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Effects of maternal separation and methamphetamine exposure on protein expression in the nucleus accumbens shell and core

J. J. Dimatelis · V. A. Russell · D. J. Stein · W. M. Daniels

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Abstract Early life adversity has been suggested to predispose an individual to later drug abuse. The core and shell sub-regions of the nucleus accumbens are differentially affected by both stressors and methamphetamine. This study aimed to characterize and quantify methamphetamine-induced protein expression in the shell and core of the nucleus accumbens in animals exposed to maternal separation during early development. Isobaric tagging (iTRAQ) which enables simultaneous identification and quantification of peptides with tandem mass spectrometry (MS/MS) was used. We found that maternal separation altered more proteins involved in structure and redox regulation in the shell than in the core of the nucleus accumbens, and that maternal separation and methamphetamine had differential effects on signaling proteins in the shell and core. Compared to maternal separation or methamphetamine alone, the maternal separation/methamphetamine combination altered more proteins involved in energy metabolism, redox regulatory processes and neurotrophic proteins. Methamphetamine treatment of rats subjected to maternal separation caused a reduction of cytoskeletal proteins in the shell and altered cytoskeletal, signaling, energy metabolism and redox proteins in the core. Comparison of maternal separation/methamphetamine to methamphetamine alone resulted in decreased cytoskeletal proteins in both the shell and core and increased neurotrophic proteins in the core. This study confirms that both early life stress and methamphetamine differentially affect the shell and core of the nucleus accumbens and demonstrates that the combination of early life adversity and later methamphetamine use results in more proteins being affected in the nucleus accumbens than either treatment alone.

Keywords Early life stress · Drugs of abuse · Ventral striatum · Proteomics

J. J. Dimatelis (✉) · V. A. Russell
Department of Human Biology, Faculty of Health Sciences,
University of Cape Town,
Observatory,
7925, Cape Town, South Africa
e-mail: jacqueline.dimatelis@uct.ac.za

D. J. Stein
Department of Psychiatry and Mental Health,
Faculty of Health Sciences, University of Cape Town,
Observatory,
7925, Cape Town, South Africa

W. M. Daniels
Discipline of Human Physiology, School of Biomedical Sciences,
Faculty of Health Sciences, University of KwaZulu-Natal,
Westville,
4001, Durban, South Africa

Introduction

Exposure to stress has been shown to affect the core and shell subregions of the nucleus accumbens differently. For example, rats that were allowed to witness other rats receiving electric footshocks had increased extracellular dopamine concentrations in the shell and not the core of the nucleus accumbens (Wu et al. 1999). Stress has in turn been found to be associated with altered subjective effects of cocaine, amphetamine and methamphetamine and to enhance drug craving in humans (Sinha et al. 1999; Weiss et al. 2001; Söderpalm et al. 2003; Hamidovic et al. 2010), possibly implicating a stress-induced dopamine increase in the nucleus accumbens shell, altering responsivity toward psychostimulants.

Methamphetamine has also been shown to differentially affect the nucleus accumbens core and shell subregions. Microdialysis studies in rodents indicated that drugs of abuse increase dopamine neurotransmission preferentially

in the shell (Imperato and Di Chara 1986; Imperato et al. 1986; Carboni et al. 1989). Similar findings were obtained in subsequent brain imaging studies in humans (Drevets et al. 2001; Leyton et al. 2002; Boileau et al. 2003). Further, reinstatement of cocaine-seeking behaviour in the rat involved activation of dopamine receptors in the shell rather than the core (Schmidt et al. 2006).

Early exposure to stress, in the form of maternal separation has been shown to alter the subsequent response to drugs of abuse, leading to enhanced cocaine self-administration and psychostimulant-induced locomotor activity in rats and mice (Brake et al. 2004; Matthews and Robbins 2003; Meaney et al. 2002; Kikusui et al. 2005). In humans, adolescence is associated with increased sensation seeking and hence increased risk of susceptibility to taking drugs (Laviola et al. 1999; Spear 2000). Previously, we showed that methamphetamine administration during adolescence resulted in altered protein expression in the frontal cortex in adulthood, including changes in proteins involved in cyto-architecture, neurotransmission and intracellular signaling in rats (Faure et al. 2009). However, few studies have examined the combined effects of early adversity and later (e.g. adolescent) methamphetamine exposure on protein expression.

Isobaric tagging of peptides (iTRAQ) enables simultaneous identification and quantification of peptides using tandem mass spectrometry (MS/MS) (Thompson et al. 2003). This approach has previously been used to study the effects of methamphetamine on the proteome (Liao et al. 2005; Iwazaki et al. 2006; 2007; 2008; Li et al. 2008; Yang et al. 2008; Faure et al. 2009). These studies indicate that methamphetamine induces alterations in proteins involved in degradation, redox regulation, neuroplasticity, cytoskeletal modifications and altered synaptic function. Maternal separation stress has similarly been shown to affect aforementioned proteins (Marais et al. 2009). The aim of the present study was to determine whether early life stress could potentiate the effect of methamphetamine exposure on the expression of these functional proteins.

Experimental procedures

Animals

Male Sprague Dawley rats were used in this experiment. Ethical approval for all experimental procedures was provided by the Committee for Experimental Animal Research of the University of Stellenbosch. Animals were housed at the Central Research Animal Facility (AAALAC accredited) of the University of Stellenbosch. All rats were housed in the same colony room separate from the experimental rooms in which the stress procedures, injections and brain dissections occurred. Animals were housed according to standard laboratory conditions as stipulated by the Ethical Guidelines of the University for the Housing of Experimental Animals. Rats were housed (2–4) in 40×25×20 cm Plexiglas cages with corncobs as bedding. The temperature was kept constant at 22 °C, humidity at 55 % and food and water was available ad libitum for the duration of the experiment.

Drugs

Methamphetamine hydrochloride, obtained from US Pharmacopeia Convention Inc. (Rockville, USA), was dissolved in 0.9 % saline and administered at a dose of 1 mg/kg intraperitoneally (i.p.).

Maternal separation paradigm

Male and female rats were paired and their offspring used for experimental purposes. The date of birth was designated as postnatal day (PND) 0. Maternal separation commenced 2 days later on PND 2 until PND 14. Rat pups were separated from their mothers for a 3 h daily period between 09 h00 and 13 h00. This protocol is in accordance with the deprivation procedures employed by Ladd et al. (2000). The pups were moved to a new cage, while the mother remained in the home cage. The cages containing the pups were moved to an isolated dedicated room where the pups were kept warm under infrared lights (30–33 °C), thereby preventing exposure to hypothermic conditions. Control litters were reared normally without separation from the dam. After maternal deprivation was completed, animals were subjected to normal housing conditions.

Experimental design

All rat pups were weaned at PND 21. Only male rats were used for the experiments. The rats were divided into four groups:

1) Control Saline (CS): animals not subjected to maternal separation and receiving 4 saline injections.
2) Maternal separation Saline (MS): animals subjected to maternal separation and 4 saline injections.
3) Control Methamphetamine (CM): animals not subjected to maternal separation and receiving 4 methamphetamine injections.
4) Maternal separation Methamphetamine (MM): animals subjected to maternal separation and methamphetamine injections.

Methamphetamine administration occurred on PND 33–36. The rationale for using 4 methamphetamine injections is firstly based on the study of Shimosato and Ohkuma (2000) who showed that 4 methamphetamine pairings (1 mg/kg) in a dual-cue CPP apparatus resulted in the highest CPP score.

Secondly, CPP is based on an associative learning paradigm and repeated pairings are necessary to form an association between the environment and the rewarding effects of the drug. Animals were decapitated on PND 52. The brains were removed and the shell and core of the nucleus accumbens were dissected according to the rat brain atlas of Paxinos and Watson (1986) and immediately frozen and stored in liquid nitrogen for later analysis.

Fractionation of striatal shell and core tissue

Three shell and core tissue samples were pooled to obtain sufficient protein for the subsequent experiments. Tissue samples were subjected to fractionation using a commercially available ProteoExtract Subcellular Proteome Extraction Kit (Merck, Calbiochem). The sample was separated into four fractions, which included cytosolic, membrane/organelle, nucleic and cytoskeletal matrix proteins. Changes in the cytosolic cellular fraction were investigated. The protein content of the cytosolic protein fractions was determined by the Bradford method according to the the ReadyPrep 2-D Cleanup Kit (Bio-Rad). After completion of the Cleanup, samples were suspended in ammonium bicarbonate (100 mM) solution and the volume reduced in a roto-evaporator (Eppendorf) to form a tight pellet for further analysis.

Sample preparation and tryptic digestion

Each sample was re-suspended in 50 μl 1 % PPS (pyridinium propyl sulfonate) silent surfactant according to the PPS silent surfactant detergent protocol (Protein Discovery, San Diego, U.S.A.) and the insoluble matter removed by centrifugation. Protein concentration was again determined, this time a nanodrop spectrophotomer was used. Equal amounts of protein were taken from each sample, to obtain the final sample of cytosolic proteins for each group of animals. The combined samples formed groups 1 to 8 respectively. Equal aliquots (100 μg) were taken from each combined group sample and digested with trypsin according to a slightly modified PPS protocol with trypsin added in a 1:10 ratio. The resultant digest was evaluated using both mass spectrometry and liquid chromatography.

Isobaric tag for relative and absolute quantitation (iTRAQ) of proteins

Peptides from the 8 groups (50 μg) were labeled using 8—plex iTRAQ labeling. The iTRAQ labeling reaction was performed according to the ABI silent protocol substituting isopropanol for ethanol (Applied Biosystems, Absciex, Framingham, U.S.A). The groups were labeled sequentially with iTRAQ labels 113 to 121, i.e. group 1 with 113, group 2 with 114 and so forth. An aliquot of each sample was mixed for confirmation of labeling by tandem mass spectrometry. The data indicated that all 8 samples had been labeled with iTRAQ tags.

Cation exchange

The mixture of labeled peptides was separated by re-suspending each sample in strong cation exchange (SCX) equilibration buffer (5 mM KH_2PO_4) (Sigma), 25 % acetonitrile (ACN) (ROMIL, Cambridge, U.K.) and applied to a pre-equilibrated SCX SPE device (Supelco SupelClean). The peptides were eluted from the device with 300 μl elution buffer (1 M HCO_2NH_4/25 % acetonitrile (Sigma, St Louis, MO, U.S.A.)). Mass spectra indicated some peptide in the flow through and wash as expected and peptides were detected in the eluate from the SPE device. The sample volume was reduced to 25 μl using a roto-evaporator (Eppendorf). The samples for MS analysis were desalted using ZipTip C18 SPE devices (Millipore, Bellerica, MA, U.S.A.).

Liquid chromatography

Peptides were separated on a Dionex Ultimate 3000 nano-LC with a C18 Pepmap column (75 μm×15 cm, LC Packings). The solvent systems were A: 2 % ACN/H_2O, 0.1 % trifluoroacetic acid (TFA); B: 80 % ACN/H_2O, 0.08 % TFA. The sample was loaded onto the column using Solvent A. Peptides were eluted with 5 % B for 5 min, 5–15 % B over 5 min, 15 %–45 % B over 70 min and 45 %–60 % B 10 min with a flow rate of 200 nL/min. The eluted peptides were spotted onto a MALDI source plate using a Probot (LC Packings) with continuous matrix addition at 600 nl/min. The matrix was 7.5 mg/ml α-cyano-4-hydroxycinmamic acid (Fluka) with 10 mM $NH_4H_2PO_4$ (Fluka) in 66 % acetonitrile, 0.1 % TFA. PepMix4 (LaserBiolabs) 5 point calibration mixture was spiked into the sample at an average of 10 fmol/μl (final quantity of 6.6 fmol total peptide/spot). Fractions were collected every 12 s and the collection started 16 min after sample injection.

Mass spectrometry

The samples collected from the chromatographic separation were mixed with MALDI matrix through an inline T connecter and spotted on a MALDI source plate. The α-cyano-4-hydroxycinnamic acid (CHCA) matrix was spiked with a 5-point internal calibration mixture. Calibration analysis showed that internal calibration was obtained in 99.6 % (1,014/1,018) of the spots and that the mass spectrometer was functioning within specifications.

Mass spectrometry was performed using an Applied Biosystems 4800 MALDI ToF/ToF. Parent ion spectra were

recorded in linear positive ion mode with 400 shots/spectrum and laser intensity of 4000 arbitrary units. The grid voltage was set to 16 kV. The spectra were processed using the PepMix4 internal calibration points. MS/MS spectra were recorded in positive mode with 1 kV deceleration voltage and a total of 600 laser shots/spectrum with the laser set to 5,000 arbitrary units.

Statistics

The mass spectral data were analyzed with ProteinPilot software (ProteinPilot™ Software 3.0, Applied Biosystems, MDS Analytical Technologies) using the *Ratus ratus* database. A Paragon Algorithm was used to determine differentially expressed proteins by calculating the average ratio of sample protein to reference protein, for each protein, along with the associated p-value and error factors. The p-value for each protein was derived from a *t*-test, where the sample size (n) was the number of peptides contributing to the identification of a specific protein and calculated as

$$t = \frac{(\text{Average of Log Ratios} - \text{Log Bias})}{\text{Standard error of Average of Log Ratios}}$$

where Log Ratio log (ratio of specific protein in test sample to specific protein in reference sample)
and Log Bias log (sample bias)

Proteins detected with >95 % confidence and those that differed significantly between the experimental groups ($p < 0.05$) are reported as fold change with respect to the appropriate reference sample. Bonferroni correction for multiple comparisons for each protein was $p < 0.0166$.

Results

Using the *Ratus ratus* database, 126 proteins (95 % confidence) were identified. The average mass deviation was −0.080 Da. The iTRAQ quantitation was performed using ProteinPilot with default settings and relative abundance expressed in terms of reporter signal. Analysis of the peptide report showed that 48.36 % (546/1,129) of the ions could be auto quantified, 26.48 % (299/1,129) were auto quantified but shared sequence data with other proteins and 24.09 % (277/1,129) were auto quantified with low confidence. In total 98.93 % (1,117/1,129) of all ions were quantified. Fragmentation data showed that 0.97 % of the ions did not contain an iTRAQ label and the experimental groups/samples examined were tagged with isobaric iTRAQ tags ranging from 113 to 121.

The sequence conversion ratio was 46.4 % with 720 distinct peptides being identified. From the 720 peptides, 303 proteins were identified with 126 detected after grouping using the Pro Group algorithm. Of the 126 proteins detected after grouping, the cytosolic proteins that were quantitatively significantly different from control groups in both shell (Table 1) and nucleus accumbens core (Table 2) fractions, are reported.

We found 27 cytosolic proteins in the shell sub-division and 24 proteins in the core subdivision of the nucleus accumbens that differed in expression between the experimental groups (CS, CM, MS, MM). We found that in comparison with CS, MS had changed more proteins in the nucleus accumbens shell than in the core (MS:CS values; Tables 1 and 2) (Figs. 1 and 2). MS decreased cytoskeletal proteins (actin cytoplasmic 2, tubulin alpha-1B chain, tubulin beta-2A chain, microtubule-associated protein 2 and tubulin alpha-4A chain) and increased peptidyl-prolyl cis-trans isomerase FKBP1A in the nucleus accumbens shell. However, MS also decreased actin cytoplasmic 2 and tubulin alpha-1B chain in the core subdivision. MS decreased proteins involved in energy metabolism (creatine kinase B-type, glyceraldehyde-3-phosphate dehyrogenase, pyruvate kinase isozymes M1/M2) in the shell and did not have an effect in the core. MS increased proSAAS and decreased importin subunit alpha-6 levels in the shell and increased importin subunit alpha-6 levels in the core. Proteins involved in redox regulation were both increased (cytochrome c, somatic) and decreased (ubiquitin carboxyl-terminal hydrolase isozyme L1, peroxiredoxin-2) in the shell of MS animals with no change in these proteins in the nucleus accumbens core. MS resulted in a minimal change in neurotrophic proteins, by decreasing gamma-enolase in the shell and no change in the core subdivision. The effect of maternal separation in rats that were subsequently treated with methamphetamine (the combination group (MM) compared to non-separated control rats that received methamphetamine (CM) Table 3), included increased signaling protein (14-3-3 protein zeta/delta) and decreased cytoskeletal proteins (actin cytoplasmic 2, tubulin beta-2A chain, tubulin beta-2C chain) in the shell. Maternal separation also increased structural (peptidyl-prolyl cis-trans isomerase A) and neurotrophic proteins (brain acid soluble protein 1) and decreased cytoskeletal (tubulin beta 2A chain) metabolic (Acyl-CoA-binding protein) and signaling (Scg2 protein) proteins in the core of rats that were subsequently treated with methamphetamine.

In comparison to non-methamphetamine treated controls, exposure to methamphetamine (CM:CS values; Tables 1 and 2) again led to differential effects in the nucleus accumbens shell and core. Methamphetamine decreased cytoskeletal proteins (actin cytoplasmic 2, tubulin alpha-1B chain, tubulin beta-2A chain, microtubule-associated protein 2) in the shell subdivision, and altered cytoskeletal proteins in the

Table 1 iTRAQ ratios for cytosolic proteins in the shell subdivision of the nucleus accumbens of maternally separated rats treated with methamphetamine in adolescence

Accession number	Protein name	MS:CS	P value	CM:CS	P value	MM:CS	P value
Cytoskeletal/structural							
sp|P63259	Actin, cytoplasmic 2	0.409	0.001[a]	0.536	0.001[a]	0.315	0.001[a]
sp|Q6P9V9	Tubulin alpha-1B chain	0.262	0.001[a]	0.483	0.001[a]	0.213	0.001[a]
sp|P85108	Tubulin beta-2A chain	0.406	0.011[a]	0.544	0.002[a]	0.300	0.002[a]
sp|Q62658	Peptidyl-prolyl cis-trans isomerase FKBP1A	1.417	0.001[a]	0.914	0.496	1.195	0.125
sp|P15146	Microtubule-associated protein 2	0.565	0.007[a]	0.557	0.001[a]	0.450	0.001[a]
sp|Q5XIF6	Tubulin alpha-4A chain	0.452	0.034	0.801	0.472	0.338	0.066
Energy metabolism							
sp|P07335	Creatine kinase B-type	0.690	0.001[a]	0.772	0.001[a]	0.663	0.001[a]
sp|P04797	Glyceraldehyde-3-phosphate dehydrogenase	0.450	0.001[a]	0.645	0.058	0.395	0.001[a]
sp|O88989	Malate dehydrogenase, cytoplasmic	0.480	0.066	0.657	0.142	0.362	0.048
sp|P11980	Pyruvate kinase isozymes M1/M2	0.473	0.006[a]	0.753	0.264	0.417	0.001[a]
sp|Q6PCU2	V-type proton ATPase subunit E 1	0.942	0.819	1.126	0.835	0.730	0.041
Neurotransmission/signalling							
sp|Q9QXU9	ProSAAS	1.378	0.047	1.215	0.249	1.301	0.357
sp|P61983	14-3-3 protein gamma	0.799	0.138	0.744	0.011[a]	0.832	0.429
sp|Q56R16	Importin subunit alpha-6	0.161	0.020	1.178	0.020	0.206	0.020
Protein fate/redox regulation							
sp|Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1	0.798	0.001[a]	0.829	0.182	0.805	0.003[a]
sp|P62898	Cytochrome c, somatic	1.516	0.039	1.310	0.440	1.673	0.008[a]
sp|P35704	Peroxiredoxin-2	0.733	0.014[a]	0.959	0.857	0.774	0.166
Protein synthesis/neurotrophic							
sp|Q05175	Brain acid soluble protein 1	1.602	0.051	1.208	0.104	1.698	0.006[a]
sp|P07323	Gamma-enolase	0.500	0.001[a]	0.607	0.007[a]	0.447	0.002[a]
sp|Q63754	Beta-synuclein	1.183	0.280	1.070	0.657	1.270	0.024
sp|P55068	Brevican core protein	0.791	0.095	0.722	0.112	0.811	0.028
Other							
tr|Q6PED0	Ribosomal protein S27a	0.714	0.002[a]	1.355	0.031	0.829	0.029
sp|P63055	Purkinje cell protein 4	1.370	0.001[a]	1.073	0.574	1.341	0.007[a]
sp|P63018	Heat shock cognate 71 kDa protein	0.377	0.013	0.729	0.004[a]	0.400	0.002[a]
sp|Q5XI72	Eukaryotic translation initiation factor 4H	1.398	0.118	1.028	0.676	1.194	0.044
sp|P62959	Histidine triad nucleotide-binding protein 1	1.213	0.346	1.286	0.128	1.415	0.037
sp|O35814	Stress-induced-phosphoprotein 1	0.814	0.259	0.906	0.601	0.787	0.045

CS are animals not subjected to maternal separation that received saline injections during adolescence. MS are the animals exposed to early life maternal separation and subjected to saline injections during adolescence. CM are the non-maternally separated rats exposed to methamphetamine injections during adolescence. MM rats were subjected to both maternal separation and methamphetamine injections

[a] Bonferonni correction applied

core, decreasing structural proteins (tubulin alpha-1B chain, tubulin beta-2A chain and microtubule-associated protein 2) and increasing microtubule-associated protein tau. Methamphetamine decreased creatine kinase B-type in the shell and had a greater effect in the core, with decreases in multiple energy-related metabolism proteins (creatine kinase B-type, Glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase isozymes M1/M2). Methamphetamine increased signaling proteins (importin subunit alpha-6) and decreased (14-3-3 protein gamma) in the shell and increased importin subunit alpha-6 and myristoylated alanine-rich C-kinase substrates in the core. Methamphetamine did not alter any proteins involved in redox regulation in the shell but increased cytochrome c oxidase subunit 5A in the core. Methamphetamine also decreased neurotrophic protein and gamma-enolase in the shell and core and increased myotrophin levels in the core. Methamphetamine treatment of rats that had been subjected to maternal separation (the combination group (MM)) compared to the maternally separated rats that were not exposed to methamphetamine (MS)

Table 2 iTRAQ ratios for cytosolic proteins in the core subdivision of the nucleus accumbens of maternally separated rats treated with methamphetamine in adolescence

Accession number	Protein name	MS:CS	P value	CM:CS	P value	MM:CS	P value
Cytoskeletal/structural							
sp|P63259	Actin, cytoplasmic 2	0.835	0.001[a]	0.672	0.001[a]	0.803	0.001[a]
sp|Q6P9V9	Tubulin alpha-1B chain	0.773	0.008[a]	0.623	0.003[a]	0.754	0.016[a]
sp|P19332	Microtubule-associated protein tau	1.082	0.417	1.171	0.016 [a]	1.067	0.479
sp|P85108	Tubulin beta-2A chain	0.800	0.059	0.662	0.001[a]	0.796	0.002[a]
sp|P62329	Thymosin beta-4	1.107	0.131	1.123	0.285	1.220	0.025
sp|P15146	Microtubule-associated protein 2	0.877	0.219	0.772	0.043	0.785	0.006[a]
Energy metabolism							
sp|P07335	Creatine kinase B-type	0.980	0.588	0.859	0.001[a]	0.870	0.001[a]
sp|P04797	Glyceraldehyde-3-phosphate dehydrogenase	0.891	0.318	0.716	0.010[a]	0.832	0.012[a]
sp|P11980	Pyruvate kinase isozymes M1/M2	0.969	0.494	0.871	0.046	0.973	0.463
Neurotransmission/signalling							
sp|P47728	Calretinin	1.043	0.434	0.933	0.108	0.816	0.033
sp|P30009	Myristoylated alanine-rich C-kinase substrate	0.914	0.262	1.186	0.002 [a]	1.106	0.364
tr|Q5FVI0	Arpp-21 protein	0.802	0.607	0.905	0.634	1.083	0.039
sp|Q56R16	Importin subunit alpha-6	2.137	0.018	2.036	0.013 [a]	1.088	0.026
Protein fate/redox regulation							
sp|P37377	Alpha-synuclein	0.972	0.802	1.082	0.187	1.153	0.030
sp|Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1	1.035	0.485	0.948	0.395	0.865	0.024
sp|P11240	Cytochrome c oxidase subunit 5A, mitochondrial	1.220	0.207	1.441	0.012 [a]	1.128	0.107
sp|P62898	Cytochrome c, somatic	0.924	0.681	1.092	0.431	1.290	0.034
Protein synthesis/neurotrophic							
sp|Q05175	Brain acid soluble protein 1	1.089	0.222	1.121	0.119	1.173	0.016 [a]
sp|P07323	Gamma-enolase	0.908	0.089	0.758	0.003[a]	0.851	0.050
sp|P62775	Myotrophin	1.014	0.799	1.114	0.006 [a]	1.087	0.352
Other							
sp|P63055	Purkinje cell protein 4	0.987	0.858	1.160	0.019	1.113	0.045
sp|P26772	10 kDa heat shock protein, mitochondrial	1.227	0.012 [a]	1.041	0.368	1.084	0.272
sp|O35814	Stress-induced-phosphoprotein 1	1.115	0.478	1.138	0.732	1.131	0.037
sp|P02688-4	Isoform 14 kDa of Myelin basic protein S	1.330	0.006 [a]	0.959	0.638	1.023	0.731

CS are animals not subjected to maternal separation that received saline injections during adolescence. MS are the animals exposed to early life maternal separation and subjected to saline injections during adolescence. CM are the non-maternally separated rats exposed to methamphetamine injections during adolescence. MM rats were subjected to both maternal separation and methamphetamine injections

[a] Bonferonni correction applied

(Table 3), resulted in decreased cytoskeletal proteins (actin cytoplasmic 2, tubulin beta-2A chain) in the shell. Methamphetamine increased peroxiredoxin-5 mitochondrial protein and decreased cytoskeletal (tubulin beta-2A chain) signaling (calretinin) and metabolic proteins (creatine kinase B-type) in the core of maternally separated rats.

In comparison to controls, the combination of maternal separation and methamphetamine exposure (MM:CS values, Tables 1 and 2) resulted in decreased expression of structural proteins (actin cytoplasmic 2, tubulin alpha-1B chain, tubulin beta-2A chain and microtubule-associated protein 2) in the shell and the core, except that thymosin beta-4 levels were increased in the core. The combination of the two stressors altered more proteins involved in energy metabolism in the nucleus accumbens shell subdivision by decreasing creatine kinase B-type, glyceraldehydes-3-phosphate dehydrogenase, malate dehydrogenase, pyruvate kinase isozyme M1/M2 and V-type proton ATPase subunit E1. In the core only creatine kinase B-type and glyceraldehydes-3-phosphate dehydrogenase were decreased in response to MS and MA treatment. The signaling protein, importin subunit alpha-6, was decreased in the shell in the MM group, while in the core; importin subunit alpha-6 and arpp-21 were increased and calretinin levels decreased. The combination of maternal separation and methamphetamine exposure also resulted in decreased ubiquitin carboxyl-terminal hydrolase isozyme and

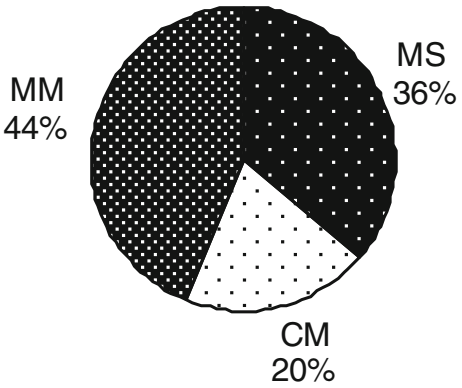

Fig. 1 Pie chart of the nucleus accumbens shell illustrating the proportions of significantly changed proteins caused by maternal separation (MS), methamphetamine (CM) and the combination of MS and CM (MM) out of the total of 27 significantly changed proteins

increased cytochrome c levels in both the shell and core while also increasing alpha-synuclein levels in the core. Maternal separation followed by methamphetamine exposure increased the neurotrophic protein brain acid soluble protein 1 and decreased gamma-enolase in both the shell and core and decreased beta-synuclein and brevican core protein in the shell.

Discussion

We found that maternal separation and methamphetamine differentially altered functional groups of proteins in the nucleus accumbens shell and core, while the combination of maternal separation and methamphetamine further altered protein expression. The proteins that were differentially expressed between the experimental groups were functionally associated with cytoskeletal modifications, energy metabolism, intracellular signaling, protein degradation and cellular growth. A subset of the altered proteins will be discussed in the context of our understanding of the pathophysiology of exposure to early maternal separation or methamphetamine exposure.

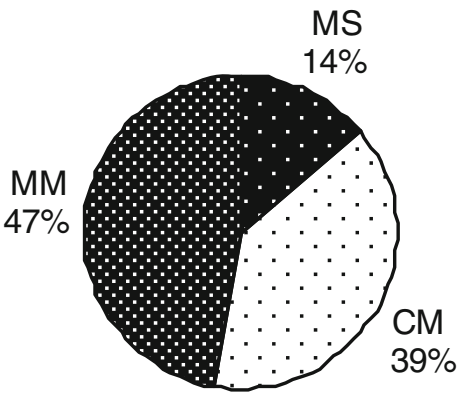

Fig. 2 Pie chart of the nucleus accumbens core illustrating the proportions of significantly changed proteins caused by maternal separation (MS), methamphetamine (CM) and the combination of MS and CM (MM) out of the total of 24 significantly changed proteins

Cytoskeletal proteins such as actin and microtubules play a key role in the maintenance of neuronal structure and function (Vale et al. 1992). Modifications to these proteins may therefore have far-reaching effects on neuron function. In the present study, a number of cytoskeletal proteins were decreased by maternal separation and by methamphetamine in the nucleus accumbens shell and core. These included actin cytoplasmic 2, tubulin alpha-1B chain and beta-2A chain, and microtubule-associated protein 2, suggesting that both maternal separation and methamphetamine caused changes in cytoskeletal structure that may have affected neurotransmission and synapse function. Maternal separation has previously been shown to alter structural proteins in the ventral hippocampus (Daniels et al. 2011), while clinical studies have also indicated prefrontal cortex and hippocampal volume reductions after early life stress consistent with a deficit in structural proteins (van Harmelen et al. 2010; Frodl et al. 2010). Cytoskeletal alterations have also been shown to occur after methamphetamine administration in various brain areas including the striatum, hippocampus, prefrontal cortex, cingulate cortex, and the amygdala (Liao et al. 2005; Iwazaki et al. 2006; 2007; 2008; Yang et al. 2008; Kobeissy et al. 2008). For instance, methamphetamine has been shown to disrupt cytoskeletal structure of dopaminergic terminals, while sparing neuronal somata (McCann and Ricaurte 2004). Maternal separation may predispose cytoskeletal structure to worsen the effects of methamphetamine destruction.

In support of disruptions in cytoskeletal integrity, thymosin beta-4 concentrations were found to be increased in the accumbens core of animals exposed to both maternal separation and methamphetamine treatment. Thymosin beta-4 forms a 1:1 complex with actin and inhibits actin polymerization (Safer et al. 1991). Thus, the spontaneous assembly of monomeric actin is prevented by thymosin, while profilin promotes barbed-end actin filament growth (Goldschmidt-Clermont et al. 1992; Pantaloni and Cartier 1993; Kang et al. 1999). Additionally, thymosin beta-4 also plays a role in motility, axonal pathfinding, differentiation, neurite formation and proliferation (Border et al. 1993; Otero et al. 1993; Molitoris 1997; Huff et al. 2001; Kobayashi et al. 2002). Upregulation of thymosin beta-4 has also been found after brain ischaemia and kainate neurotoxicity (Vartiainen et al. 1996; Carpintero et al. 1999; Popoli et al. 2007). Our findings are therefore in line with previous evidence indicating alterations in the expression of proteins related to plasticity, under conditions of stress and toxicity, and with the hypothesis of the combined effect of early adversity and exposure to substances.

Table 3 iTRAQ ratios for cytosolic proteins in the shell and core subdivision of the nucleus accumbens of animals subjected to maternal separation and treated with methamphetamine in adolescence when compared to either treatment alone

Nucleus accumbens shell			
Accession number	Protein name	MM:MS	P value
sp|P63259|	Actin, cytoplasmic 2	0.363	0.015[a]
sp|P85108|	Tubulin beta-2A chain	0.751	0.003[a]
Accession number	Protein Name	MM:CM	P Value
sp|P63102|	14-3-3 protein zeta/delta	1.836	0.041
sp|P63259|	Actin, cytoplasmic 2	0.205	0.001[a]
sp|P85108|	Tubulin beta-2A chain	0.660	0.002[a]
sp|Q6P9T8|	Tubulin beta-2C chain	0.190	0.046
Nucleus accumbens core			
Accession number	Protein Name	MM:MS	P Value
sp|P85108|	Tubulin beta-2A chain	0.862	0.028
sp|P47728|	Calretinin	0.666	0.037
sp|P07335|	Creatine kinase B-type	0.711	0.047
sp|Q9R063|	Peroxiredoxin-5, mitochondrial	5.754	0.002[a]
Accession number	Protein Name	MM:CM	P Value
sp|P85108|	Tubulin beta-2A chain	0.946	0.011[a]
sp|P10111|	Peptidyl-prolyl cis-trans isomerase A	1.086	0.008[a]
sp|Q05175|	Brain acid soluble protein 1	1.106	0.040
sp|P11030|	Acyl-CoA-binding protein	0.575	0.042
tr|Q6P7R4|	Scg2 protein	0.630	0.046

MS are the animals exposed to early life maternal separation and subjected to saline injections during adolescence. CM are the non-maternally separated rats exposed to methamphetamine injections during adolescence. MM rats were subjected to both maternal separation and methamphetamine injections

[a] Bonferonni correction applied

The present study demonstrated a general decrease in proteins involved in energy metabolism in animals treated with methamphetamine. This reduction in protein expression was observed in both the shell and core subregions of the nucleus accumbens. These proteins included glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase isozyme M1/M2 and V-type proton ATPase subunit E1. The core was affected more than the shell in the methamphetamine treated group, and a greater number of proteins involved in metabolism was reduced in the shell following maternal separation combined with later methamphetamine treatment. The methamphetamine findings are in line with previous findings demonstrating that repeated methamphetamine use decreased energy metabolism in the hippocampus, dorsal raphe nucleus and amygdala in rats (Huang et al. 1999; Iwazaki et al. 2008). In accordance with these reports, methamphetamine reduced striatal ATP which paralleled methamphetamine-induced dopamine depletion (Chan et al. 1994). Similarly, clinical studies have reported cerebral glucose hypometabolism in human methamphetamine abusers (Kim et al. 2005). The finding that combined maternal separation and methamphetamine further reduce expression of proteins involved in energy metabolism is consistent with previous work indicating that maternal separation reduced metabolic protein levels in ventral hippocampus (Marais et al. 2009).

Significant differences in the expression of proteins that are members of signal transduction or neurotransmission pathways were found in the nucleus accumbens shell and core in animals exposed to maternal separation, methamphetamine and the combination of maternal separation plus methamphetamine. For example, in the shell sub-region, ProSAAS was increased after maternal separation compared to non-maternally separated rats and 14-3-3 protein gamma was decreased after methamphetamine treatment compared to non-methamphetamine treated animals. ProSAAS is a granin-like protein which inhibits the action of prohormone convertase (PC) 1. Convertases usually mediate the proteolytic cleavage of many peptide precursors via the regulated/constitutive secretory pathway (Fricker et al. 2000; Qian et al. 2000). However, in the brain, proSAAS itself is cleaved into a smaller peptide that is unable to inhibit PC1 (Mzhavia et al. 2001). It has been suggested that cleaved proSAAS may act as a neuropeptide in the brain (Mzhavia et al. 2002). The increase in proSAAS expression after maternal separation in the shell sub-region could possibly be related to the role neuropeptides play in the stress response. For instance, it is known that neuropeptide Y can activate the hypothalamic-pituitary-adrenal (HPA) axis in response to maternal separation (Schmidt et al. 2008). The increase in proSAAS in the maternally separated animals may reflect regulation of HPA axis activity. The 14-3-3 protein gamma is known to regulate signal transduction pathways which are involved in cell proliferation, differentiation and survival (Jin et al. 2004; Bridges and Moorhead 2005; Aitken 2006; Chen et al. 2006; Ajjappala et al. 2009), and 14-3-3 protein gamma activates tyrosine and tryptophan hydroxylases, protein kinase C (PKC) and Raf-1 in the mitogen activated protein kinase (MAPK) signal transduction pathway (Aitken 1995). The decrease in

14-3-3 protein gamma expression in shell after methamphetamine exposure is consistent with altered signal transduction and may also reflect regulation of the HPA axis, since DARPP-32 deficient mice which is also a protein that mediates multiple signaling cascades do not show activation of the HPA axis following binge cocaine exposure (Zhou et al. 1999).

Increased expression of signal transduction proteins in the accumbens core, e.g. myristoylated alanine-rich C-kinase substrate (MARCKS), Arpp-21 and alpha-synuclein after methamphetamine treatment and the combination of maternal separation followed by methamphetamine are notable, as each plays a key role in important cellular processes that may be altered by exposure to methamphetamine. In particular, the core is of importance, since food- (i.e. natural reward) conditioned stimuli increases dopamine in the core while drug-conditioned stimuli increases dopamine in the shell (Bassareo et al. 2011) and hence if methamphetamine alters core signaling proteins, could possibly interfere with the rewarding properties of natural rewards and contribute to addiction pathology.

MARCKS is phosphorylated by PKC and also targeted by calmodulin (CaM) which translocates MARCKS from the plasma membrane, while the return of MARCKS back to the plasma membrane is mediated by dephosphorylation by calcineurin or the lowering of intracellular calcium (Thelen et al. 1991; Clarke et al. 1993; Seki et al. 1995; Arbuzova et al. 1998; Ohmori et al. 2000; Arbuzova et al. 2002). It is proposed that MARCKS mediates cross-talk between the PKC and CaM signal transduction pathways (Arbuzova et al. 2002). MARCKS has been found to regulate many processes including endocytosis, exocytosis and neurosecretion (Aderem 1992; Blackshear 1993), which may then be altered by exposure to methamphetamine.

Arpp-21 is a neuronal phosphoprotein which occurs in high concentrations in the nucleus accumbens (Ouimet et al. 1989). Arpp-21 is a substrate for cAMP-dependent protein kinase (Walaas et al. 1983) and is suggested to act as a third messenger in the intracellular cascade involving adenylate cyclase (AC). One of the first messengers activating AC includes dopamine binding to dopamine D1 receptors (Ivkovic et al. 1996). The limbic striatum is highly enriched with dopamine D1 and D2 receptors that either stimulate or inhibit AC depending on the G-protein linked to the receptor, thereby modulating cAMP levels in this brain region (Stoof and Kebabian 1981; Levey et al. 1993; Missale et al. 1998; Zhuang et al. 2000). Furthermore, Caporaso et al. (2000) found Arpp-21 phosphorylation increased after activation of D1 receptors in the striatum, while D2 activation via quinpirole reduced Arpp-21 phosphorylation. Since the rewarding effects of psychostimulants are mediated by increased dopamine transmission in the mesolimbic dopaminergic pathway (Koob et al. 1998), it was proposed that phosphorylation of Arpp-21 is involved in methamphetamine-induced intracellular signal transduction.

Increased alpha-synuclein levels in the accumbens core in the present study after combined maternal separation and methamphetamine treatment is consistent with previous findings whereby methamphetamine has been linked to altered alpha-synuclein levels. For example, methamphetamine neurotoxicity has been found to lead to the formation of inclusion bodies in substantia nigra and striatal neurons (Lotharius and Brundin 2002) in both the soma and terminal endings (Fornai et al. 2004a; Brenz Verca et al. 2003). These cytoplasmic inclusions have been found to contain α-synuclein and ubiquitin (Lowe et al. 1990; Spillantini et al. 1997; Chung et al. 2001; Fornai et al. 2004b). Alpha-synuclein has been found to possess protective qualities since it prevented further oxidative damage by interacting with degradation products of dopamine (Sulzer 2001; Conway et al. 2001; Machida et al. 2005). It has been suggested that increased alpha-synuclein may be a compensatory mechanism to protect neurons against oxidative damage induced by methamphetamine (Li et al. 2008).

A number of proteins involved in protein synthesis or neurotrophic functions were differentially expressed in the shell and core. Increased expression of brain acid soluble protein 1 (BASP1) was seen after maternal separation in the shell and, when exposed to further methamphetamine treatment, this rise was evident in both the shell and core. BASP1 forms part of a family of growth-associated proteins and increased levels are found in neurons during nerve regeneration (Mosevitsky et al. 1994; Iino and Maekawa 1999; Frey et al. 2000). This effect is apparently dependent on the localization of BASP1 at the plasma membrane (Korshunova et al. 2008) and therefore alterations in its expression may have implications for the regulation of actin dynamics and membrane structure (Wiederkehr et al. 1997). An increase in myotrophin levels was also observed in the core after methamphetamine treatment. This protein has been shown to regulate protein synthesis (Taoka et al. 1992; 1994; Fujigasaki et al. 1996), and to control the expression of catecholaminergic enzymes. Overexpression of myotrophin results in increased tyrosine hydroxylase, aromatic L-amino acid decarboxylase and dopamine β-hydroxylase mRNA levels in neuronal cells, with subsequent stimulation of catecholamine synthesis (Yamakuni et al. 1998). The increase in catecholamines has been postulated to be one of the mechanisms by which methamphetamine elicits its reinforcing effects (Koob et al. 1998). In agreement with our finding, increased myotrophin was also identified in a recent proteomic study in the hippocampus of methamphetamine-treated rats (Li et al. 2008). Increased levels of BASP1 and myotrophin in the present study may represent a compensatory mechanism to protect neurons against the damaging effects of methamphetamine and early

maternal separation stress. Gamma-enolase, beta-synuclein and prosaposin are neurotrophic proteins (Hattori et al. 1994; Misasi et al. 2001; Hashimoto et al. 2001; 2004; Sorice et al. 2008) and were decreased after the combination of early maternal separation and later methamphetamine treatment. These changes may reflect the detrimental effect of this combination on neuronal function.

In summary, the present study quantitatively identified a variety of cytosolic proteins in the shell and core of the nucleus accumbens in animals subjected to maternal separation and methamphetamine treatment, independently or in combination. This study confirms that both early life stress and methamphetamine differentially affect the shell and core of the nucleus, and demonstrates that the combination of early life adversity and later methamphetamine use results in greater changes in protein levels in the nucleus accumbens than either alone. A detailed understanding of changes in protein levels in the nucleus accumbens may be of value in fully understanding the behavioural changes seen in the many individuals exposed to both early adversity and subsequent methamphetamine use.

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Conflict of interest The authors declare that they have no conflict of interest.

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