing Tris-HCl (50 mM, pH 8.8, Sigma), urea (6 M), SDS (2% w/v, Sigma), glycerol (30% w/v, Sigma), a trace of bromophenol blue (Sigma) and DTT (1% w/v) for 15 min, followed by equilibration for 15 min in the same buffer containing iodoacetamide (2.5% w/v; BioRad) instead of DTT. Equilibrated IPGs were transferred to 9–16% polyacrylamide gradient gels as described previously [27]. Electrophoresis was performed for 6–7 h in a BioRad Protean II xi chamber (BioRad) with current limited to 40 mA *per* gel. A standard protein marker (LMW, Amersham Pharmacia Biotech), covering range of 14.4–97 kDa, was loaded at the right side of gels.

2.2.2 Silver staining

Gels were fixed overnight in a 30% v/v ethanol, 10% v/v acetic acid solution. After three washes of 20 min, in 30% v/v ethanol solution, gels were sensitised for 1 min in 0.2% w/v sodium thiosulphate solution (Sigma) and washed 3 × 30 s in water. The gels were then soaked in 0.2% w/v silver nitrate (Sigma), 0.02% v/v formaldehyde (37% *per* litre, Sigma) for 20 min. The gels were rinsed for 3 × 30 s in water before the development step. Gels were incubated for 3–10 min in 3% w/v sodium carbonate (Sigma), 0.05% v/v formaldehyde (37% *per* litre) and 0.0005% w/v thiosulphate sodium. After two washes in water for 2 × 30 s, the reaction was stopped by placing gels in 0.5% w/v glycine (Sigma) for 30 min. The gels were rinsed twice for 30 min and stored in water until image acquisition.

2.2.3 2-D gel image analysis

Silver-stained gels were digitised using a high resolution scanner (Molecular Dynamics Personal Densitometer, Amersham Pharmacia Biotech) and analysed using the PDQuest 7.2.0 software (BioRad). Standard criteria of this software were used to automatically locate spots, subtract levels of background intensity and match detected spots among all gels. Automatically matched spots were confirmed and edited manually. Protein spots were normalised using the parameter 'total of all valid spots' provided by the software. Finally, the intensity of the spots on the gels was compared between the two groups. Differences in spot quantity between experimental and control groups were analysed using *t*-test provided by the PDQuest software. All results were shown as mean ± SD. Differentially expressed protein spots were excised and stored at −70°C for identification by MS.

2.2.4 In-gel digestion and MALDI-TOF-MS analysis

Excised plugs from silver-stained gels were prepared as described previously by Gharahdaghi *et al*. [28] and Shevchenko *et al*. [29]. Proteins were digested overnight at 37°C by sequencing-grade trypsin (12.5 μg/mL; Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The resulting peptides were extracted with 10 μL 50% ACN/0.1% TFA (Sigma), lyophilised, resuspended in 10 μL 0.1% TFA and desalted on ZipTip C18-microcolumns (Millipore, Bedford, USA). Elution was performed with CHCA (3 mg/mL) in 50% v/v ACN directly onto the MALDI target. An Ettan MALDI-TOF/Pro mass spectrometer (Amersham Biosciences) was used to obtain PMF. Spectra were acquired in the delayed extraction (DE), reflectron R mode. To produce final spectra, 100–300 scans were averaged. Spectra were calibrated using the monoisotopic MH^+ ion from three peptide standards (trypsin autodigestion products: 842.510, 1045.564 and 2211.1046 Da). PMF database searching was carried out using Profound (http://prowl.rockefeller.edu/), Peptident (www.expasy.org) and MASCOT (http://www.matrixscience.com/) software available online.

The identity of proteins was annotated using the Swiss-Prot and TrEMBL database. The identification was based on five or more matches. For two low molecular weight proteins (spots P10818 and P02401), broken into a small number of peptides, the identification was based on three or four matching peptides. MS identification was repeated three times using spots from three different gels.

2.2.5 Western blot

Frontal cortex samples ($n = 4$ *per* group) were prepared as previously described [30]. Ten micrograms of protein lysate were loaded in each well and separated using 12% SDS-PAGE in a Mini PROTEAN cell (BioRad). Proteins were transferred to an NC membrane (0.45-μm, Amersham Pharmacia, Saclay, France) by semidry blotting (0.8 mA/cm^2) and saturated with 5% nonfat dried milk. Western blot was performed using primary antibodies diluted in PBS containing 0.2% v/v Tween-20 with 5% nonfat dried milk and a HRP-conjugated secondary antibody. Mouse anti-GAPDH (Chemicon), rabbit anti-GAP-43 (Chemicon) and mouse anti-drebrin (MBL) antibodies were used at the dilutions 1/5000, 1/2000 and 1/1000, respectively. Anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibodies were diluted at 1/5000. Blots were visualised with a commercially available ECL kit (Roche, Basel, Switzerland). Nonsaturated autoradiographs were digitised using a high resolution scanner (Molecular Dynamics Personal Densitometer, Amersham Pharmacia Biotech) and analysed using Syngene software. Quantitation of differences in protein expression between

control and experimental groups was performed using GAPDH as an internal calibrator and staining proteins of interest and the calibrator on the same blot. Western blots for each protein of interest were performed at least in duplicate and, when two independent assays produced identical results, bands were quantified. Only one assay for each antibody is shown in Section 3.

2.3 Protein data mining

As the primary analysis, biological interpretation of pooled and area-linked data from the 2-DE was performed using a Java/Perl application, Predictsearch™, developed by GenSodi (www.gensodi.com). Briefly, protein aliases corresponding to the selected proteins were first retrieved from public database source (http://bioinfo.weizmann.ac.il/cards/index.shtml) and were submitted as queries to PubMed in order to collect titles and abstracts of all related publications. Trivial terms (mainly terms deprived of biological significance) were discarded and the remaining words were stored in a database that was later used as a library in which co-occurrences of every pair of proteins could be extracted. The retrieved document set was then filtered by the user with specific terms. From this library, a matrix was established based on joint and individual occurrence statistics for every pair of proteins. The nodes, edges and edge values were passed to a Java applet which was executed in an HTML document containing a table that links pairs of proteins to those MedLine documents that generated the edge between the pair. A 2-D graph was generated from this matrix, with node and edge inclusion being determined by a user-defined threshold. The generated graphs allowed us to visualise co-occurrences between distinct proteins within abstracts as well as co-occurrences between proteins and specific words.

3 Results

3.1 DVD deficiency induces a long term dysregulation of proteins involved in mitochondrial functioning, cytoskeleton maintenance and neurotransmission

In order to analyse differences in brain protein expression between control and experimental groups, a comparative proteomic study was performed. We examined protein expression in two brain areas – the frontal cortex and the hippocampus. 643 and 681 spots were analysed from the hippocampal and cortical areas, respectively. For each spot, the mean value in each group was calculated and proteins whose expression was found to be significantly altered (*t*-test, $p<0.05$) were registered on a candidate list.

A representative 2-D gel is shown in Fig. 1. Within the frontal cortex samples, the expression of 22 spots was significantly changed in the experimental group ($n = 6$) when compared to the control group ($n = 4$). These 22 spots, listed in Table 1, correspond to 17 distinct proteins. Within the hippocampus samples, the intensity of 28 spots was significantly altered in the experimental group ($n = 4$) when compared to the control group ($n = 2$). These 28 spots, listed in Table 1, correspond to 23 distinct proteins.

As shown in Table 1, some dysregulated proteins within each area were found in duplicate or triplicate on 2-D gels. These different spots represent isoforms of the same protein and may correspond to various post-translational states of the same protein. Therefore, the numbers of misexpressed proteins in the frontal cortex and the hippocampus were 17 and 23, respectively. Four of them (Hs7C, Tbb1, EnoG, ATPb) were found in both areas, making a combined total of 36 misexpressed proteins in the two areas under scrutiny. The vast majority (89%, 32/36) of the abnormally expressed proteins were down-regulated.

A quick and restricted validation of our results was performed using Western blotting. We targeted two proteins of interest – Drebrin and Gap43 – with readily available antibodies. Figure 2 shows a clear down-regulation of Drebrin (34 ± 6.87 *vs.* 78.35 ± 3.46, $p<0.05$) and Gap43 (34.9 ± 14.93 *vs.* 111.17 ± 2.95, $p<0.005$).

The 36 dysregulated proteins were analysed using Predictsearch software. The main disrupted functions were: oxidative phosphorylation, redox balance, cytoskeleton maintenance, synaptic plasticity, calcium homeostasis, chaperoning, PTM and neurotransmission (Table 1). Seven of these eight functions are disturbed in both frontal cortex and hippocampus. However, dissimilarities between these two brain areas were noted. Expression of astrocyte-specific metabolites (Gfap, Pea15), calcium-binding proteins (Calm, Vis1) and thioredoxin-related molecules were specifically altered in the hippocampus while production of actin-associated proteins (Dreb, RhoA) or synaptic vesicle-attached molecules (Syn2) was distinctively modified in the frontal cortex.

As shown in Fig. 3, DVD deficiency primarily affected proteins from mitochondria, cytoskeleton and synapses. More than two thirds (78%, 28/36) of the affected proteins are related to at least one of these three subcellular compartments. Within this pool, 14 proteins are linked to all three subcellular compartments. Seven proteins are associated to two of these three compartments. Finally, five and two proteins are specifically related to mitochondria and cytoskeleton, respectively.

3.2 Pathway analysis and systems biology

The list of dysregulated proteins generated by our experiments was analysed using Predictsearch library. More than 5000 articles with at least one co-occurrence were retrieved as described in Section 2. Careful reading and exclusion of irrelevant articles led to the selection of about 500 papers. This restricted library was then used to draw metabolic pathways and correlate protein dysregulation with brain pathologies.

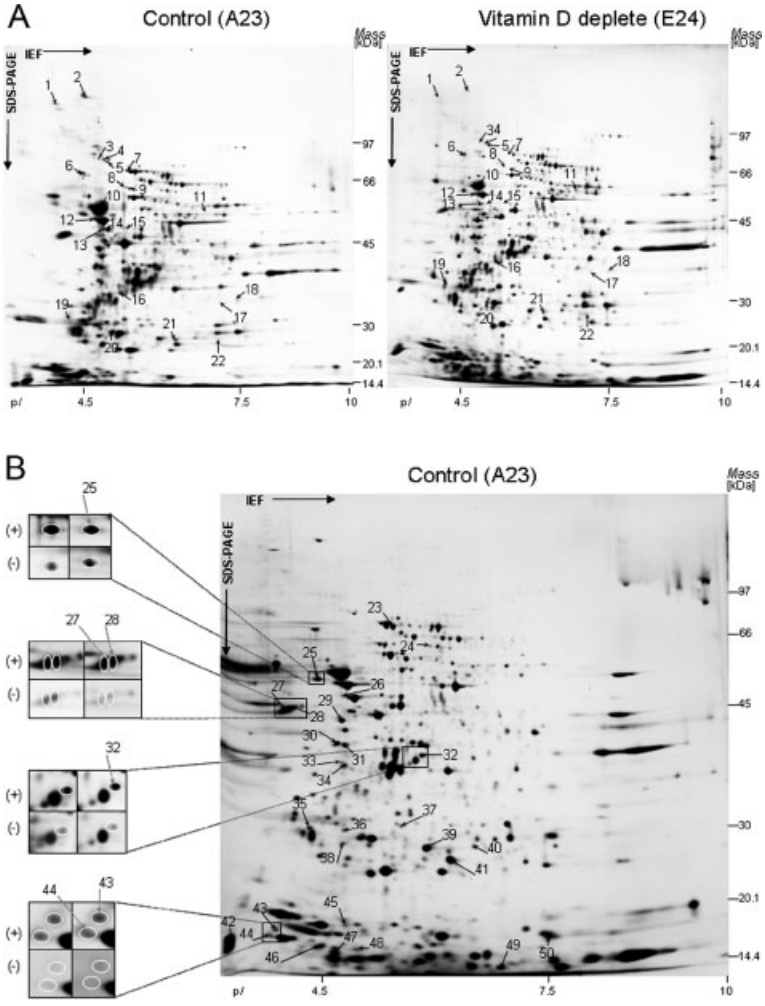

Figure 1. Representative silver-stained 2-D gels from control (left) and DVD-deficient (right) frontal cortex lysates (A) and an example of a hippocampal lysate (B). Note differential spot intensity of four regions, (+) experimental *vs.* (−), control in hippocampal lysates. Altered spot protein expression, analysed with PDQuest software, are annotated on the gels. The 22 annotated spots in the cortex gels and 28 in the hippocampus gels were identified by PMF and the corresponding proteins are reported in Table 1.

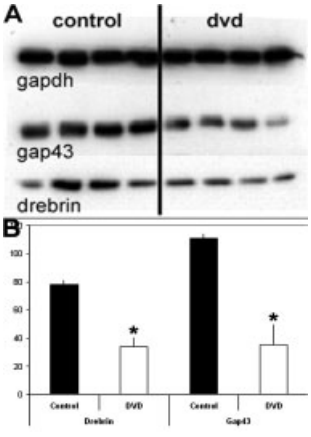

Figure 2. Quantification of protein dysregulation using Western blotting. (A) Four frontal cortex protein samples from each group (control *vs.* DVD) were run on gel and stained with antibodies raised against two proteins of interest (Drebrin and Gap43). (B) Gel spots were quantified and normalised using Gapdh as a housekeeping protein. The fold change between groups is 2.3 for Drebrin and 3 for Gap43 (*, $p<0.05$).

Figure 4 indicates the main interactions between dysregulated proteins. Following DVD deficiency, four molecules involved in neurotransmission were disrupted, three involved in synaptic plasticity were disrupted and eight involved in PTMs were disrupted (Fig. 4A).

Figure 4B shows that six identified proteins are closely associated with the main mitochondrial functions. The DVD deficiency group exhibited misexpression of (i) three components of the oxidative phosphorylation system (Cox6a1, Etfa, ATPase); (ii) two agents involved in the maintenance of cellular redox balance (Pdx5, Pdx6); (iii) one membrane channel playing a role in calcium homeostasis and ATP transport (Vdac2). In addition to the mitochondrial compounds, the cell redox homeostasis is disturbed as well by six non-mitochondrial misexpressed molecules involved in protein chaperoning and proteasome activity (Grp78, Hs7c, Hs70p5, Uchl1, Psa2, Psa5). Furthermore, expression of four molecules implicated in organelle transport along the cystokeletal filaments (Tpm1, 143G, RhoA, Dreb) is disrupted (Fig. 4).

Using Predictsearch, we searched for molecules dysregulated in our animal model that have also been shown to be

Table 1. Altered proteins in two brain areas from DVD-deficient rats

Spot number	Accession number (Swiss-Prot/ TrEMBL)	Abbr. name	Protein name	Theor. M_r/p*I*	Observ. M_r/p*I*	MALDI-MS			Intensity (mean ± SD)		Fold change
						Peptides		Sequence coverage (%)			
						Matching	Total		Control	Depleted	
Frontal cortex											
Oxidative phosphorylation											
12	P10719***	ATPB	ATP synthase β-chain, mitochondrial (precursor)	51.71/4.9	52/4.6	14	35	39	2072 ± 450	793 ± 347	−2.44
17	P13803***	ETFA	Electron transfer flavoprotein α-subunit, mitochondrial (precursor)	34.98/8.7	35/7.2	6	30	15	311 ± 134	805 ± 151	+2.59
Cytoskeleton maintenance											
2	P12839***	NEF3	Neurofilament triplet M protein (160 kDa neurofilament protein) (Neurofilament medium polypeptide) (NF-M)	95.66/4.8	117/4.4	8	23	15	2179 ± 335	400 ± 209	−5.56
6	P19527***	NFL	Neurofilament triplet L protein	61.20/4.6	71/4.4	15	26	29	2352 ± 538	871 ± 313	−2.70
8	P23565**	NF66	α-internexin	56.11/5.2	65/5.3	17	23	33	679 ± 174	320 ± 183	−2.13
9	P23565**	NF66	α-internexin	56.11/5.2	65/5.3	15	43	31	958 ± 236	343 ± 250	−2.78
10	P04691**	TBB1	Tubulin β-chain	49.96/4.8	55/4.6	9	14	27	40 140 ± 9614	18 979 ± 6289	−2.13
15	P04691*	TBB1	Tubulin β-chain	49.96/4.8	50/5.3	12	49	26	851 ± 257	387 ± 105	−2.22
16	P04691**	TBB1	Tubulin β-chain	49.96/4.8	35/5.2	14	49	32	1429 ± 181	833 ± 284	−1.72
19	P04692*	TPM1	Splice isoform 5 of tropomyosin 1 α-chain (α-tropomyosin)	28.34/4.7	32/4.1	8	27	28	1000 ± 253	2088 ± 466	+2.09
21	P61589***	RHOA	Transforming protein RhoA	21.44/5.3	27/6.2	9	24	41	1642 ± 376	830 ± 172	−1.96
Synapse plasticity											
1	Q07266***	DREB	Drebrin (developmentally regulated brain protein)	77.47/4.5	109/3.8	9	29	19	1414 ± 481	224 ± 156	−6.25
Neurotransmission											
11	Q63537-2***	SYN2	Synapsin IIb	52.45/8.0	56/6.8	5	10	12	252 ± 133	693 ± 171	+2.75
13	P07323*	ENOG	γ enolase	47.01/5.0	50/4.8	14	25	41	543 ± 138	212 ± 128	−2.56
14	P07323***	ENOG	γ enolase	47.01/5.0	48/4.9	9	20	25	2218 ± 701	513 ± 452	−4.35
Calcium homeostasis											
18	P81155*	VDAC2	Voltage-dependent anion-selective channel protein 2	31.71/7.4	35/7.5	6	23	29	1140 ± 472	2983 ± 1133	+2.62
Chaperoning											
3	Q9DC41***	HS70P5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kD)	72.42/5.0	78/4.6	15	25	27	476 ± 89	176 ± 73	−2.70
4	P06761*	GRP78	78 kDa glucose-regulated protein (precursor)	70.47/5.0	78/4.7	6	20	15	920 ± 268	383 ± 169	−2.38
5	P06761**	GRP78	78 kDa glucose-regulated protein (precursor)	70.47/5.0	77/4.7	15	24	27	492 ± 148	120 ± 101	−3.45
7	P08109***	HS7C	Heat shock cognate 71 kDa protein	70.87/5.4	74/5.3	10	22	20	1259 ± 315	296 ± 130	−4.35
PTM											
20	Q00981***	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	24.78/5.1	29/5.0	8	29	44	1550 ± 354	674 ± 237	−2.33
22	P17220*	PSA2	Proteasome subunit α type 2	25.80/7.1	27/7.0	10	18	55	357 ± 103	655 ± 144	+1.83
Hippocampus											
Oxidative phosphorylation											
26	P10719	ATPB	ATP synthase β-chain, mitochondrial (precursor)	51.71/4.9	48/5.0	10	14	29	568 ± 53	293 ± 99	−1.96
49	P10818	COX6a1	Cytochrome *c* oxidase polypeptide Via-liver, mitochondrial (precursor)	12.30/9.3	14/7.0	3	16	35	1605 ± 107	771 ± 192	−2.08
Redox balance											
39	O35244	PDX6	Peroxiredoxin 6	24.69/5.7	28/6.0	5	19	26	3160 ± 547	1229 ± 405	−2.56

Table 1. Continued

Spot number	Accession number (Swiss-Prot/ TrEMBL)	Abbr. name	Protein name	Theor. M_r/p*I*	Observ. M_r/p*I*	MALDI-MS			Intensity (mean ± SD)		Fold change
						Peptides		Sequence coverage (%)			
						Matching	Total		Control	Depleted	
50	Q9R063	PDX5	Peroxiredoxin 5, mitochondrial (precursor)	22.18/8.9	17/7.6	6	29	30	2772 ± 314	1374 ± 424	−2.04
Cytoskeleton maintenance											
32	P04691	TBB1	Tubulin β-chain	49.96/4.8	38/6.0	7	26	22	1335 ± 236	619 ± 93	−2.17
36	P47819	GFAP	Glial fibrillary acidic protein, astrocyte	49.94/5.3	31/4.9	18	35	40	711 ± 57	359 ± 123	−1.96
37	Q63939	MHC	Myosin heavy chain (fragment)	54.65/5.5	31/5.7	11	35	19	599 ± 33	347 ± 21	−1.72
43	Q6AY56	TBA8	Tubulin α-8 chain	50.04/5.0	18/3.8	6	14	18	1016 ± 101	390 ± 156	−2.63
46	P04691	TBB1	Tubulin β-chain	49.96/4.8	16/4.5	6	25	18	2645 ± 178	1412 ± 268	−1.89
Synapse plasticity											
24	Q91XM8	CRMP4	Collapsin response mediator protein 4	61.97/6.0	61/6.2	9	46	22	328 ± 29	155 ± 43	−2.13
27	P07936	GAP43	Neuromodulin	23.60/4.6	45/4.1	9	36	40	1813 ± 377	861 ± 194	−2.08
28	P07936	GAP43	Neuromodulin	23.60/4.6	45/4.3	6	21	37	1035 ± 45	319 ± 56	−3.23
Neurotransmission											
30	P07323	ENOG	γ enolase	47.01/5.0	40/4.8	5	10	18	2057 ± 211	933 ± 237	−2.22
31	P07323	ENOG	γ enolase	47.16/5.0	40/5.0	10	32	32	2074 ± 402	678 ± 215	−3.13
34	P07323	ENOG	γ enolase	47.01/5.0	37/4.9	13	34	33	1331 ± 34	525 ± 278	−2.56
38	P61983	143G	14-3-3 protein γ	28.17/4.8	29/4.9	8	43	19	1101 ± 73	511 ± 196	−2.17
45	P62762	VIS1	Visinin-like protein 1	22.01/5.0	19/4.9	14	34	62	777 ± 23	207 ± 48	−3.70
Calcium homeostasis											
42	P02593	CALM	Calmodulin	16.71/4.1	19/3.2	7	29	59	3819 ± 674	1028 ± 474	−3.70
Chaperoning											
23	P08109	HS7C	Heat shock cognate 71 kDa protein	70.87/5.4	69/5.6	7	10	15	1899 ± 306	905 ± 76	−2.13
29	P08109	HS7C	Heat shock cognate 71 kDa protein	70.87/5.4	43/4.9	6	18	10	1874 ± 166	688 ± 147	−2.70
PTM											
35	P34064	PSA5	Proteasome subunit α type 5	26.39/4.8	31/4.5	7	40	44	2990 ± 325	1197 ± 332	−2.50
40	P48500	TPI1	Triosephosphate isomerase	26.79/6.5	28/6.7	10	28	38	877 ± 74	487 ± 93	−1.79
44	P02401	RPLP2	60S acidic ribosomal protein P2	11.69/4.4	17/3.7	4	15	39	1130 ± 98	355 ± 198	−3.23
48	Q9DAK9	PHP1	14 kDa phosphohistidine phosphatase	14.00/5.3	16/5.0	9	26	53	2001 ± 410	613 ± 170	−3.22
Lipids synthesis											
33	P02650	APOE	Apolipoprotein E (precursor)	35.75/5.2	38/4.9	10	40	38	404 ± 24	180 ± 70	−2.72
Others											
25	O35568	FBL3	EGF-containing fibulin-like extracellular matrix protein 1 (precursor)	54.60/5.0	52/4.6	12	46	30	2382 ± 59	728 ± 357	−3.23
41	O88767	PARK7	CAP1 protein	19.97/6.3	27/6.4	10	48	60	4892 ± 978	1755 ± 403	−2.78
47	Q9Z297	PEA15	Astrocytic phosphoprotein PEA-15	15.04/4.9	15/4.7	5	19	42	2372 ± 434	1210 ± 242	−1.96

Proteins from frontal cortex (first half of the table) and hippocampus (second half of the table) were separated by 2-D electrophoresis and were identified by MALDI-TOF MS, following in-gel digestion with trypsin as described in Section 2.7. The spot numbers correspond to the same as indicated on the gels in Fig 1. The identities of the spots, their accession number, the theoretical and approximate M_r/p*I* values observed, as well as the matching and total peptides and percentage sequence coverage by matching peptides are listed. The protein spots were quantified from silver-stained gels using PDQuest software. Frontal cortex proteins that were statistically significantly (Student's *t*-test) altered were reported and are represented by asterisks (*, $p<0.05$; **, $p<0.02$; ***, $p<0.01$). The table includes dysregulated hippocampal proteins but without *t*-test values ($n = 2$ in the control group). Spot intensities and variations indicated, respectively, by mean ± SD and by fold change (expressed as ratio of DVD-deficient to controls), are listed in the last columns. Proteins are clustered by main biological functions.

misexpressed in either schizophrenia or multiple sclerosis (references are indicated in Table S1 of Supporting Information). Figure 4C indicates that 15 of the proteins that are abnormally expressed in our animal model are also dysregulated in at least one of the two diseases. Of these, eight molecules are common to the two diseases.

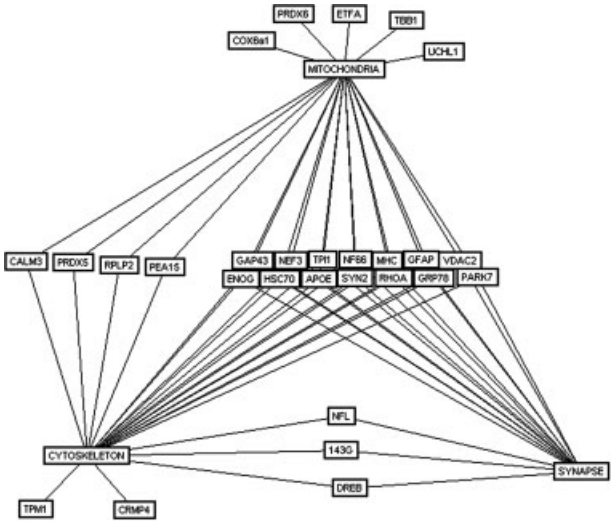

Figure 3. Schematic view of main protein functions. The list of dysregulated proteins in the experimental group was blasted using Predictsearch matrix. A 2-D graph was generated from this matrix, with node and edge inclusion being determined by a user-defined threshold. Mitochondria, cytoskeleton and synapses are the subcellular compartments that are primarily affected by the DVD deficiency. More than two thirds (28/36) of the affected proteins are related to at least one of these three subcellular compartments. Abbreviations are listed in Table 1.

4 Discussion

We show here that DVD deficiency leads to long-lasting alterations in protein expression involved in mitochondrial, cytoskeletal and neuronal function evident in the adult cortex and hippocampus. Specifically, 36 protein molecules were identified which are involved in oxidative phosphorylation, redox balance, cytoskeleton maintenance, calcium homeostasis, synaptic plasticity, neurotransmission, chaperoning and PTMs. These findings compliment findings from previous studies using the same animal model which demonstrates that an alteration in the foetal environment can produce long-lasting changes in adult brain structure and function as a result of prenatal vitamin D deficiency [20, 21, 23, 25].

4.1 DVD deficiency induces persistent changes in vitamin D-regulated proteins

There was no alteration in the VDR in the DVD-deficient adult brain, consistent with earlier findings [21, 22], but several proteins in this study were identified previously as genes/proteins whose expressions were altered by dietary or genetic vitamin D modification, namely cytochrome *c* oxidase, Na^+ K^+ ATPase, thioredoxin reductase (also named peroxiredoxin 5, Pdx5), calmodulin, tubulins, Gfap, neurofilaments, tropomyosin, RhoA, various HSPs and Ubqc [31–46]. A recent *in silico* survey on vitamin D target genes identified a putative vitamin D responsive element (VDRE) in the promoter region of 6 of these 36 proteins [47].

4.2 DVD deficiency induces persistent changes in cytoskeletal maintenance, mitochondrial function and synaptic function

The Predictsearch algorithm identified that 14 of the 36 affected proteins were commonly involved in cytoskeletal, mitochondrial and synaptic activities, which are central to neuronal function. Although the number of studies assessing the role of vitamin D on brain development and functioning is increasing, systems biology is at the present time too complex and it is not possible to identify the directions of causative pathways. However, these effects may be interrelated and are consistent with certain molecular changes previously described in the DVD-deficient adult rat brain.

Reduced energy production from suboptimal mitochondrial function could slow down the growth of the cytoskeleton. Therefore, reduced energy production may be 'upstream' for many of these changes. Alternatively, as vitamin D is also well known to regulate the expression of NGF, neurotrophin 3, neurotrophin 4 and GDNF [48–51], it may be possible that alterations in mitochondrial function may be a 'down-stream' consequence of reduced expression of such agents, a factor which we have observed previously [21, 22].

Otherwise, DVD deficiency may affect cytoskeleton maintenance and cell morphology *via* effectors such as small GTPases. RhoA, which was significantly down-regulated in our study, is part of the Rho family of GTPases which regulate a wide variety of biological processes including actin cytoskeleton rearrangements and microtubule dynamics [52, 53]. *In vivo* rat studies have demonstrated that vitamin D induces the expression of RhoA [45, 54]. RhoA plays a key role in the distribution and motility of mitochondria within the cell [55] and mediates neuronal growth cone collapse *via* its interaction with Semaphorin 3A [56]. RhoA and GFAP are well known for their roles in cytoskeleton maintenance [57, 58]. These two proteins are representative of a wider cytoskeletal alteration in the present study where nine proteins involved in cytoskeleton organisation have been altered, including neurofilament L (NFl) which has been shown to be down-regulated in a previous study of the DVD-deficient rat [22].

Drebrin emerges as another key molecule linking cytoskeleton and synaptic function. Drebrin is an actin-binding protein that is developmentally regulated in the process of neuronal growth and aids in dendritic spine formation. Interestingly, rats with drebrin knockdown displayed an increase in locomotor activity, faster learning and perseverative behaviour [59, 60]. These impairments are similar to those previously reported in the DVD-deficient adult rat, that is (i) hyperlocomotion [23]; (ii) impaired latent inhibition and increased maintenance of learnt rules [25] and (iii) enhanced learning (Abreu *et al.*, manuscript in preparation).

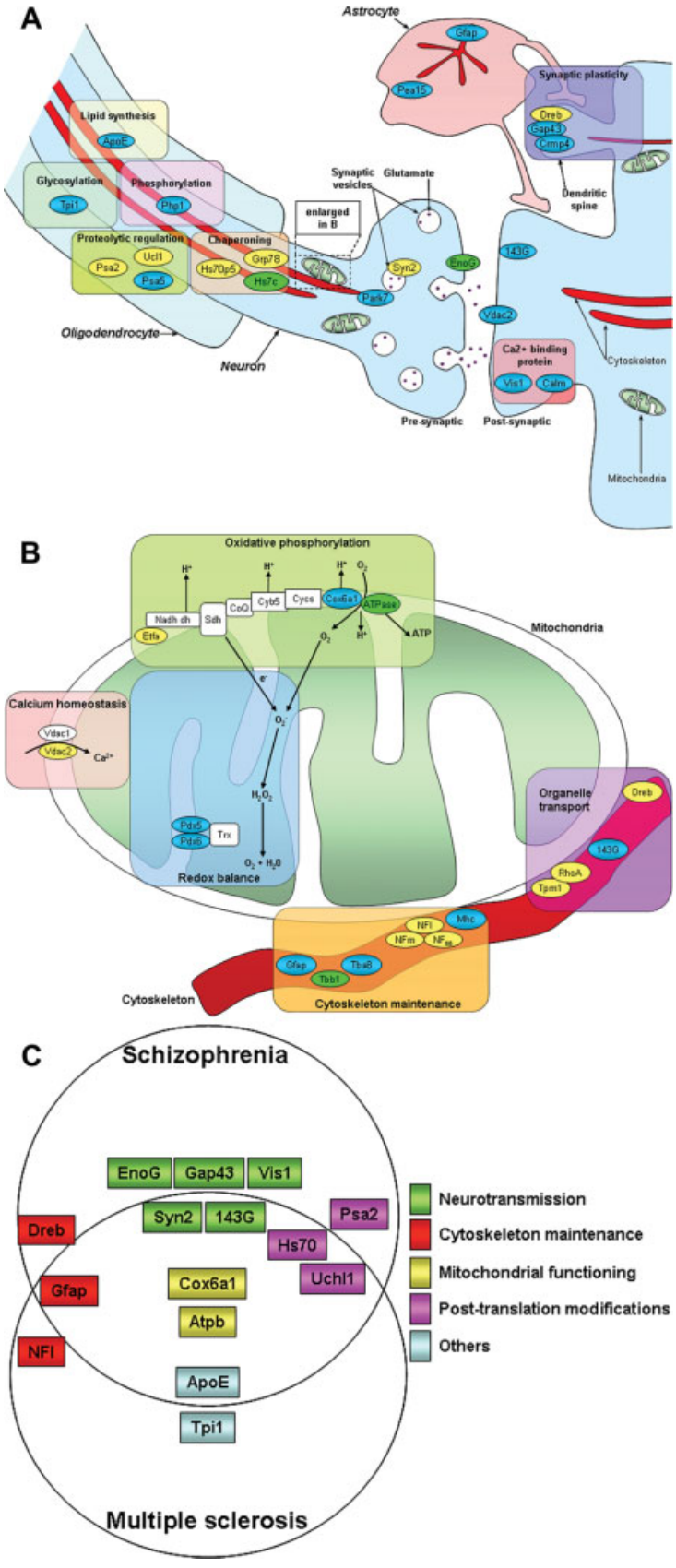

Figure 4. Schematic view of protein locations with known interactions. The list of dysregulated proteins in the experimental group was blasted using Predictsearch library. (A) Most of the abnormally expressed proteins are located in neurons and astrocytes. Within the pool of neuron-specific dysregulated metabolites, four and three are involved in neurotransmission and synaptic plasticity, respectively. (B) Six misexpressed proteins are associated with four main mitochondrial functions: oxidative phosphorylation, redox balance, calcium/ATP homeostasis and organelle transport. Dysregulated proteins in the frontal cortex are annotated in yellow oval boxes and dysregulated proteins in the hippocampus are annotated in blue oval boxes. When a protein is misexpressed in both the frontal cortex and the hippocampus, the name appears in a green oval box. Known partners of identified dysregulated molecules are indicated in white boxes. (C) Scheme of shared molecular anomalies between our animal model and two brain diseases with latitude-related prevalence. In total, 15 of our abnormally expressed metabolites are also dysregulated in either schizophrenia or multiple sclerosis. The common denominator for the two pathologies and our animal model is composed of eight molecules. Dysregulated molecules are clustered by main biological function, using a colour code. Abbreviations are listed in Table 1.

4.3 DVD deficiency and dopamine

All brains examined in this study were collected from animals in which locomotor behaviour has been assessed. Using two separate paradigms (open field and elevated plus maze), we showed that DVD deplete animals displayed spontaneous hyperlocomotion [23]. We have also recently observed that both spontaneous and psychomimetic-induced locomotion in DVD animals is selectively sensitive to dopamine blocking agents [61]. Therefore, it is of special interest to note that, in an animal model using meth-amphetamine to induce dopamine toxicity, of the five proteins that could be identified as being altered in the striatum, three were shown to be similarly altered in the present study. The expression of ATPB, UCHL1 and an unspecified peroxiredoxin were all reduced by acute meth-amphetamine treatment [62]. As previously mentioned, these three proteins have all been shown to be regulated by vitamin D. Whether the down-regulation of these three proteins reflects dopamine dysfunction or toxicity in DVD-deficient animals is presently unknown.

4.4 DVD deficiency and adult brain diseases

With respect to human psychiatric and neurological disorders, many of the proteins found to be dysregulated in the current model have also been reported in postmortem and genetic studies in patients with schizophrenia or multiple sclerosis. The most prominent of these is the dysregulation of mitochondrial proteins. In schizophrenia, ATP production in the frontal and left temporal lobes is reduced [63, 64] and mitochondrial density is reduced in several brain regions, a factor that can be reversed by antipsychotic treatment [65–67]. Importantly, a recent genomic/proteomic study of frontal lobes from patients with schizophrenia revealed that genes connected to mitochondrial function were among the most dysregulated [68]. In plaques in chronic multiple sclerosis, there is oxidative damage to mitochondrial DNA [69] and the number of mitochondria *per* axon is increased in animal models of demyelination [70, 71]. In addition, it has been recently shown that axonal degeneration in multiple sclerosis patients is caused by mitochondrial dysfunction [72].

With respect to schizophrenia specifically, gene expression profiling of prefrontal cortex demonstrated that transcripts encoding proteins involved in the regulation of presynaptic function were decreased in all subjects with schizophrenia compared to controls [73]. One of the most consistently changed transcripts, synapsin-2, was also misexpressed at the protein level in our DVD model. Other neurotransmission-associated molecules misexpressed in schizophrenia and in DVD-deficient animals are Gap43, Vis1 and 143G (references in Table 2). The gene for RhoA in humans is located at 3p21.3, a region associated with schizophrenia in several linkage studies (see meta-analysis [74]). The role of RhoA in growth cone collapse is mediated *via* Semaphorin 3A, which was altered in postmortem brains from patients with schizophrenia [75]. Finally, ApoE, identified in the DVD-deficient brain was also identified as a schizophrenia-susceptibility locus in linkage and association studies [76].

With respect to multiple sclerosis, specific antigenicity against GFAP, NFl, actin and triosephosphate isomerase was found in the brain of multiple sclerosis patients [27]. The expression of all these proteins was reduced in the DVD-deficient rat brain.

4.5 Caveats

Brain tissue from rat control animals was alsoused in a previous study (Feron *et al.*, 2005 [22]) and a smaller number of hippocampi were available. We have included hippocampal data because protein misexpression closely resembles the changes seen in the prefrontal cortex. However, given the number limitation in control sampling, hippocampal results should be regarded as preliminary.

The 2-D gel comparisons undertaken in this study were not corrected for multiple comparisons. However, the finding that the vast majority of the abnormally expressed proteins (89%, 32/36) were down-regulated lends further weight to the growing body of structural and behavioural evidence demonstrating that low prenatal vitamin D alters adult brain function [21–22, 61]. More studies including restricted grey matter from both genders are needed to confirm the results of the current study.

5 Conclusions

Exposure to certain environmental conditions during prenatal and early life has been shown to subtly alter the risk of adverse adult health outcomes [77, 78]. In particular, early nutrition can permanently alter adult metabolism in mammals. For example, the phenotype of the genetically obese agouti mouse was dependent on whether the pregnant mother was fed with a methyl-rich diet [79], presumably as a result of altering the methylation status of the DNA of the developing foetus. The current study provides evidence that DVD deficiency alters brain development leading to alterations in mitochondrial, cytoskeletal and synaptic function in the adult brain, presumably *via* long-lasting changes in gene regulation. We speculate that these changes could lay the foundation (or 'first hit') for a range of adult disorders. Combined with specific susceptibility genes, and additional specific postnatal exposures ('second hits' such as infection, substance abuse, stress, *etc.*), DVD deficiency may contribute to a previously unrecognised range of adverse health outcomes. The treatment of low vitamin D in pregnant women is safe and cheap, and could be addressed within the framework of recommendations for the consumption of other key nutrients during pregnancy (*e.g.* iron to reduce the risk of maternal anemia, folate to reduce the risk of spina bifida in

the offspring). The findings from the current study provide an informative framework for future research to examine these hypotheses in more detail.

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