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ORIGINAL ARTICLE

# A proteomic analysis of the ventral hippocampus of rats subjected to maternal separation and escitalopram treatment

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**Abstract** Early life stress is known to predispose humans to the development of depression. Developmental stress has been shown to cause various changes in neurotransmitter systems, neurotrophin expression and the hypothalamic pituitary adrenal-axis in the rat brain. The aim of this study was to identify which cytosolic proteins are altered by maternal separation, as a model for depression, as well as by chronic antidepressant treatment. Rats were maternally separated from postnatal day 2–14 for 3 h per day while control rats were normally reared. Both groups were divided and received either escitalopram or saline injections for 6 weeks starting from postnatal day 40. The ventral hippocampal tissue was fractionated and the cytosolic fraction used for 2-D-gel electrophoresis and liquid chromatography coupled to mass spectrometry analyses to identify peptides. Mascot database searches were done to identify proteins that were differentially expressed between the groups. Proteins that were significantly changed by maternal separation included amongst others: molecular chaperones and proteins related to energy metabolism; neuroplasticity; oxidative stress regulation; and protein metabolism. Treatment with escitalopram, a selective-serotonin reuptake inhibitor, induced changes in a different group of proteins, except for a few involved in energy metabolism and neuro-

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protective pathways. The results indicate which cytosolic proteins are changed by early life stress and may therefore be involved in the development of depression.

**Keywords** Proteomics · Early life stress · Maternal separation · Rat model for depression

## Introduction

It is well known that children subjected to early life stress, such as physical or sexual abuse, or neglect, are predisposed to developing depression or anxiety disorders during adolescence or adulthood (Kessler and Magee 1993; Pelcovitz et al. 1994; Gilmer and McKinney 2003). Stressful encounters during this critical developmental stage of the brain may induce various changes in the hypothalamic-pituitary adrenal axis (HPA-axis), neurotransmitter and neurotrophin levels.

A rat model for early life stress, maternal separation (ms), has been used in various laboratories to study the effects of stressful experiences during childhood on the brain. We have also successfully used this rat model to study dysregulation of the HPA system (Daniels et al. 2004; Marais et al. 2008), which may be relevant to understanding depression in humans in terms of changes in the neurobiological systems. For instance, HPA-axis activity is dysregulated in patients with depression, as evidenced by increases in basal cortisol levels (Heim and Nemeroff 1999), and a blunted ACTH response following intravenous injection of corticotrophin releasing factor (Holsboer et al. 1986). Wong et al. (2000) observed increased basal plasma cortisol levels but no difference in basal ACTH levels during a 30 h period in patients with depression compared to normal controls. Our maternally separated rats showed similar endocrine changes with elevated baseline corticosterone levels and a blunted ACTH response after acute restraint stress was induced (Marais et al. 2008; Daniels et al. 2004). Increased cortisol levels can affect aerobic energy metabolism pathways as the binding of cortisol to the glucocorticoid receptor (GR) regulates transcription of proteins including BAX (Bcl-2 associated X protein), which binds to the mitochondrial membrane. The GR-complex also binds to the membrane and influences the membrane potential, which can lead to increased cytochrome c release and apoptosis (Iijima 2006; Zhang et al. 2006).

Dysregulation in the serotonergic system also plays a role in the development of depression and selective serotonin reuptake inhibitors (SSRI's) achieve their therapeutic effect by increasing synaptic serotonin levels (Blier et al. 1987). Clinical studies have shown low cerebrospinal fluid serotonin levels in female patients with depression and in suicidal patients with depression (Hou et al. 2006) and a decreased number of serotonin $(5\text{-HT})_1$ receptors in the hippocampus of depressive patients (Cheetham et al. 1990). In addition, the prolactin response to intravenous citalopram, an SSRI, injection was shown to be blunted in depressive patients indicating decreased availability of serotonin in the brain (Bhagwagar et al. 2002). Similarly, ms rats had decreased serotonin levels in the dorsal hippocampus and medial prefrontal cortex (Matthews et al. 2001) and the administration of SSRI's (citalopram, escitalopram and fluoxetine) to normally reared or ms rats exhibited anti-depressant effects (Kuśmider et al. 2007; El Khoury et al. 2006; Leventopoulos et al. 2009).

Quantitative autoradiography showed that rats subjected to early life stress had decreased 5-$HT_{1A}$ receptor binding in their brains and which was increased by chronic fluoxetine treatment (Leventopoulos et al. 2009) and 5-$HT_{1A}$ receptor agonists have antidepressant effects since it decreased the immobility time of rats in the forced swim test (Detke et al. 1995).

Neurotrophins, which are important for cell survival and neuroplasticity (Hennigan et al. 2007), are also affected by early life stress, since decreased levels of nerve growth factor (NGF) and neurotrophin-3 (NT-3) were measured in the ventral hippocampus of ms rats (Marais et al. 2008) and brain derived neurotrophic factor (BDNF) mRNA decreased in whole hippocampi (Kuma et al. 2004). These reductions in neurotrophin levels can cause decreased proliferation or increased neuronal death of hippocampal neurons, such as the reduction in hippocampal volume as seen patients with depression (Bremner et. al. 2000; Sheline et al. 1999). Indeed, it was found that serum or plasma BDNF levels were significantly lower in patients with depression than in controls (Karege et al. 2002; Lee et al. 2007).

It is evident from previous studies that the development of depression after a stressful event is not caused by one specific alteration in the brain, but rather by many included in downstream pathways of neurotransmitter or neurotrophin binding that in turn affect the expression of signalling proteins (Duman et al. 1997). The differential expression of cytosolic proteins may also participate in the pathogenesis of depression. To test this hypothesis, we subjected rats to ms and compared the expression profile of proteins in their ventral hippocampi to that of normally reared animals. The ventral hippocampus was selected because our previous work showed ms to induce significant reductions in neurotrophin levels in this brain region (Marais et al. 2008).

Using proteomic techniques (2-Dimensional gel electrophoresis coupled to mass spectrometry) we wanted to identify which proteins were upregulated or down-regulated by early life stress, suggesting their involvement in the development of depression. Furthermore, a comparison was made between rats that have been treated with escitalopram and appropriate controls to establish whether proteins that are changed by ms were in fact targeted by an SSRI.

## Materials and methods

This project was approved by the Committee for Experimental Animal Research of the University of Stellenbosch (project number: P04/10/020). The experiments were performed in the Central Research Facility of the University of Stellenbosch. Male Sprague-Dawley rats were used for experiments. Rats were housed under standard laboratory conditions (12 h/12 h light/dark cycle; lights on at 6:00am; food and water *ad libitum*).

### Maternal separation

Rat pups in the ms group ($n$=9) were separated from their dams between postnatal days 2–14 for 3 h per day in the morning (Marais et al. 2008). For this procedure, the respective dams was removed from the home cage and pups carried to an isolated

room. During separation, the pups were placed under infrared lights that maintained the ambient temperature at 30–33°C. Another group of rats ($n$=9) were normally reared (nr) with their mothers and served as controls. The cages were all cleaned twice a week and no culling was performed on the litters. All pups were weaned on postnatal day 21, after which the males were kept in pairs for further experimentation.

## Escitalopram treatment

Both ms and nr rats were divided into 2 groups: escitalopram treated ($n$=3 for each group) and saline treated ($n$=6 for each group). Escitalopram dissolved in saline (5 mg/kg/day; Uys et al. 2006) or saline only was administered via intra-peritoneal injection for 6 weeks from day 40–82. Rats were killed on postnatal day 83 and ventral hippocampal tissue collected (the bottom1/3 of both hippocampi) and stored in liquid nitrogen until analysis. The ventral hippocampus was used since we have previously found significant alterations in the neurotrophin levels of maternally separated rats in this brain region (Marais et al. 2008).

## 2-Dimensional (2-D) gel electrophoresis

Ventral hippocampi were fractionated using a Calbiochem Proteoextract subcellular proteome extraction kit (Merck). The protein concentrations of each of the cytosolic fractions were then determined with a Bradford assay (Bradford 1976). A ReadyPrep 2-D clean-up kit (Bio-Rad) was used to remove substances from the sample that are known to interfere with Isoelectric focusing (IEF)/2-D-gel electrophoresis. The precipitated sample was resuspended in 2-D sample/rehydration buffer (Bio-Rad) and protein concentrations determined with a RC/DC protein assay (Bio-Rad). Isoelectric focusing was done on an 11 cm pH 5-8 IEF strip (Bio-Rad) with 150 µg of protein in a volume of 200 µl. These strips and the protein concentration were chosen based on previous work done in our laboratory (Uys et al. 2008). Strips were rehydrated for 12 h (2 ml mineral oil added on top after an hour) and focusing done for 40 000 Vh in a Protean IEF cell (Bio-Rad). Mineral oil was removed using blotting paper and strips were then incubated for 15 min with equilibration buffer I and II (Bio-Rad) with 2.5% w/v iodoacetamide (Sigma) added to buffer II. Strips were run on pre-cast Bis-Tris 4–12% Criterion XT gels (Bio-Rad) and these were fixed in a 40% methanol and 7% acetic acid solution for an hour. Three rats from each experimental group were run in triplicate against their respective control groups. Three comparisons were done namely: (1) ms rats were compared with nr rats (control group); (2) ms rats injected with escitalopram were compared to ms rats injected with saline (control group) and (3) nr rats injected with escitalopram were compared with nr rats injected with saline (control group). Gels were stained overnight in Coomassie Colloidal Blue (Sigma) and destained in 25% methanol for 2.5 h. Gels were scanned on a GS-800 densitometer (Bio-Rad) and differentially expressed protein spots ($p<0.05$; t-test) were identified using the PD Quest Advanced, version 8.0.1 software package (Bio-Rad). Gels were stored in a 25% ammonium sulphate solution until spots were manually excised into a 96-well microtitre plate. Spots from all 6 gels were cut out for each protein and placed in the same well to increase the concentration of the protein.

Destaining, digestion and peptide extraction

Spots were destained twice using 50% acetonitrile in 100 mM ammonium bicarbonate, rinsed with acetonitrile and allowed to air dry for 10 min. The spots were the reduced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 30 min followed by alkylation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. The gel spots were then rinsed with acetonitrile and 100 mM ammonium bicarbonate followed by acetonitrile for a further 3 washes. A 25 µl aliquot of 6 ng/µl trypsin was added to each sample and allowed to incubate at 37°C for 4.5 h. The resulting peptides were initially extracted using 30 µl of an aqueous solution containing 2% acetonitrile and 1% formic acid. A second extraction using 15 µl of an aqueous solution containing 51% acetonitrile and 0.5% formic acid was then performed and combined with the first extraction in a cooled second 96-well plate. At this stage, and if necessary, the extractions were stored at −80°C prior to analysis by mass spectrometry.

Peptide separation by in-line liquid chromatography (LC) and electrospray ionisation mass spectrometry (ESI-MS)

The extracted tryptic peptides were resolved using an in-line NanoAcquity LC and autosampler system. LC solvents were supplied by Mallinckrodt Baker, Inc. A 4.9 µl aliquot of each sample was injected onto a nanoACQUITY UPLC™ trapping column 10kpsi Symmetry C18 180 µm×20 mm 5 µm (Waters) equilibrated in 3% aqueous acetonitrile containing 0.1% formic acid and the column flushed with 1% aqueous acetonitrile/0.1% formic acid at 15 $\mu Lmin^{-1}$ for 1 min. The peptides were then eluted onto a nanoACQUITY UPLC BEH C18 Column, 1.7 µm, 100 µm× 100 mm, 10 K psi (Waters) at 1.2 $\mu Lmin^{-1}$ using a linear gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile) and run over 20 min. The eluted peptides were analysed on a Micromass Q-Tof Global Ultima mass spectrometer fitted with a nano-LC emitter (New Objective) with an applied capillary voltage of 3–4 kV. The instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of [$glu^1$]-fibrinopeptide B (GFP—Sigma-Aldrich F3261). A calibration was accepted when the average error obtained on a subsequent acquisition was <10 ppm. Sensitivity was assessed by an injection of 50 fmol of a phosphorylase B tryptic digest giving a base peak intensity >1000 counts per sec in MS mode on the most intense peptide. The instrument was operated in data dependent acquisition (DDA) mode over the mass/charge (*m/z*) range of 50–2000. During the DDA analysis, both MS and tandem mass spectrometry (CID) were performed on the three most intense peptides as they eluted from the column. The uninterpreted MS/MS data were processed using the Waters ProteinLynx Global Server v2.3 software package (smoothed, background subtracted, centred and deisotoped) then mass corrected against the doubly charged GFP peptide. A peak list file was created and subjected to Mascot using MS/MS Ion search and the SwissProt database to identify proteins (www.matrixscience.com). Search parameters specified were: fixed modifications of carbamidomethyl (C); variable modifications of oxidation (M) and phosphorylation (ST, Y); 1 missed trypsin cleavage was allowed for; peptide tolerance was set at

0.2 Da and MS/MS tolerance was 0.5 Da and peptide charge was +2 and +3. Mono-isotopic mass was used and proteins identified were significant ($p<0.05$) according to the probability-based MOWSE scores.

## Results

The expression of a number of cytosolic proteins in the ventral hippocampus was affected by maternal separation or escitalopram treatment. Tables 1 and 2 lists the proteins and their functions upregulated and downregulated respectively in ms rats compared to nr rats (control group). Tables 3 and 4 lists the proteins and their functions that are upregulated and downregulated respectively in escitalopram ms rats vs. saline treated ms rats (control group). Tables 5 and 6 lists the proteins and their functions that are upregulated and downregulated respectively in escitalopram nr vs. saline treated nr rats (control group). Proteins that were identified in more than one experimental analysis, i.e. in more than one rat of the same group, are indicated with an asterisk in the results tables.

## Discussion

In the present study, the effects of ms on cytosolic protein levels in ventral hippocampal tissue were assessed with 2D-gel electrophoresis and mass-spectrometry techniques. Previous studies have mainly focused on the effect of ms on neurotransmitters (Matthews et al. 2001; Daniels et al. 2004) or neurotrophins (Marais et al. 2008), which subsequently alter signalling pathways. We wanted to acquire more information about the effect of early life stress on the expression of cytosolic proteins to identify specific proteins that may be involved in the development of depression.

### Maternal separation

Proteins that increased in response to ms, include several heat shock proteins or molecular chaperones that are known to be induced by stress. These are heat shock proteins 60, 70, 71 and stress-induced phosphoprotein 1, a co-chaperone that links heat shock proteins 70 and 90. These molecular chaperones usually function in transport of proteins within the cell and prevent misfolding and aggregation of old and new proteins under stressful conditions (Walter and Buchner 2002).

Maternal separation increased a number of proteins that appear to have beneficial effects in neurons, for example dihidropyrimidase-related protein (DRP)-2 which is involved in neuroplasticity and specifically axonal outgrowth and regeneration (Minturn et al. 1995; Inagaki et al. 2001), and aminoacylase-1, which is known to influence the activity and cellular location of sphingosine kinase type 1, a promoter of cell growth and inhibitor of apoptosis (Maceyka et al. 2004; Xia et al. 2002). These proteins are possibly upregulated in response to stress as a compensatory mechanism of the brain to protect against the adverse effects of ms.

**Table 1** Proteins upregulated in the ventral hippocampus of maternally separated rats compared to normally reared rats (control)

| Protein | Accession no. | Mass (Da) | Ratio to control | Mascot score | Sequence coverage (%) | Isoelectric point (pI) | Function |
|---|---|---|---|---|---|---|---|
| 60 kDa heat shock protein, mitochondrial | P63039 | 61088 | 1.48 | 129 | 12 | 5.91 | Molecular chaperone |
| Aminoacylase-1A | Q6AYS7 | 46060 | 1.17 | 185 | 30 | 6.03 | Differentiation of neurons |
| ATP synthase subunit alpha, mitochondrial | P15999 | 59831 | 3.26 | 55 | 4 | 9.22 | ATP synthesis |
| ATP synthase subunit d, mitochondrial | P31399 | 18809 | 1.24 | 100 | 38 | 6.17 | ATP synthesis |
| Carbonic anhydrase 2 | P27139 | 29267 | 1.68 | 112 | 19 | 6.89 | Calcium regulation |
| D-3-phosphoglycerate dehydrogenase | O08651 | 57256 | 1.87 | 370 | 23 | 6.28 | L-serine synthesis |
| Dihydrolipoyl dehydrogenase, mitochondrial | Q6P6R2 | 54574 | 1.62 | 142 | 16 | 7.96 | Pyruvate carboxylation |
| Dihydropyrimidinase-related protein 2 | P47942 | 62638 | 1.89 | 64 | 1 | 5.95 | Neuroplasticity |
| Ferritin heavy chain | P19132 | 21113 | 1.14 | 87 | 22 | 5.85 | Protects against oxidative stress |
| Glutamate dehydrogenase 1, mitochondrial | P10860 | 61719 | 2.66 | 174 | 26 | 8.05 | Glutamate metabolism |
| Heat shock 70 kDa protein 4 | O88600 | 94795 | 1.23 | 251 | 16 | 5.13 | Molecular chaperone |
| Heat shock cognate 71 kDa protein | P63018 | 71055 | 1.62 | 343 | 24 | 5.37 | Molecular chaperone |
| Nucleoside diphosphate kinase B | P19804 | 17386 | 1.6 | 201 | 50 | 6.92 | nucleoside triphosphate synthesis |
| Phosphatidylethanolamine-binding protein 1 | P31044 | 20788 | 2.1 | 156 | 22 | 5.48 | Serine protease inhibitor |
| Proteasome subunit beta type-7[a] | Q9JHW0 | 30250 | 1.38 | 104 | 12 | 8.13 | Protein catabolism |
| Protein disulfide-isomerase A3 | P11598 | 57044 | 1.44 | 298 | 36 | 5.88 | Formation of disulfide bonds in proteins |
| Stress-induced-phosphoprotein 1 | O35814 | 63158 | 1.67 | 203 | 18 | 6.4 | Co-chaperone linking Hsp-70/Hsp-90 |
| Transitional endoplasmic reticulum ATPase | P46462 | 89977 | 1.21 | 398 | 31 | 5.14 | Vesicle formation in endoplasmic reticulum |

$n=3$; [a] proteins identified in more than one experimental analysis of the same groups

**Table 2** Proteins downregulated in the ventral hippocampus of maternally separated rats compared to normally reared rats (control)

| Protein | Accession no. | Mass (Da) | Ratio to control | Mascot score | Sequence coverage (%) | Isoelectric point (pI) | Function |
|---|---|---|---|---|---|---|---|
| [Protein ADP-ribosylarginine] hydrolase | Q02589 | 40220 | 0.4 | 91 | 13 | 5.62 | Catalyzes de-ADP-ribosylation of proteins |
| Aldehyde dehydrogenase, mitochondrial | P11884 | 54813 | 0.83 | 196 | 23 | 5.83 | Converts aldehydes to acids |
| Amphiphysin 1 | O08839 | 64493 | 0.48 | 119 | 15 | 4.95 | Neurotransmitter recycling |
| Annexin-5 | P14668 | 35779 | 0.3 | 226 | 30 | 4.93 | Apoptosis |
| COP9 signalosome complex subunit 4 | Q68FS2 | 46546 | 0.48 | 366 | 21 | 5.6 | Ubiquitin-dependant protein degradation |
| Elongation factor Tu, mitochondrial | P85834 | 49890 | 0.66 | 245 | 32 | 7.23 | Protein synthesis |
| Glutathione S-transferase P | P10299 | 23652 | 0.64 | 83 | 13 | 6.89 | Conjugates glutathione to targets, reduces oxidative stress |
| Glyceraldehyde-3-phosphate dehydrogenase | P04797 | 35805 | 0.89 | 121 | 9 | 8.14 | Glycolysis |
| Glyoxalase domain-containing protein 4 | Q5I0D1 | 33532 | 0.63 | 145 | 31 | 5.11 | Removes methylglyoxal from mitochondria |
| Inositol monophosphatase | P97697 | 30834 | 0.56 | 211 | 21 | 5.17 | Generation of myo-inositol |
| Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial | Q99NA5 | 40044 | 0.75 | 171 | 19 | 6.47 | Citric acid cycle |
| N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 | O08557 | 31805 | 0.79 | 228 | 23 | 5.75 | Hydrolyses NOS inhibitors |
| Neurogranin | Q04940 | 7720 | 0.86 | 179 | 26 | 6.54 | Neuroplasticity |
| Nucleoside diphosphate kinase A | Q05982 | 17296 | 0.84 | 169 | 51 | 5.96 | Nucleoside triphosphate synthesis |
| Phosphoglycerate mutase 1 | P25113 | 28928 | 0.88 | 304 | 47 | 6.67 | Glycolysis |
| Protein DJ-1 | O88767 | 20190 | 0.74 | 131 | 19 | 6.32 | Protection against oxidative stress |
| Stress-70 protein, mitochondrial | P48721 | 74097 | 0.84 | 545 | 37 | 5.97 | Molecular chaperone |
| Triosephosphate isomerase[a] | P48500 | 27345 | 0.78 | 197 | 20 | 6.89 | Glycolysis |

$n=3$; [a] proteins identified in more than one experimental analysis of the same groups

**Table 3** Proteins upregulated in the ventral hippocampus of maternally separated rats after treatment with escitalopram vs. saline treated maternally separated rats (control)

| Protein | Accession no. | Mass (Da) | Ratio to control | Mascot score | Sequence coverage (%) | Isoelec-tric point (pI) | Function |
|---|---|---|---|---|---|---|---|
| Alpha-enolase | P04764 | 47.128 | 1.43 | 82 | 8 | 6.16 | Glycolysis |
| Complexin-1/Complexin-2 | P63041/ P84087 | 15.12/ 15.394 | 1.29/ 1.62 | 51 | 8 | 4.93/ 5.06 | Neurotransmitter release |
| Cytosol aminopeptidase | Q68FS4 | 56.15 | 1.08 | 201 | 7 | 6.77 | Protein catabolism |
| D-3-phosphoglycerate dehydrogenase | O08651 | 56.493 | 1.43 | 112 | 4 | 6.28 | Amino-acid synthesis |
| Dihydrolipoyllysine-residue acetyltransferase, mitochondrial | P08461 | 67.166 | 1.22 | 82 | 3 | 8.76 | Pyruvate decarboxylation |
| Dihydrolipoyllysine-residue succinyltransferase, mitochondrial | Q01205 | 48.925 | 1.32 | 203 | 8 | 8.89 | Pyruvate decarboxylation |
| EF-hand domain-containing protein D2 | Q4FZY0 | 26.759 | 1.22 | 76 | 23 | 5.01 | Calcium regulation |
| Glyoxalase 1 | Q6P7Q4 | 20.82 | 1.51 | 91 | 14 | 5.12 | Detoxification of methylglyoxal |
| Heat shock protein 105 kDa | Q66HA8 | 96.419 | 1.32 | 313 | 10 | 5.4 | Molecular chaperone |
| Hypoxanthine-guanine phosphoribosyltransferase | P27605 | 24.477 | 1.54 | 88 | 5 | 6.07 | Purine salvage pathway |
| Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial | Q99NA5 | 39.614 | 1.39 | 150 | 8 | 6.47 | Citric acid cycle |
| Malate dehydrogenase, cytoplasmic | O88989 | 36.483 | 1.47 | 141 | 9 | 6.16 | Citric acid cycle |
| N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 | O08557 | 31.426 | 1.24 | 142 | 11 | 5.75 | Hydrolyses NOS inhibitors |
| Prohibitin | P67779 | 29.82 | 1.23 | 121 | 12 | 5.57 | Inhibits DNA synthesis |
| Protein-L-isoaspartate(D-aspartate) O-methyltransferase | P22062 | 24.641 | 1.2 | 143 | 12 | 7.14 | Protein repair |
| Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | P49432 | 38.982 | 1.28 | 146 | 8 | 6.2 | Pyruvate decarboxylation |
| Thioredoxin-dependent peroxide reductase, mitochondrial | Q9Z0V6 | 28.295 | 1.56 | 94 | 8 | 7.14 | Redox regulation |
| Triosephosphate isomerase[a] | P48500 | 26.849 | 5.57 | 261 | 25 | 6.89 | Glycolysis |
| Ubiquitin carboxyl-terminal hydrolase isozyme L1 | Q00981 | 24.838 | 1.29 | 155 | 33 | 5.14 | Protein catabolism |
| Voltage-dependent anion-selective channel protein 2 | P81155 | 31.746 | 1.27 | 157 | 13 | 7.44 | Apoptosis |

$n=3$; [a] proteins identified in more than one experimental analysis of the same groups

**Table 4** Proteins downregulated in the ventral hippocampus of maternally separated rats after treatment with escitalopram vs. saline treated maternally separated rats (control)

| Protein | Accession no. | Mass (Da) | Ratio to control | Mascot score | Sequence coverage (%) | Isoelec-tric point (pI) | Function |
|---|---|---|---|---|---|---|---|
| Aconitate hydratase, mitochondrial | Q9ER34 | 85.433 | 0.32 | 245 | 5 | 7.87 | Citric acid cycle |
| Alcohol dehydrogenase [NADP+] | P51635 | 36.506 | 0.66 | 124 | 19 | 6.84 | Converts alcohols to aldehydes |
| Aminoacylase-1A | Q6AYS7 | 45.804 | 0.5 | 163 | 7 | 6.03 | Differentiation of neurons |
| Annexin A5 | P14668 | 35.745 | 0.61 | 80 | 4 | 4.93 | Apoptosis |
| Carbonic anhydrase 2 | P27139 | 29.114 | 0.23 | 69 | 6 | 6.89 | Calcium regulation |
| Cytosolic acyl coenzyme A thioester hydrolase | Q64559 | 42.735 | 0.46 | 121 | 6 | 8.8 | Fatty acid synthesis |
| Dihydropyrimidinase-related protein 2[a] | P47942 | 62.278 | 0.65 | 367 | 18 | 5.95 | Neuroplasticity |
| Dihydropyrimidinase-related protein 4[a] | Q62951 | 61.086 | 0.49 | 263 | 14 | 6.3 | Neuroplasticity |
| Dihydropyrimidinase-related protein 5[a] | Q9JHU0 | 61.54 | 0.67 | 348 | 13 | 6.6 | Neuroplasticity |
| Dynactin subunit 2 | Q6AYH5 | 44.148 | 0.71 | 51 | 5 | 5.14 | Mitosis |
| Fructose-bisphosphate aldolase C | P09117 | 39.284 | 0.43 | 228 | 15 | 6.67 | Glycolysis |
| Glutamate dehydrogenase 1, mitochondrial | P10860 | 61.416 | 0.74 | 103 | 4 | 8.05 | Glutamate metabolism |
| GTP-binding nuclear protein Ran | P62828 | 24.423 | 0.42 | 118 | 12 | 7.01 | Protein transport |
| Phosphoglycerate mutase 1 | P25113 | 28.832 | 0.28 | 59 | 11 | 6.67 | Glycolysis |
| Synapsin-2 | Q63537 | 63.457 | 0.41 | 79 | 5 | 8.73 | Neurotransmitter release |

$n=3$; [a] proteins identified in more than one experimental analysis of the same groups

**Table 5** Proteins upregulated in the ventral hippocampus of normally reared rats after treatment with escitalopram vs. saline treated rats (control)

| Protein | Accession no. | Mass (Da) | Ratio to control | Mascot score | Sequence coverage (%) | Isolelectric point (PI) | Function |
|---|---|---|---|---|---|---|---|
| ATP synthase subunit d, mitochondrial | P31399 | 18.763 | 1.91 | 105 | 30 | 6.17 | ATP synthesis |
| Bcl-2-like protein 11 | O88498 | 22.056 | 3.35 | 35 | 6 | 6.1 | Apoptosis |
| Creatine kinase B-type | P07335 | 42.725 | 1.43 | 293 | 20 | 5.39 | Phosphate transfer |
| Dihydrolipoyllysine-residue acetyltransferase, mitochondrial | P08461 | 67.166 | 1.32 | 254 | 12 | 8.76 | Pyruvate decarboxylation |
| Endoplasmic reticulum protein ERp29 | P52555 | 28.575 | 1.47 | 217 | 13 | 6.23 | Protein transport |
| Eukaryotic translation initiation factor 4H | Q5XI72 | 27.324 | 1.49 | 74 | 5 | 6.67 | Translation |
| Fructose-bisphosphate aldolase C | P09117 | 39.284 | 1.29 | 517 | 33 | 6.67 | Glycolysis |
| Glutamine synthetase[a] | P09606 | 42.268 | 2.31 | 198 | 18 | 6.64 | Protein catabolism |
| Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1/beta-2 | P54311/ P54313 | 37.377/ 37.331 | 1.49 | 115/124 | 13 | 5.6 | Initiates signal transduction pathways |
| Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial | Q99NA5 | 39.614 | 2.16 | 343 | 20 | 6.47 | Citric acid cycle |
| Neuronal protein 22 | P37805 | 22.501 | 1.44 | 217 | 32 | 6.84 | Microtubule structure |
| Pyruvate dehydrogenase E1 component subunit beta, mitochondrial[a] | P49432 | 38.982 | 1.32 | 177 | 9 | 6.2 | Pyruvate decarboxylation |
| Triosephosphate isomerase | P48500 | 26.849 | 1.64 | 403 | 51 | 6.89 | Glycolysis |

$n$=3; [a] proteins identified in more than one experimental analysis of the same groups

**Table 6** Proteins downregulated in the ventral hippocampus of normally reared rats after treatment with escitalopram vs. saline treated rats (control) $n=3$

| Protein | Accession no. | Mass (Da) | Ratio to control | Mascot score | Sequence coverage (%) | Isoelectric point (PI) | Function |
|---|---|---|---|---|---|---|---|
| 78 kDa glucose-regulated protein | P06761 | 72.347 | 0.47 | 234 | 12 | 5.07 | Molecular chaperone |
| Alpha-enolase | P04764 | 47.128 | 0.43 | 324 | 20 | 6.16 | Glycolysis |
| Fascin | P85845 | 21.781 | 0.72 | 97 | 15 | 5.86 | Microtubule structure |
| Glutathione S-transferase P | P10299 | 23.901 | 0.67 | 105 | 13 | 6.89 | Conjugates glutathione to targets, reduces oxidative stress |
| Glyceraldehyde-3-phosphate dehydrogenase | P04797 | 35.828 | 0.63 | 96 | 26 | 8.14 | Glycolysis |
| Malate dehydrogenase, cytoplasmic | O88989 | 36.483 | 0.53 | 85 | 12 | 6.16 | Citric acid cycle |
| Phosphoglycerate kinase 1 | P16617 | 44.538 | 0.64 | 314 | 25 | 8.02 | Glycolysis |
| Proteasome subunit beta type-4 | P34067 | 29.197 | 0.71 | 155 | 12 | 6.45 | Protein catabolism |
| Protein-L-isoaspartate(D-aspartate) O-methyltransferase | P22062 | 24.641 | 0.74 | 99 | 7 | 7.14 | Protein repair or degradation |
| Pyridoxine-5'-phosphate oxidase | O88794 | 30.184 | 0.56 | 174 | 9 | 8.66 | Vit. B6 synthesis |
| Pyruvate kinase isozymes M1/M2 | P11980 | 57.818 | 0.64 | 106 | 5 | 6.63 | Glycolysis |
| Tubulin alpha-1A chain | P68370 | 50.136 | 0.34 | 189 | 13 | 4.94 | Microtubule structure |
| UMP-CMP kinase | Q4KM73 | 22.169 | 0.75 | 158 | 22 | 5.66 | Phosphate transfer |

Additionally, proteins related to the protection of neurons to oxidative stress were increased. Increased ferritin heavy chain suggests protection of neurons by sequestering iron in ferrous form (Theil 1987), resulting in a reduction in the formation of superoxide free radicals via the fenton reaction (Kitahara et al. 1995). In a similar, protective way, increased levels of carbonic anhydrase 2 maintain the pH balance of cells by catalyzing the reaction of carbon dioxide ($CO_2$) hydration, reducing intracellular $CO_2$ levels (Pocker and Sarkanen 1978). A high amount of $CO_2$ increase acidity and induce oxidative stress in cells (Bentes de Souza et al. 2004).

A number of proteins upregulated by ms are involved in amino acid or protein metabolism. D-3-phosphoglycerate dehydrogenase is involved in the metabolic pathway of the biosynthesis of L-serine, and is therefore essential for neuronal cell proliferation (De Koning et al. 2003) and dendritic and axonal growth in neuronal cell cultures (Savoca et al. 1995). Protein disulfide-isomerase catalyzes the formation of disulfide bonds in proteins and is involved in reactivation of denatured proteins (Yao et al. 1997). Upregulation of these proteins may therefore reflect the attempts of the brain to restore damaged neurons and proteins in ms rats.

Several of the upregulated proteins are involved in energy metabolism pathways, for example the subunits of adenosine triphosphate (ATP)-synthase enzyme complex and dihidrolipoyl dehydrogenase that forms part of the pyruvate dehydrogenase complex that decarboxylates pyruvate into acetyl-CoA (Lissens et al. 2000). The increased levels of these proteins indicate that ms induced an increase in aerobic metabolism and production of ATP. This suggestion is supported by the increase in nucleoside diphosphate kinase B since this enzyme is involved in the synthesis of nucleoside triphosphates including ATP. Interestingly, nucleoside diphosphate kinase B also regulates transcription and deoxyribonucleic acid (DNA) binding (Kimura 2003). On the contrary, nucleoside diphosphate kinase A levels were decreased after ms.

Other proteins involved in glycolysis and the citric acid cycle were also decreased indicating a reduction in the activity of these pathways. Triosephosphate isomerase is involved in glycolysis where it interconverts dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). Decreased levels of this enzyme increase DHAP which is spontaneously converted to methylglyoxal. Methylglyoxal in turn modifies DNA and increases neuronal death, and it is therefore indicated in neurodegenerative diseases (Gnerer et al. 2006; Oláh et al. 2005). Glyoxalase domain-containing protein 4, part of the glyoxalase 1 family, contributes to the detoxification of methylglyoxal (Thornally 2003), and the reduction of this protein may lead to increased cell death. Interestingly, glyoxalase 1 mRNA has been found to be decreased in patients with major depressive disorder (Fujimoto et al. 2008); suggesting that decreased levels of this protein can be used as a biomarker for depression.

Inositol monophosphatase is another enzyme with significant clinical relevance. Decreased levels of this enzyme were been observed in our ms rats as well in the brains of patients with depression (Coupland et al. 2005), while treatment with myo-inositol has been shown to decrease depression scores in patients (Levine et al. 1995). The reduction in inositol monophosphatase will cause decreased production of myo-inositol, which is part of the phosphatidylinositol signaling pathway. This

pathway is activated by monoamine receptor binding, which serves to increase $Ca^{2+}$ release and entry into the cell, and replenishes phosphatidylinositol biphosphate in the cell membrane (Harvey et al. 2002; Berridge and Irvine 1984). Alternatively, since this pathway is closely linked to intracellular $Ca^{2+}$ levels, an alteration in its function may lead to dysregulation of $Ca^{2+}$ homeostasis. Interestingly, ms also decreased neurogranin, which is implicated in neuroplasticity of dendritic spines and axons, by regulating calmodulin availability and $Ca^{2+}$ immobilization in response to neurotransmitter receptor binding (Gerendasy and Sutcliffe 1997). Calmodulin-dependent kinase II is involved in the modulation of neurotransmission and synaptic plasticity (Braun and Schulman 1995). Another protein relating to neurotransmission is Amphiphysin 1, which is important for synaptic vesicle endocytosis and neurotransmitter recycling (Di Paolo et al. 2002) and was found to be downregulated in ms rats. In addition, DJ-1 mutations are normally found in patients with Parkinson's disease and the function of this protein is to reduce reactive oxygen species formed by dopamine catabolism and is therefore neuroprotective (Hedrich et al. 2004; Lev et al. 2009). It may therefore also be possible that decreased levels of DJ-1 found in our ms rats reflect a disturbance in the dopaminergic system.

## Escitalopram treatment

Escitalopram treatment affected the expression of a number of different proteins in ms rats as opposed to nr rats. Only 4 of the proteins related to energy metabolism were similarly upregulated in both ms and nr rats chronically treated with escitalopram in comparison to their saline injected controls. These were dihydrolipoyllysine-residue acetyltransferase component of the pyruvate dehydrogenase complex, pyruvate dehydrogenase E1 component subunit beta, isocitrate dehydrogenase [NAD] subunit alpha, and triosephosphate isomerase. As previously discussed, these proteins all function in aerobic energy metabolism pathways, the pyruvate dehydrogenase complex, the citric acid cycle and glycolysis to produce ATP.

Rats chronically treated with fluoxetine or venlafaxine showed similar upregulation of hippocampal proteins as our escitalopram treated groups (Khawaja et al. 2004). These proteins were alpha-enolase and pyruvate dehydrogenase E1 component subunit beta involved in energy metabolism and ATP production. In addition, dimethylarginine dimethylaminohydrolase 1 (DDAH1), which regulates nitric oxide synthase (NOS) activity by hydrolyzing asymmetrically methylated arginine residues, competitive inhibitors of NOS, to citrulline was upregulated (Ogawa et al. 1989; Tran et al. 2000).

Chronic escitalopram treatment increased the expression of some of the cytosolic proteins in the ventral hippocampus that were downregulated after ms. The increase in triosephosphate isomerase is important due to its function in reducing the concentration of methylglyoxal in neurons. Glyoxalase 1 was also upregulated in ms rats treated with escitalopram, which also functions in detoxification of methylglyoxal (Thornally 2003). DDAH1 and isocitrate dehydrogenase [NAD] subunit alpha were also upregulated after escitalopram treatment.

The expression of a number of proteins that were not affected by ms was also significantly altered in the treatment groups. In nr rats, treatment increased levels of

neuronal protein 22, which is related to neuronal morphology, which binds to the cytoskeleton, and is also implicated in neuroplasticity since increased levels have been found during synaptogenesis (Mori et al. 2004; De la Heras et al. 2007; Depaz and Wilce 2006). However, the reduction of DRP-2, 4 and 5, which are involved in axonal growth (Minturn et al. 1995; Inagaki et al. 2001) was observed after treatment in ms rats. A previous study also indicated that DRP-2 is related to escitalopram resistance in a chronic mild stress model for depression, since it was differentially regulated between responders and non-responders to escitalopram treatment (Bisgaard et al. 2007).

Escitalopram increased complexin in ms rats, a protein which facilitates $Ca^{2+}$—triggered neurotransmitter release at synapses as observed in complexin-knockout mice where the synaptic activity of neurons was decreased (Xue et al. 2008). The β-subunits of guanine nucleotide-binding protein (G-protein) were increased in nr rats treated with escitalopram. When neurotransmitters bind to their G-protein coupled receptors, various intracellular signalling pathways are activated (Gilman 1987). Usually the β-subunit of G-proteins specifically activates phospholipase C and adenylyl cyclase 2 (Boyer et al. 1992; Chen et al. 1997), but it has also been shown to be incorporated into microtubules and is thought to play a regulatory role in cytoskeletal structure (Wu et al. 1998).

## Conclusions

Maternal separation predisposes rat pups to develop depressive-like behaviour (Marais et al. 2008). The results of the present study indicate that the development of this behavioural abnormality may be associated with the alteration in the expression of a large number of cytosolic proteins in the ventral hippocampus. Chronic treatment with escitalopram only affects the expression of a few of the proteins that were altered by ms and it therefore is likely that escitalopram targets another group of cytosolic proteins to achieve its therapeutic effect. A limitation of this study is that it does not confirm the observations with another method such as western blotting, and this could be considered in future studies. The current data obtained with ms as a rat model for depression may be important as it indicates pathways and specific proteins that are potentially involved in the development of depression, thereby providing greater insight into the pathogenesis of the disorder.

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