A proteomic and neurochemical analysis of the effects of early life stress on drug addiction and post abuse therapeutic interventions: an animal study

by

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DECLARATION

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Abstract

Psychosocial stressors have frequently been associated with an increased risk for developing depression, anxiety or substance abuse in adult life. Animal studies have also suggested that stressful experiences may result in altered behavioural responses to drugs of abuse as evidenced by enhanced cocaine self-administration and psychostimulant-induced hyperlocomotor activity.

The main aim of our study was to establish whether adversity early in life would render individuals more vulnerable to later drug usage. We adopted maternal separation as our animal model of early life adversity and treated these animals with methamphetamine during the adolescent stage of their life. A conditioned place preference (CPP) paradigm was subsequently used to determine the rewarding effects of methamphetamine. To obtain an understanding of the underlying molecular mechanisms of methamphetamine-induced behaviour, we measured neurochemical changes on a neuroendocrine, neurotrophic, neurotransmitter and proteome level.

Firstly, we established that methamphetamine-induced place preference behaviour lasted for at least 2 weeks after the last methamphetamine administration. Contrary to expectation, this behaviour was not affected by prior exposure to maternal separation. However, rats subjected to maternal separation caused a decrease in apomorphine-induced locomotor behaviour in methamphetamine-treated rats. Maternal separation therefore preferentially affected the behavioural repertoire of the dorsal striatum rather than that of the ventral striatum.

A general down regulation of neuroendocrine activity (ACTH and corticosterone levels) was observed in animals subjected to maternal separation or methamphetamine treatment, as well as those subjected to the combination of the two interventions. Increased concentrations of plasma prolactin levels in maternally separated as well as normally reared animals subjected to methamphetamine-CPP were found which suggested a reduction in dopamine inhibition.

Maternal separation resulted in increased NGF levels in the ventral hippocampus of methamphetamine treated rats. This suggested that the ventral hippocampus may particularly be vulnerable to the effects of early life stress. The increased neurotrophin concentrations may reflect a compensatory response to stress and drug exposure.

The contributions of the cholinergic (Lobeline) and opioid (Naltrexone) systems in place preference behaviour were determined by employing a post-methamphetamine pharmacological treatment strategy. These two treatments failed to reverse the methamphetamine-induced place preference. However, administration of the drugs did lead to alterations in striatal dopamine and serotonin levels which may infer beneficial effects against the biochemical alterations induced by methamphetamine.

We used both 2-D gel-based proteomics and isobaric tagging for relative and absolute quantitation (iTRAQ) to identify proteins in the frontal cortex, and nucleus accumbens shell and core of rats that were subjected to maternal separation, methamphetamine or both regimes. The proteins were associated with cytoskeletal modifications, altered energy metabolism, degenerative processes, interruptions in normal neurotransmission and enhanced intracellular signalling. We found that more proteins were quantitatively expressed in rats that were exposed to maternal separation followed by methamphetamine treatment than those animals subjected to the individual interventions independently. Additional proteins recruited by the combination of MS followed by MA which remained unchanged with independent treatments included malate dehydrogenase, V-type proton ATPase subunit E1, beta-synuclein, brevican core protein, eukaryotic translation initiation factor 4H, histidine triad nucleotide binding protein 1 and stress-induced phosphoprotein in the nucleus accumbens shell sub-region. Additional proteins recruited in the core subregion with the combination treatment included thymosin beta-4, calretinin, Arpp-21 protein, alpha-synuclein, ubiquitin carboxyl-terminal hydrolase isozyme L1, cytochrome c, brain acid soluble protein 1, prosaposin and stress-induced phosphoprotein 1. Although, on a behavioural level via the use of CPP we found that MS did not exacerbate the rewarding effects of MA, the proteomic data does infer a role played by early life stress by the recruitment of additional proteins. We therefore propose that the molecular mechanisms by which early adverse events predispose animals to the addictive state may involve a complex assembly of cellular processes within the brain.

Opsomming

Psigososiale stressors word gereeld geassosieer met ‘n verhoogde risiko vir die ontwikkeling van depressie, angs en dwelm misbruik in volwassenheid. Diere studies het ook al bewys dat vroeë lewensstres in die vorm van moederlike skeiding lei tot veranderde gedrag teenoor dwelm misbruik. Hierdie veranderde gedrag veroorsaak deur moederlike skeiding sluit die verhoodge kokaïne toediening en psigostimulant geinduseerde verhoging in lokomotoriese aktiwiteit in.

Die hoofdoel van die studie was om vas te stel of vroeë lewensstres mense meer vatbaar laat vir latere dwelm misbruik. ‘n Moederlike skeidings diere model was gebruik om vroeë lewensstres voor te stel and het verder hierdie diere behandel met metamfetamiene gedurende adolesensie. Die gekondisioneerde plek voorkeur model was gebruik om die euforiese / verslawende effekte van metamfetamiene te bepaal. Om die onderliggende molekulêre meganismes van metamfetamien geinduseerde gedrag te verstaan het ons neurochemiese veranderinge op ‘n neuroendokriene, neurotrofiese, neurotransmissie en proteinvlak vasgestel.

Eerstens het ons was gestel dat metamfetamien geinduseerde plek voorkeur vir ten minste twee weke na die laaste metafetamien toediening voortduur. In teenoorstelling met verwagting, het moederlike skeiding nie metamfetamien geinduseerde plek voorkeur beinvloed nie, maar eerder apo-morfien geinduseerde lokomotoriese aktiwiteit geaffekteer. Moederlike skeiding stres het by voorkeur die gedrags funksie van die dorsale striatum beinvloed eerder as die ventrale gedragsfunksie.

‘n Algemene afregulering van neuroendokriene aktiwiteit was waargeneem (adrenokortikotrofiene en kortikosteroon vlakke) in diere wat aan moederlike skeiding of metafetamien behandeling sowel as die kombinasie behandeling blootgestel was. Verhoogde plasma prolaktien vlakke was gevind in moederlike skeidings rotte sowel as kontrole diere wat verder blootgestel is aan metamfetamien behandeling wat ‘n inhibisie van die dopamiene sisteem toon.

Moederlike skeiding het ook ‘n verhooging in neurotrofiene (NGF) in die ventrale hippokampus van metamfetamien behandelde rotte veroorsaak. Hierdie bevinding stel voor dat die ventrale hippokampus veral vatbaar is vir die effekte van vroeë lewensstres. ‘n

Verhoging in neurotrofien konsentrasies mag ‘n kompenserende teenslag van die brein wees teen stres en dwelm blootstelling.

Die bydrae van die cholinergiese (Lobeline) en opiaat (Naltrexone) sisteme in plek voorkeur gedrag was bepaal deur farmaseutiese behandeling te volg na metamftemien toediening. Lobeline en naltrexone was egter nie suksesvol om die metamfetamien geinduseerde plek voorkeur te wysig nie. Alhoewel die toediening van die twee behandelings het tot veranderinge in neurotransmissie (dopamiene en serotoniene) gelei wat moontlik tot voordelige effekte teen die biochemiese veranderinge van metamfetamien kan lei.

Om veranderinge op proteinvlak in die frontale korteks en nukleus akkumbens middel en buitenste subareas vas te stel het ons gebruik gemaak van twee-dimensie gel elektroforese en isobariese merkers vir relatiewe en absolute kwantifisering (iTRAQ) gevolg deur massa spektrofotometrie. Geindentifiseerde proteine was geassosieer met sitoskeletale modifikasies, veranderde energie metabolisme, afbrekende prosesse, onderbrekings met normale neurotransmissie en intrasellulêre seintransduksie. Meer proteine was beduidend in die diere wat aan beide moederlike skeiding en metamfetamien behandeling blootgestel was. Addisionele proteine wat deur die kombinasie behandeling geaffekteer is in die buitenste subarea van die nukleus akkumbens sluit ‘malate dehydrogenase’, ‘V-type proton ATPase subunit E1’, ‘beta-synuclein’, ‘brevican core protein’, ‘eukaryotic translation initiation factor 4H’, ‘histidine triad nucleotide binding protein 1’ en ‘stress-induced phosphoprotein’ in. Additionele proteine geaffekteer in die middelste subarea van die nukleus akkumbens sluit ‘thymosin beta-4’, ‘calretinin’, ‘Arpp-21 protein’, ‘alpha-synuclein’, ‘ubiquitin carboxyl-terminal hydrolase isozyme L1’, ‘cytochrome c’, ‘brain acid soluble protein 1’, ‘prosaposin’ en ‘stress-induced phosphoprotein 1’ in. Vanuit ‘n gedrags benadering deur die gebruik van metamfetamien geinduseerde plek voorkeur het moederlike skeiding nie diere meer vatbaar gemaak vir die effekte van metamfetamien nie, maar die protein data wys wel dat vroeë lewens stres ‘n rol speel deur dat meer proteine geaffekteer word deur die kombinasie van moederlike skeiding gevolg deur later metamfetamien toediening. Ons stel voor dat die molekulêre meganismes waardeur vroeë lewensstres diere meer vatbaar maak vir die verslawende effekte van stimulante behels ‘n komplekse samestelling van sellulêre prosesse in die brein.

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Abbreviations

MA	methamphetamine
MS	maternal separation
CPP	conditioned place preference
iTRAQ	isobaric tagging for relative and absolute quantitation
SACENDU	South African Community Epidemiology Network on Drug Use
HPA axis	hypothalamic-pituitary-adrenal axis
VMAT2	vesicular monoamine transporter 2
5-HT	5-hydroxytryptamine; serotonin
VTA	ventral tegmental area
DSM	Diagnostic and Statistical Manual of Mental Disorders
SUD	substance use disorders
NAc	nucleus accumbens
PFC	prefrontal cortex
ACC	anterior cingulate
OFC	oribitofrontal cortex
CuZnSOD	copper/zinc-superoxide dismutase
OCD	obsessive-compulsive disorder
PET	positron emission tomography
MRI	magnetic resonance imaging
BDNF	brain-derived neurotrophic factor
GDNF	glial cell-derived neurotrophic factor
D1 / D2 / D4	dopamine receptor type 1 / 2 / 4
rCBF	regional cerebral blood flow
MRS	magnetic resonance spectroscopy
NAA	N-acetylaspartate
Cr	creatine
PCr	phosphocreatine
Cho	choline-containing compounds
MI	myoinositol
PVC	primary visual cortex
MPEP	2-methyl-6-(phenylethynyl) pyridine
DAT	dopamine amine transporter

Ub	ubiquitin
PD	Parkinson's disease
MAO	monoamine oxidase
COMT	catechol-o- methyltranferase
bp	base-pair
VNTR	variable number tandem repeat
UTR	untranslated region
SPECT	single photon emission computed tomography
OCT3	organic cation transporter 3
SNP	single nucleotide polymorphism
PICK1	protein interacting with C-kinase-1
GABA	gamma-aminobutyric acid
SERT	serotonin transporter
mGluR2/3	metabotropic glutamate receptor 2 / 3
NMDA	N-methyl-d-aspartate
NAC	N-acetylcysteine
BBB	blood brain barrier
ROS	reactive oxygen species
HBMVEC	human brain microvascular endothelial cells
P-gp	P-glycoprotein
GlyTs	glycine transporters
NE	norepinephrine
NET	norepinephrine transporters
ADHD	attention-deficit hyperactivity disorder
PTSD	post-traumatic stress disorder
PRL	prolactin
TIDA	tuberinfundibular dopaminergic systems
THDA	tuberohypophysial dopaminergic system
AAALAC	association for assessment and accreditation of laboratory animal care
PND	postnatal day
s.c.	subcutaneous
i.p.	intra-peritoneally
ACTH	adrenocorticotropin hormone
IRMA	immunoradiometric assay

EIA	enzyme immunoassay
ANOVA	analysis of variance
SPSS	statistical package for the social sciences
SEM	standard error of the mean
HVA	homovanillic acid
DOPAC	3,4-dihydroxyphenylacetic acid
NGF	nerve growth factor
PMSF	phenylmethylsulfonyl fluoride
NT-3	neurotropin-3
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
2-DE	2-dimensional gel electrophoresis
IEF	iso-electric focusing
IPG	immobilized pH gradient
LC	liquid chromatography
DDA	data dependent acquisition
CID	collisionally induced decomposition
m/z	mass/charge ratio
MS/MS	tandem mass spectrometry
pkl	peak list file
GFP	green fluorescent protein
DRP 2	dihydropyrimidase-related protein 2
UCH-L1	ubiquitin carboxyl-terminal hydrolase isozyme L1
ROS	reactive oxygen species
GRP78	78 kDa glucose-regulated protein
PEBP1	phosphatidylethanolamine-binding protein 1
HCNP	hippocampal cholinergic neurostimulating peptide
MARCKS	myristoylated alanine-rich C-kinase substrate
BASP1	brain acid soluble protein
SCX	strong cation exchange
ACN	acetonitrile
TFA	trifluoroacetic acid
MALDI	matrix assisted laser desorption ionization
PPS	pyridinium propyl sulfonate

CHCA	α-cyano-4-hydroxycinnamic acid
ToF/ToF	time of flight / time of flight
ATP	adenosine triphosphate
PC	prohormone convertase
PKC	protein kinase C
MAPK	mitogen activated protein kinase
CaM	calmodulin
AC	adenylate cyclase
cAMP	cyclic adenosine monophosphate
mRNA	messenger ribonucleic acid
ESI-Quad-TOF	electronspray ionization quadrupole time of flight
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors

CHAPTER 1

Introduction to the study

Methamphetamine (MA), also known on the streets as “speed”, “meth”, “chalk” or in the smoke-able form as “crank”, “ice, “crystal”, “glass” or in the Western Cape lingo as ‘tik-tik”. MA is a very addictive psycho-stimulant and is easily synthesized in clandestine laboratories from relatively inexpensive ingredients, making the drug low in cost and highly available. For these reasons, methamphetamine has become the drug of choice for abuse in South Africa, and hence methamphetamine dependence is growing at alarming proportions. Reports from the local Medical Research Council’s South African Community Epidemiology Network on Drug Use (SACENDU) have shown that in the Western Cape the most common abused substance amongst the youth, is methamphetamine (Plüddemann et al., 2009). These alarming statistics have kindled our interest in studying environmental factors that may predispose individuals to methamphetamine abuse, as well as investigating the underlying molecular mechanisms that may be involved in the drug’s addictive action.

Stress has been shown to lead to increased susceptibility towards substance abuse and contributes to relapse in addicted individuals (Sinha, 2001; Gordon, 2002). This has been evidenced for instance by association studies linking psychosocial stressors with an increased risk for developing depression, anxiety or substance abuse in adult life (Kendler et al., 2000). This phenomenon has been clearly demonstrated in animal studies where stressful experiences early in life such as maternal separation (MS), resulted in altered behavioural responses to drugs of abuse that included enhanced cocaine self-administration and psychostimulant induced locomotor activity (Brake et al., 2004; Matthews et al., 2003; Meaney et al., 2002; Kikusui et al., 2005).

We have previously shown that animals subjected to trauma and stress early in life display behavioural abnormalities at a later stage in life. These changes in adult behaviour were associated with a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and alterations in the expression of neurotrophic factors (Daniels et al., 2004; Uys et al., 2006; Faure et al., 2006; 2007; Marais et al., 2008). These findings confirmed the concept that adverse events early in life may affect the development and maturation of the brain negatively. Complementary reports were obtained from clinical studies showing significant

correlations between patients with early trauma and the subsequent development of depression and anxiety disorders (Heit et al., 1997; Ladd et al., 2000). In these cases the behavioural abnormalities were also coupled to endocrine dysfunction.

The rewarding effects of MA are mainly mediated by the mesolimbic dopaminergic system (Kalivas and Stewart, 1991). MA's primary mechanism of action is to increase the release of dopamine into the synapse via its action on vesicular monoamine transporters (VMAT2) (Sulzer and Rayport, 1990; Pifl et al., 1995; Wilhelm et al., 2004; Sulzer et al., 2005). Other neurotransmitter systems may also modulate dopaminergic activity in the brain by interacting with its circuitry. For instance, serotonin (5-HT) modulates dopaminergic neurotransmission by inhibiting the activity of dopaminergic neurons in the ventral tegmental area (VTA) (Brodie and Bunney, 1996). Furthermore, it has been suggested that MS alters neurochemical role players such as decreased dopamine transporter levels in the nucleus accumbens and caudate putamen and an enhanced mesolimbic dopamine response to amphetamines (Hall et al., 1999; Meaney et al., 2002; Brake et al., 2004) that are critical in the development of addictive processes.

In the current study, we combined maternal separation with methamphetamine-induced conditioned place preference, in order to determine whether animals subjected to adverse conditions early in life would demonstrate enhanced place preference for methamphetamine associated environmental stimuli. In addition, we characterized this model of addictive behaviour by determining the duration of place preference behaviour, as well as assessing associated neuroendocrine abnormalities in these animals. As such, we evaluated the activity of the HPA axis activity and measured neurotrophin concentrations in the hippocampus. Two pharmacological interventions (Lobeline and Naltrexone) were used to probe the contributions of the cholinergic and opioid systems in place preference behaviour. We also wanted to determine whether these agents were able to reverse methamphetamine-induced place preference behaviour and whether their effects resulted from altering striatal dopamine and serotonin levels. Finally, we employed proteomic techniques to generate a global overview of protein changes in the frontal cortex and nucleus accumbens shell and core of maternally separated and methamphetamine-treated animals.

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CHAPTER 2

Methamphetamine (MA / "tik-tik") addiction – a review of the literature

Methamphetamine (MA / "tik-tik") addiction – a review of the literature

Introduction

Methamphetamine is a very addictive psycho-stimulant and is easily synthesized in clandestine laboratories from relatively inexpensive ingredients. Thus, methamphetamine is low in cost and highly available. When methamphetamine is consumed it produces a high that lasts 4 to 24 hours (Cook et al., 1993) and the additional methyl group makes it more potent than its counterparts, amphetamine and dextro-amphetamine (Fasciano et al., 1997). In addition, tolerance for the drug is achieved quite rapidly, resulting in 'binging' of the drug to achieve the same euphoric effects, hence an escalation in consumption (Angrist et al., 1987; Brauer et al., 1996).

Recent reports indicate that methamphetamine abuse and addiction has grown to a major illicit drug problem in the USA (http://www.usdoj.gov/ndic/pubs11/13846/meth.htm). Similar concerns exist in countries such as UK, Finland and Australia. In South Africa reports from the local Medical Research Council's South African Community Epidemiology Network on Drug Use (SACENDU) have shown that in the Western Cape the most common abused substance is methamphetamine and treated 2807 patients across 24 treatment centres in the last 6 months of 2008 with 35% reporting methamphetamine as their primary substance of abuse (Plüddemann et al., 2009). In light of these alarming statistics, more research is needed that focusses on this topic.

Defining methamphetamine addiction

No clear cut definition of addiction has been formulated and various fields of expertise focus on different aspects for the terminology of addiction. The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV TR) does not include the word 'addiction.' Rather they term the phenomenon as 'substance dependence' and this together with substance abuse is classified as Substance Use Disorders (SUD). Substance dependence is defined as an individual continuing using a substance despite negative consequences relating to the substance. Furthermore, the compulsive and repetitive usage of the substance may lead to tolerance to the effects of the drug and the development of withdrawal symptoms after

discontinuation of the substance (American Psychiatric Association, 2000). Addiction, from a psychiatric point of view, is referred to as psychological dependence, and from a physiological standpoint, is referred to as physical dependence. For the purpose of this review we will side with the physiological point of view and focus particularly on brain neurochemistry and structures involved in addiction.

Methamphetamine dependence or addiction is a brain disorder which is caused by the repeated usage of methamphetamine despite negative consequences and resultant loss of control. Long-term brain alterations that interfere with the individual's ability to exercise cognitive control, is thought to underlie drug seeking behaviour and hence addiction (Kalivas and Volkow, 2005). Goldstein and Volkow (2002) have also proposed that drug addiction can be viewed as a syndrome that is characterised by impaired response inhibition and abnormalities in reward processing. Natural reinforcers are no longer seen as rewarding and are over powered by affects of abused drugs and drug related cues by altering the threshold of the reward circuitry. In addition, the addict's main motivation becomes the "wanting" of the drug on which they have become dependent (Robinson and Berridge, 2003). Addiction is also referred to as a chronic relapsing disease since more than half of the addicts seeking treatment relapses into drug usage again (Miller, 1996). One of the hypotheses forwarded for this phenomenon states that brain functions responsible for self-control and normal cognitive functioning are impaired following chronic methamphetamine abuse.

Epidemiology

According to the United Nations Office on Drug and Crime, more than 34 million individuals use amphetamines world wide (United Nations Office on Drug and Crime, 2003). The present statistics in South Africa show that methamphetamine as the drug of choice for abuse and addiction is growing at alarming proportions. The Medical Research Council's South African Community Epidemiology Network on Drug Use found the abuse of MA is especially evident in the Western Cape and have shown that the number of patients that use MA as their primary substance of abuse have increased dramatically at specialist treatment centres (Plüddemann et al., 2009). Other studies have found the age of onset of female MA users to be much younger than that of their male counterparts. Females had an average age of onset of 19.2 years for MA use and males first encounter with using MA on average were 20.6 years (Hser et al., 2005).

Pharmaco-chemistry

MA is also known on the streets as “speed”, “meth”, “chalk” or in the smoke-able form as “crank”, “ice, “crystal”, “glass” or as in the Western Cape lingo as ‘tik-tik” (www.whitehousedrugpolicy.gov/streetterms; Plüddemann et al., 2009). MA, an N-methyl homologue of amphetamine, is a synthetic drug which is synthesized from the ephedra plant *Ephedra vulgaris*, the active ingredient being ephedrine (Barker and Antia, 2007). It is a white, crystalline, bitter-tasting, odourless powder that easily dissolves in water or alcohol (Gulaboski et al., 2007). Methods of MA consumption include the injection, smoking, snorting or oral ingestion of the drug (Anglin et al., 2000).

MA has a plasma half-life of 12 hours generating effects that may last up to 24 hours (Cook et al., 1993). MA is classified as a psychostimulant agent causing psychosis similar to certain psychiatric disorders, such as paranoid psychosis and sometimes even schizophrenia-like or manic bipolar disorder-like symptoms (Snyder, 1972; Schmidt et al., 1985; Iwanami et al., 1994; Brauer and de Wit, 1996, 1997; Bufferstein et al., 1999; Lee et al., 2000; Shoptaw et al., 2003; Nejtek et al., 2008). These behavioural symptoms can occur acutely after MA use and persists long after drug cessation (Sato, 1992; Iwanami et al., 1994; Zweben et al., 2004).

The “high” induced by psychostimulant drugs have been correlated with increases in brain dopamine levels as well as a relationship has been established between the intensity of the “high” and the occupancy of dopamine D2 receptors (Volkow et al., 1999a). MA also affects other neurotransmitters system, which include the serotonergic (5-HT) system and abnormalities in this particular system has been linked to the behavioural deficits experienced by MA abusers (Schmidt et al., 1985; Ezaki et al., 2008).

Brain areas and circuitry involved in MA addiction

Ventral tegmental area and prefrontal cortex: Dopaminergic projection

During various stages of drug addiction, drugs of abuse induce differential neuroplastic changes. The development of these alterations at the synaptic level over time is suggested to underlie the establishment of addictive behaviour.

Initially, a few brain areas and neurotransmitter systems are involved in eliciting the rewarding effects of drugs of abuse. The early rewarding experiences are caused by dopaminergic projections of the mesocorticolimbic system, particularly those that originate in the ventral tegmental area (VTA) and innervate the nucleus accumbens (NAc), specifically the shell subregion (Wise, 1981; Carboni et al., 1989; Koob, 1992; Bassareo and Di Chiara, 1997; Drevets et al., 1999; Bradberry et al., 2000). Another brain area that plays an important role during the acute stages of drug taking is the prefrontal cortex (PFC) (Kalivas et al., 2005). The VTA is the area of the brain that detects motivationally relevant stimuli, i.e. drug of choice, and responds to the stimulus by releasing dopamine (Robinson and Berridge, 1993; McClure et al., 2003) to the PFC and NAc shell (Bassareo and Di Chiara, 1999a; Berns et al., 2001; Volkow et al., 2003; Sellings and Clarke, 2003). The anterior cingulate (ACC) and the oribitofrontal cortex (OFC), both PFC regions, are of importance during motivational salient events. These brain regions are active especially if the reward is predictable (Berns et al., 2001; Volkow et al., 2003). They appear to determine the intensity of responding to drug stimuli (Jentsch and Taylor, 1999; Bush et al., 2002).

The shell of the NAc has a strong reciprocal dopamine innervation to the VTA and thus aids in modulating motivational salient stimuli. This projection participates in the learning of associations between motivational relevant stimuli and environmental perceptions (Bassareo and Di Chiara, 1999; Sellings and Clarke, 2003) and hence plays a role in Pavlovian incentive learning by using dopamine as a substrate (Bassareo and Di Chiara, 1997; Bassareo and Di Chiara, 1999a, 1999b, Bassareo et al., 2002). Dopamine release is the trigger to direct behavioural responses toward motivational salient stimuli and leads to cellular adaptations that facilitate learning of associations between stimuli and the event (Jay, 2003). Interestingly, as the anticipated reward becomes familiar, dopamine is not released upon exposure to the reward (Schultz, 1998). Drug-induced dopamine release is 3-5 times more when compared to dopamine release upon a natural reward (Wise, 2002). This demonstrates the powerful effects elicited by drugs of abuse and highlights the difficulty of drug cessation.

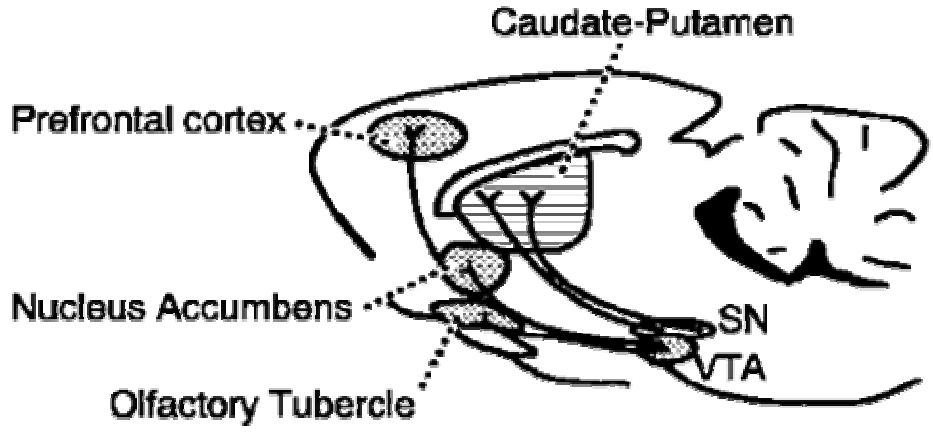

Figure 1: Rat brain illustrating some of the main brain areas involved in methamphetamine addiction (www.cellscience.com/CCA.htm).

The nucleus accumbens

The NAc core is primarily involved with the expression of motivation or learned behavioural responses (Kelley, 2004; Di Ciano and Everitt, 2001) or habit learning (Ito et al., 2002). This brain area is also connected to the ACC and OFC and expresses adaptive learned behaviour by glutamatergic innervation from the PFC (Di Ciano et al., 2001). Thus, behavioural expression occurs via glutamatergic afferents from the PFC and directing this input towards the accumbens-thalamocortical circuitry (Jog et al., 1999; Haber, 2003). The NAc is therefore proposed to act as an interface between motivation and action (Mogenson and Yang, 1991).

During the early stages of drug taking, dopamine from the VTA and glutamate from the PFC are released in the NAc (Wise and Rompre, 1989; Koob and Le Moal, 2001). It has been proposed that these two events are responsible for the synaptic changes at the level of the NAc that drives drug seeking behaviour (Cornish et al., 1999; Cornish and Kalivas, 2000; Kalivas and Volkow, 2005; Kalivas et al., 2005). Since glutamate plays such an important role in learning and neuronal plasticity it is plausible to propose that glutamate transmission is pivotal to the conditioning process and is suggested that addiction might involve glutamate-dependent neuroplasticity in limbic cortico-striatal circuits (Everitt and Wolf, 2002).

The amygdala

Some studies suggest that reinstatement of drug seeking also require the release of dopamine into the amygdala (See et al., 2001; McFarland and Kalivas, 2001; Capriles et al., 2003; McFarland et al., 2004). The amygdala functions in recognising cues that are associated with the drug or with using the drug and plays a primary role in conditioned associations and the learning of these associations (Everitt et al., 2003). Interestingly, the basolateral nucleus and the central nucleus of the amygdala have differential roles in addiction. In order for learned associations to influence future more complex behavioural responses towards conditioned association, glutamatergic neurotransmission from the basolateral amygdala back to the PFC and NAc is necessary (Cardinal et al., 2002). This reciprocal projection is involved in cue-primed drug seeking (Meil and See, 1997; Erb and Stewart, 1999), since rats with lesioned basolateral amygdala were unsuccessful in establishing cue-induced cocaine seeking (Whitelaw et al., 1996).

Frontal cortical areas

The frontal and prefrontal brain areas are primarily involved in executive functioning which includes higher-order cognition, motivational functions, memory, impulse control, problem solving and decision making (Royall et al., 2002). The frontal cortex suppresses inappropriate fast behavioural responses in order to allow slower decision making processes to influence behaviour (Friedman and Miyake, 2004). Psychostimulant abusers display distorted patterns of expectancy, compulsion and decision making (Paulus et al., 2002; London et al., 1999) as evidenced by drug-induced functional changes in forebrain structures. Ventromedial prefrontal cortical lesions have resulted in patients lacking inhibitory control in decision making when measuring immediate reward against long-term negative consequences (Bechara et al., 2000). On a microscopic level, increases in dendritic branching and dendritic spine density in the PFC (Robinson et al., 2001) reflects altered synaptic connectivity that influence decision making and cognitive control over drug taking (Volkow et al., 2003). Some of the most prominent frontal brain areas are compromised in addiction and include the dorsolateral prefrontal cortex, the OFC, the ACC and the ventromedial cortex. The OFC and the ACC are anatomically connected to the limbic structures and show activation during periods of drug using, craving and binging and deactivated during drug withdrawal (Goldstein and Volkow, 2002). This has been demonstrated by studies using blood-oxygenation-level-dependent responses during cocaine intoxication (Breiter et al., 1997), decreased relative

cerebral blood flow and lower brain glucose metabolism during periods of drug withdrawal (Volkow et al., 1988; Volkow et al., 1992).

The OFC is specifically associated with assessing reward value and reinforcing properties which include the processing of short- and long-term gains or losses (Bechara, 2001; O'Doherty et al., 2000; Rolls, 2000). Damage to the OFC has been shown to lead to compulsive behavioural responses to engage in drug seeking in the face of negative consequences or unpleasurable drug effects (Rolls, 2000). Similarly, this effect is also seen in other compulsive disorders as in the case of obsessive-compulsive disorder (OCD) (Insel, 1992).

The ACC has also been implicated in processes of cognitive control (Vogt et al., 1992) of which the dysfunction has been associated with relapse in MA abusers. Positron emission tomography (PET) studies have shown that the ACC plays a role in emotional processing (George et al., 1993; Lane et al., 1997) and the orchestration of appropriate behavioural responses (Paus et al., 1993). Dysfunctionality of the ACC has serious implications in addiction pathology, since psychostimulant abusers show impaired cognitive control over appropriate behavioural responses. In fact, their behaviour is dominated by a sensitization toward responses directed by drug-related cues (Aron and Paulus, 2007).

The posterior cingulate cortex is involved in evaluative processes (Vogt et al., 1992) and has been implicated in the making of risky choices (McCoy and Platt, 2005). This region evaluates the degree to which actions lead to predictable versus unpredictable behavioural consequences (Blakemore et al., 1998) as well as controlled versus uncontrolled movements (Schubert et al., 1998). Increased neuronal activity in the posterior cingulate cortex has been connected to relapse in cocaine addicts and this correlated with poorer treatment outcomes (Kosten et al., 2006). Observations such as these have led to the posterior cingulated cortex being associated with cocaine-induced craving (Garavan et al., 2000; Kilts et al., 2004).

The parietal cortex

The parietal cortex is important for higher brain functions such as attentional processes, memory, skills learning and spatial perception and imagery (Cabeza and Nyberg, 2000). The parietal cortex has therefore been linked to addictive behaviour due to these behavioural

functions conducted by the parietal cortex. For instance, the right inferior parietal cortex directs attention towards selected actions or stimuli and plays a critical role during assessment processes and decision making (Aron and Paulus, 2007), sustained and selective attention (Coull et al., 1996), control of voluntary attention (Hopfinger et al., 2000) and inhibitory control (Garavan et al., 1999). The precuneus or the posteromedial parietal cortex is involved in imagery, episodic memory retrieval and consciousness of self (Kjaer et al., 2002). Decreased activation of the precuneus has been observed in MA subjects when engaged in a decision making task (Paulus et al., 2003).

Pallidal structures

The ventral pallidum forms part of the limbic cortical-ventral striatopallidal circuitry that is implicated in drug seeking and reinstatement. The ventral striatopallidal system plays a major role in translating a motivational state into a behavioural output (Kalivas and Nakamura, 1999; Kelley and Berridge, 2002; Kelley, 2004). In general, behavioural output is orchestrated by the shell of the NAc via the ventral pallidum and mediodorsal thalamus to motor areas. This circuit usually drives the execution of behaviour in response to rewarding stimuli (Waraczynski, 2006). The NAc shell also connects to the hypothalamus via the ventral pallidum and this projection serves to modulate responding to natural rewards (Kelley, 2004). The ventral pallidum may therefore be seen as a relay center for the execution of behavioural responses to rewarding or reinforcing stimuli. In addition, this brain area performs other complex actions by modulating neurotransmitter input from the NAc and midbrain regions, especially via μ opioid receptors, while also modulating the effectiveness of glutamatergic inputs from the PFC and amygdala (Napier and Mitrovic, 1999).

The limbic region

The limbic and paralimbic brain regions play a critical role in the development of addiction because of its role in the processing of emotions. The insula is a structure deeply nestled into the frontal, temporal and parietal lobes within the lateral fissure and is highly connected to the PFC and amygdala (Augustine, 1996). Interestingly, the activation of the left insula was inversely correlated with outcome predictability in MA users (Paulus et al., 2003). The reduced activation of the insula is proposed to affect response inhibition of behaviour and

decision making, since stimulant abusers are less concerned with processing success or failure of behavioural responses (Paulus et al., 2005).

The hippocampus is involved in memory consolidation and is instrumental in learned associations necessary for addiction development. The hippocampus is involved in conditioning to contextual or spatial stimuli, unlike the amygdala that is more involved in conditioning to discrete stimuli (Selden et al., 1991; McDonald and White, 1993). Theta burst stimulation of the hippocampus has been demonstrated to reinstate extinguished cocaine seeking behaviour which occurred only during glutamate neurotransmission in the VTA. This increase in neuronal activity has been correlated with context-dependent reinstatement of drug taking (Vorel et al., 2001). Reinstatement or relapse has been closely linked to craving (Anton, 1999; Hyman and Malenka, 2001) and memory consolidation needed for craving is thought to be mediated by the hippocampus in conjunction with the amygdala. The experience of craving is however thought to be mediated by the ACC and the thalamo-orbitofrontal circuitry (Goldstein and Volkow, 2002).

Methamphetamine-induced neurotoxicity

Role of dopamine

Despite the fact that a non-toxic dose was administered to rats in the present study, neurotoxicity induced by repeated MA abuse is very prevalent and clinically revelant for discussion in this review. One of the major contributing factors of MA toxicity is that MA has a very similar chemical structure to that of the body's natural occuring dopamine and this allows MA to enter axons (Iversen, 2006). Toxicity occurs as a result of both endogenous dopamine accumulation as well as high extracellular dopamine levels released from nerve terminals (Krasnova and Cadet, 2009). Accumulated dopamine rapidly auto-oxidizes and results in the formation of oxygen free radical species, which include superoxide radicals, hydroxyl radicals, hydrogen peroxide and dopamine quinones (Cubells et al., 1994; Acikgoz et al., 1998; LaVoie and Hastings, 1999; Larsen et al., 2002; Miyazaki et al., 2006; Lazzeri et al., 2007). Increased dopamine oxidation has been suggested to be one of the leading contributing factors to MA-induced dopaminergic terminal damage (LaVoie and Hastings, 1999). MA has also been found to tip the balance in favour of reactive oxygen species (ROS)

production than that of antioxidant potential to scavenge ROS. This has been demonstrated by MA reducing copper/zinc-superoxide dismutase (CuZnSOD), glutathione and peroxiredoxins which were accompanied by high levels of lipid peroxidation and protein carbonyls (Jayanthi et al., 1998; Harold et al., 2000; Gluck et al., 2001; Iwazaki et al., 2006; Chen et al., 2007; Kobeissy et al., 2008; Li et al., 2008). When ROS overrides the anti-oxidant capacity of the cells it gives rise to MA-induced terminal degeneration and neuronal apoptosis (Potashkin and Meredith, 2006). The inhibition of tyrosine hydroxylase via α-methyl-ρ-tyrosine has been demonstrated to guard against MA-induced toxicity in the striatum (Wagner et al., 1980; Axt et al., 1990; Thomas et al., 2008). In addition, the administration of clorgyline, a monoamine oxidase inhibitor (Wagner and Walsh, 1991; Kita et al., 1995; Thomas et al., 2008) and reserpine, an irreversible inhibitor of vesicular transport (Wagner et al., 1983; Albers and Sonsalla, 1995; Thomas et al., 2008; Kuhn et al., 2008), which results in increased cytoplasmic dopamine concentrations, worsens MA toxicity. This is evidence in favour of endogenous dopamine levels contributing to MA-induced dopamine terminal degeneration. Dopamine released from the terminals has also been shown to contribute to striatal MA toxicity, since the DAT inhibitor, amphonelic acid, also protects against damage induced to dopamine axons. Similarly, the activation of post-synaptic dopamine D1 receptors has been shown to result in degeneration of dopamine terminals, since dopamine receptor antagonists prevent terminal damage (Sonsalla et al., 1986; O'Dell et al., 1993; Jayanthi et al., 2005; Xu et al., 2005), however, partially depend on changes in dopamine release (Krasnova and Cadet, 2009). Activation of post-synaptic dopamine receptors also results in the activation of transcription factors and Fas/FasL death pathway leading to increased expression of FasL and caspase-3 in rat striatal cells, which was inhibited by the pre-treatment of SCH23390, a dopamine D1 receptor antagonist (Jayanthi et al., 2005).

Role of glutamate

Dopamine and glutamate are thought to interact to cause neurotoxicity in striatal dopamine terminals, since glutamate receptor antagonists decrease MA-induced degeneration of both dopamine and serotonin terminals in various brain areas (Sonsalla et al., 1991; Fuller et al., 1992; Battaglia et al., 2002; Chipana et al., 2008). Also, the involvement of glutamate was supported by the fact that MA results in the release of glutamate in the brain, including that of the striatum and nucleus accumbens (Baldwin et al., 1993; Abekawa et al., 1994; Mark et al., 2004; 2007). Glutamate toxicity has been found to result from MA-induced release of large

amounts of glutamate activating N-methyl-d-aspartate (NMDA) receptors and resulting in formation of reactive oxygen species including nitric oxide (Lafon-Cazal et al., 1993; Gunasekar et al., 1995). Not only is nitric oxide a contributing factor towards glutamate neurotoxicity, nitric oxide also reacts with superoxide radicals forming peroxynitrite (Pacher et al., 2007). Increases in 3-nitrotyrosine induced by MA administration, which is a marker for peroxinitrite production, is inhibited by antioxidants and blocks MA-induced dopamine depletion (Imam et al., 2001).

Morphological alterations in the brain induced by MA

Magnetic resonance imaging (MRI) and PET studies have been used extensively to evaluate structural brain changes induced by MA. The basal ganglia have been shown to be particularly vulnerable to the effects of MA, due to its enriched innervations of dopamine. The neurotoxic effects of MA have been found to be evident even after long periods of abstinence (McCann et al., 1998; Ernst et al., 2000; Volkow et al., 2001a; Chang et al., 2005; Sekine et al., 2006). Some of the early brain structural changes observed in abstinent adult MA users included greater basal ganglia volumes of all its subdivisions, i.e. the caudate, globus pallidus and putamen, as well as the parietal cortex (Chang et al., 2005; Jernigan et al., 2005). Interestingly, after longer periods of abstinence these volume differences seem to normalise. The enlarged striatum in abstinent MA abusers has been attributed to increased water content, MA-induced inflammation and/or reactive gliosis (Chang et al., 2005). A possible mechanism for MA inflammation proposes that MA stimulates matrix degrading proteinases (Conant et al., 2004) that compromise the blood-brain-barrier (BBB) resulting in the access of pro-inflammatory cytokines, chemokines and macrophages into the brain (Chang et al., 2007). An alternative mechanism suggested that the enlarged striatum resulted from the influence of glial-mediated neurotrophic factors, since brain-derived neurotrophic factor (BDNF) has been shown to be elevated in MA users (Kim et al., 2005). This view was supported by animal studies that indicated that BDNF and glial cell-derived neurotrophic factor (GDNF) secreted by microglia and macrophages, stimulated sprouting of dopaminergic neurons (Batchelor et al., 1999). In addition, dopamine too possesses neurotrophic properties as evidenced by increased striatal dopamine neuron spine density after cocaine administration (Lee et al., 2006). Since treatment of schizophrenic patients with a typical antipsychotic also yielded increased striatal volumes (Corson et al., 1999; Lieberman et al., 2005), the

occupancy of D1 or D2 dopaminergic receptors has been suggested to mediate the change in striatal size. Therefore, it seems that after the initial increase in basal ganglia during early abstinence from MA use, normalization of the volumes of the basal ganglia occurs which compares with control volumes during longer periods of abstinence. Therefore, abusing MA for longer periods, eventually lead to greater basal ganglia neuronal damage and decreases in volume (Chang et al., 2005), which are usually accompanied by cognitive deficits.

Volume decreases in abstinent MA users were not confined to the striatum, but also observed in other brain areas. Using MRI and new computational brain-mapping techniques, Thompson and colleagues (2004), found chronic MA consumption to result in severe gray matter deficits in the cingulate, limbic and paralimbic cortices, and expanded ventricles. In particular, the hippocampus was 7.8 % smaller in volume with a 7 % increase in white matter in the temporal regions surrounding the hippocampus. Decreased hippocampal volumes were correlated to impaired memory performance on a word-recall test. MA users also performed poorly on test of verbal memory, perceptual motor speed and executive functioning (Simon et al., 2002), as well as on tasks requiring response inhibition and decision making (Salo et al., 2002; Kalechstein et al., 2003; Paulus et al., 2002). The finding of atrophy of the right cingulate in chronic MA abusers resulted in the cancellation of normal asymmetry of the right > left gray matter in normal healthy subjects (Thompson et al., 2004). White matter hypertrophy was suggested to result from altered myelination or gliosis in response to repeated drug exposures. Overall, chronic MA abuse resulted in specific cerebral deterioration with main effects found in the medial temporal lobe and limbic cortices (Thompson et al., 2004).

Structural abnormalities of the corpus callosum have also been observed in abstinent MA abusers (Oh et al., 2005). A few studies have demonstrated that white matter tracts within the corpus callosum are influenced by substance abuse (Pfefferbaum et al., 2006a, 2006b). Automated shape analysis experiments and diffusion tensor imaging studies revealed greater curvature of the genu and smaller width of the posterior midbody and isthmus. The abnormalities of the genu and the posterior midbody were well correlated with lifetime usage of MA. These shape abnormalities of the corpus callosum complimented observed structural and functional abnormalities in both the frontal and parietal cortices of MA abusers (Oh et al., 2005) and correlated with poorer cognitive control (Salo et al., 2009). The frontal white matter at the anterior commissure-posterior commissure plane was also decreased as

measured by fractional anisotropy. The population of MA abusers in which this decrease was observed made significantly more errors in a Wisconsin Card Sorting test when compared to healthy controls (Chung et al., 2007). Here too, altered myelination (Alberson et al., 2004; Melo et al., 2006) and MA-induced degeneration of axons has been forwarded as possible reasons for the structural modifications. Interestingly, a separate analysis found that decreased frontal white matter and poorer performance on the Wisconsin Card Sorting test were confined to the male MA subjects of the study and were not found in the female MA abusers. This observation made the authors suggest that estrogen may offer protection against the neurotoxic effects of MA (Chung et al., 2007).

MA has consistently been shown to result in reduced gray matter density in all cortical lobes, which included the temporal, frontal, occipital and parietal (Bartzokis et al., 2000; Thompson et al., 2004; Kim et al., 2006), and increases in white matter (Thompson et al., 2004; Chang et al., 2005; Bae et al., 2006), although mixed results for MA effect on white matter have also been reported (Oh et al., 2005; Schlaepfer et al., 2006; Chung et al., 2007). Usually gray matter reduces and white matter increases during normal aging (Bartzokis et al., 2001). This means that it is important to note the time of abuse onset and age of test subjects when conducting imaging studies. Abuse potential has been shown to decline with age (Berman et al., 2008) and therefore ascribing structural abnormalities to the actions of psychostimulants need to be done with caution (Bartzokis et al., 2002).

Regional cerebral blood flow (rCBF) and hence perfusion rates in brain areas, is often used as an indicator of the functionality of that particular brain region. For instance, decreased regional cerebral blood flow was noted in the ACC when compared to normal healthy subjects (Hwang et al., 2004). Similarly male MA abusers tend to have hypoperfused regions in the frontal and parietal cortex. In contrast, female MA abusers present more with hyperperfusion of the parietal and occipital cortex (Chang et al., 2002). Hyperperfusion in females is suggested to be a protective mechanism of the brain that may decrease neuronal injury (Chang et al., 2002). However, it has been suggested that increases in regional cerebral blood flow may perhaps be attributed to glial activation and astrogliosis in MA addicts (Thompson et al., 2004).

Other studies have found that cerebral blood flow abnormalities overlap with deficits in glucose metabolism of regions investigated (Iyo et al., 1997; Chang et al., 2002). Cerebral

glucose metabolism is considered an index of brain function. Significant increases in glucose metabolism were noted over the first month of abstaining from MA use (Berman et al., 2008). This increase in global glucose metabolism was 10.9 %. Closer investigation showed that the increased was more than 20 % in the parietal cortex suggesting that posterior cortical mechanisms are the driving force behind the global glucose metabolism. Increases in the cortical, limbic, striatal and thalamic regions were found to be modest (Berman et al., 2008). Volkow et al. (2001a) emphasized that elevations in cerebral glucose metabolism may merely result from gliosis. Glial responses have been associated with hypermetabolism (Roh et al., 1998).

Metabolic markers

Magnetic resonance spectroscopy (MRS) is a technique that assesses the level of specific brain metabolites to detect neuronal damage (Sager et al., 2001). Brain metabolite levels are therefore indicators of the structural integrity and function of neurons. N-acetyl compounds, e.g. N-acetylaspartate (NAA), high energy metabolic compounds like creatine (Cr) and phosphocreatine (PCr), cell membrane synthesis or degradation markers e.g. choline-containing compounds (Cho), and myoinositol have been measured in MA studies. The proton spectrum NAA peak is a measure of neuronal loss or neuronal damage since NAA is present almost exclusively in neurons and their dendritic and axonal processes (Simmons et al., 1991; Tsai and Coyle, 1995; Gonen et al., 2000). Disorders that have been associated with neuronal loss or damage include Alzheimer's disease, brain tumors and head trauma have all showed good correlation with NAA peaks (Ernst et al., 1997; Pfefferbaum et al., 1999; Ott et al., 1993; Friedman et al., 1999). The creatine and phosphocreatine (Cr + PCr) peak of the proton spectrum reflects high-energy phosphate metabolism (Chang et al., 1996; Tedeschi et al., 1995). It is often assumed that the creatine peak is constant and used as a reference for other peaks, however, gray matter has been found to have higher Cr values than white matter (Narayana et al., 1989; Doyle et al., 1995; Chang et al., 1996; Lim et al., 1998). Because of this discrepancy the creatine peak has become useful in the study of disorders such as MA addiction, where there are discrepant gray and white matter alterations. Membrane synthesis and turnover results in increased intensity of choline signal (Miller et al., 1999; Tedeschi et al., 1996). It is suggested that Cho can act as a marker to determine the extent of neuronal recovery after drug cessation. Myoinositol (MI) is only present in glial cells and can be considered a glial marker (Brand et al., 1993).

Metabolic abnormalities in abstinent MA users were investigated in the frontal cortex, frontal white and gray matter and the basal ganglia (Ernst et al., 2000). Researchers found NAA concentrations reduced in the basal ganglia, frontal lobe and frontal white matter of MA users. Decreased NAA levels are indicative of neuronal loss or persistent neuronal damage inflicted by MA. Total Cr was reported to be decreased in the basal ganglia, while Cho and MI concentrations were increased in the frontal gray matter. According to Ernst et al. (2000) the combination of decreased NAA levels and elevated Cho and MI reflected glial proliferation in response to neuronal damage resulting from MA exposure.

Nordahl et al. (2005) measured the metabolite concentrations in the ACC of recently abstinent (1 – 6 months), sustained abstinent (1-5 years) MA users and healthy control subjects. These authors assessed the metabolite levels in the primary visual cortex (PVC) which served as a control, since this brain area receives minimal dopamine innervation (Hall et al., 1994; Eberling et al., 2002). Proton MRS showed decreased NAA levels in the ACC with no change in the PVC. The Cho/NAA concentrations in recently abstinent users were higher in the ACC, but were normal in long-term abstinent MA users. It was suggested that after drug cessation this normalization of metabolite levels may relate to adaptive structural and functional changes taking place in the ACC (Nordahl et al., 2005). Animal and human studies have shown that neuronal changes accompanying MA exposure are not always permanent and that partial recovery may occur with prolonged abstinence (Melega et al., 1996; Wilson et al., 1996; Harvey et al., 2000; Volkow et al., 2001b; Wang et al., 2004). Similarly to the suggestion of Ernst et al. (2000), this study concluded that increased Cho concentrations in early abstinence resulted from either acute damage to membranes, gliosis and membrane biosynthesis or axonal sprouting. The normalization of the Cho metabolite after a longer period of drug cessation probably occurs as a reflection of a subsequent period of axonal pruning of misguided axons (Gao et al., 1999).

In another study, the relationship between metabolite concentrations in the frontal gray and white matter of abstinent MA users and their clinical characteristics (abstinence duration and total cumulative dose of greater or less than 100g lifetime) were tested. Decreased NAA concentrations in the frontal white matter that were exposed to more than 100g MA in their lifetime were found in abstinent male MA users and hence this finding was correlated with a cumulative MA dose. MI concentrations were also significantly reduced in the frontal white

matter of MA abusers (Sung et al., 2007). Reduced NAA levels were ascribed to frontal dopaminergic nerve fibre degeneration induced by MA as was documented in a T2 white matter hyperintensity study (Bae et al., 2006). Increased MI levels were once again ascribed to glial cell proliferation in response to toxic damage inflicted by MA (Sheng et al., 1994). These findings were not present in the frontal gray matter and it has been suggested that gray matter may recover from neurotoxic effects of MA over longer periods of abstinence.

The role of glutamate

A number of animal studies have focused on the modulation of MA-induced dopamine release by glutamate. In vivo PET experiments conducted on primates and rodents, have demonstrated a suppression of MA-mediated dopamine secretion by blocking the group I metabotrophic glutamate receptor with the antagonist 2-methyl-6-(phenylethynyl) pyridine (MPEP) (Tokunaga et al., 2009). In line with this evidence, pre-treatment with the NMDA glutamate receptor antagonist, MK-801, has been shown to attenuate the toxic effects of repeated MA doses which included the activation of microglial and the depletion of both dopamine and the dopamine transporters (DAT) in the terminal striatal neurons (Thomas and Kuhn, 2005a). These results suggested that glutamate antagonism may possibly hold beneficial effects for MA addicts (Chang et al., 2007; Blandini et al., 1996; Izumi et al., 2009). Furthermore, this is in line with the need for glutamatergic neurotransmission during cue-associated learning and memory processes in the development of addictive behaviour.

The role of inflammatory processes

Microglial cells form part of the body's immune response and their activation has been associated with neurodegenerative disorders such as (Kreutzberg, 1996) Alzheimer's disease and Parkinson's disease (Banati et al., 2000; Cagnin et al., 2001; Ouchi et al., 2005). Chronic self administration of MA in abusers has also been shown to result in increased activation of microglia in the midbrain, striatum, thalamus, orbitofrontal cortex and insular cortex. All these brain areas are areas innervated by dopaminergic and serotonergic projections from the hindbrain. Activation of microglia during MA exposure appears to dissipate over long periods of drug abstinence (Sekine et al., 2008; Thomas and Kuhn, 2005b). Administration of anti-inflammatory agents, ketoprofen and minocycline, attenuate the neurotoxic effects of MA (Asanuma et al., 2003; Thomas and Kuhn, 2005a; Hashimoto et al., 2007) and therefore this

approach has also been suggested as a therapeutic intervention for MA-abusers (Sekine et al., 2008).

The role of misfolded proteins

Ubiquitin (Ub) is involved in the ubiquitin proteosome pathway and plays a key role in the proteolytic degradation of misfolded or damaged proteins via the 26S proteosomal pathway (Dickson et al., 1990; Alves-Rodrigues et al., 1998; Coleman and Ribchester, 2004). Ubiquitin plays a major pathological role in neurodegenerative diseases such as Parkinson's disease (PD). This neurodegenerative disorder is characterized by substantial decreases in dopamine concentrations in the striatum due to the loss of dopaminergic neurons in the substantia nigra pars compacta (Lotharius and Brundin, 2002). Interestingly, PD is also identified by the accumulation of Lewy bodies, which are cytoplasmic inclusions containing the proteins α-synuclein and ubiquitin (Lowe et al., 1990; Spillantini et al., 1997; Chung et al., 2001; Fornai et al., 2004a). MA has neurotoxic effects on particularly dopaminergic neurons (Sonsalla et al., 1992; Frost and Cadet, 2000; Iman et al., 2001; Guilarte, 2001). MA disrupts the metabolic integrity of the cell, leading to increased oxidative stress, and ultimately apoptotisis and/or necrotisis (Davidson et al., 2001; Fornai et al., 2003; Fornai et al., 2004b). Oxidative damage to neurons leads to misfolded proteins and degradation via the UP pathway (Iwai et al., 2003). Neurons therefore degenerate when the ubiquitin proteosome pathway is unable to cope with the onslaught of misfolded proteins. Similar to PD, MA neurotoxicity also leads to the formation of inclusion bodies in nigral and striatal neurons (Lotharius and Brundin, 2002). PC12 cells treated with MA show that the formed inclusions or membranous whorls contain α-synuclein which co-localizes with ubiquitin within the whorl (Fornai et al., 2004b). Others have shown that ubiquitination occurs in nigral dopaminergic neurons and periaqueductal gray matter in the midbrain of MA abusers and in MA fatality victims. Subsequently it has been postulated that dysfunction of these neurons may be primarily responsible for clinical symptoms displayed by MA users (Quan et al., 2005).

Genes and proteins affected by MA: possible therapeutic targets

Dopaminergic system

MA affects various neurotransmitter systems involved in reward and addiction. Initially, MA affects dopaminergic pathways in order to illicit rewarding or reinforcing effects, however this subsequently also alters the transmission of various other neurotransmitter pathways (Everitt and Wolf, 2002; Di Chiara et al., 2004; Pierce and Kumaresan, 2006). MA acts by entering the neuron at the dopaminergic amine transporter (DAT) which is located on the surface membrane of the neuron. In doing so, it reverses the function of the transporter (Zahniser and Sorkin, 2004; Wilhelm et al., 2006) and acts as an ion channel leading to non-exocytotic amine release into the synapse. This increases extracellular synaptic cleft dopamine availability. In addition, MA affects dopamine concentrations by interacting with the vesicular monoamine transporter 2 (VMAT2) and reverses the VMAT2 transporter function (Sulzer and Rayport, 1990; Pifl et al., 1995; Wilhelm et al., 2004; Sulzer et al., 2005). The VMAT2 transporter is responsible for the intracellular vesicle storage of dopamine, serotonin, norepinephrine and histamine (Erickson et al., 1992; Weihe and Eiden, 2000). The enzymes responsible for the intracellular and extracellular break down of monoamines, monoamine oxidase (MAO) and catechol-o- methyltranferase (COMT), are inhibited by MA (Robinson, 1985; Kita et al., 1995). This effect of MA on these enzymes ensures greater dopamine levels in response to MA.

The intensity of the “high” experienced by the MA user was found to be directly related to greater dopamine concentration levels and the occupancy of dopamine receptor type 2 (D2) (Laruelle et al., 1995; Volkow et al., 1999a; Drevets et al., 2001). D2 receptors are highly expressed in the shell subregion of the NAc (Larson and Ariano, 1995). Down regulation of D2 was observed in the striatum of MA users (Chang and Haning, 2006) and such reductions of striatal D2 receptors correlated well with increased metabolic activity of the OFC (Volkow et al., 2001c). Interestingly, decreased D2 brain levels has been associated with feelings of pleasure after methylphenidate administration in non-drug users, while increased D2 levels produced feelings of aversion (Volkow et al., 1999b). D2 receptor gene polymorphisms have been studied in substance abusers (Young et al., 2004). Here, it was suggested that the low functionality D2- TaqI A1 allele may serve as a risk factor in addictive behavioural disorders (Blum et al., 1996; Harano et al., 2001; Volkow et al., 2001c). Other studies have shown a greater prevalence of the D2- TaqI A1 allele polymorphism in MA abusers. These individuals also presented with greater novelty seeking behaviour and a trend towards decreased frontal executive functioning (Han et al., 2008). Similarly, greater TaqI A1 allele and the TaqI B1

allele of the D2 receptor gene were documented in Caucasian poly-drug users, one of the drugs being amphetamine (Smith et al., 1992; O'Hara et al., 1993). This was confirmed in a separate study of cocaine dependent individuals (Noble et al., 1993).

The dopamine receptor type 4 (D4) gene, which forms part of the D2 receptor family, and the COMT gene was also investigated for possible association with MA abuse (Li et al., 2004). Here it was noted that the 120-base-pair (bp) variable number tandem repeat (VNTR) polymorphism in the promoter of the D4 gene did not result in a significant association with MA abuse. However, an interaction between the Val158Met polymorphism in the COMT gene and D4 120 bp promoter polymorphism was reported as well as between COMT 158 Val/Met and the 48 bp VNTR polymorphism of D4 receptor gene. The researchers concluded that these genetic variations may constitute important risk factors for MA dependence (Li et al., 2004). This view was supported by reports of significant associations between MA abuse and the D4 and COMT genes (Bousman et al., 2009).

PET studies have indicated that chronic MA abuse is characterised by decreased DAT in the striatum (McCann et al., 1998; Sekine et al., 2001; Volkow et al., 2001d), OFC and dorsolateral PFC and the amygdala (Sekine et al., 2003). The SLC6A3 gene (or also referred to as the hDAT1 gene) encodes the dopamine transporter and found that MA psychosis was associated with a significant excess of the 9- or fewer-repeat alleles of the VNTR in 3' untranslated region (UTR) of the SLC6A3 gene (Ujike et al., 2003). A single photon emission computed tomography (SPECT) in vivo study demonstrated that a 22% reduction of DAT protein in the putamen of individuals possessing the 9-repeat/10-repeat genotype when compared to individuals with the 10-repeat homozygosity (Heinz et al., 2000). It has been suggested that genotypes with 9- or fewer-repeat alleles of the VNTR have decreased transcriptional activity of the SLC6A3 gene and that these individuals might be more susceptible to the neurotoxic effects of MA (Ujike et al., 2003). However, the 40-bp VNTR polymorphism of the 3'UTR of the DAT gene and allele frequencies for DAT repeats 11, 10 and 9 were found to have no significant associations with the clinical outcome of MA abusers (Liu et al., 2004). Similarly, no association between the DAT 3'-VNTR and MA dependence were also found in another study (Hong et al., 2003).

DAT inhibitors have been suggested to have possible therapeutic benefits in MA dependence, since chronic MA use results in low dopaminergic tone (Volkow et al., 2001c) and the

antidepressant, bupropion, may aid in restoring dopamine homeostasis. Bupropion has been shown to decrease subjective effects of MA and reduce craving (Newton et al., 2006). The drug also increased abstinence in males with low to moderate MA dependence (Shoptaw et al., 2008). In addition, animal studies have indicated that bupropion reduces the neurotoxic effects of MA in rats (Montoya and Vocci, 2008), thereby supporting the drug's potential benefit as a therapeutic agent in substance abuse.

The neuronal specific, vesicular monamine transporter 2 (VMAT2), transports monoamines from the cytosol into intracellular secretory vesicles for storage (Schuldiner, 1994). This transporter is therefore essential in preventing dopaminergic accumulation in the cytosol and reducing possible neurotoxic effects of dopamine break down. VMAT2 has been implicated in MA dependence because of MA's reversal of the transporter function, leading to the promotion of the increases in cytosolic dopamine concentrations (Sulzer and Rayport, 1990; Erickson et al., 1992; Pifl et al., 1995; Weihe and Eiden, 2000; Wilhelm et al., 2004; Sulzer et al., 2005). It has been demonstrated that MA neurotoxicity results from decreased VMAT2 uptake capacity in the striatum and this MA effect was blocked by the prior use of DAT inhibitors (Fleckenstein and Hanson, 2003). Heterozygous VMAT2 knockout mice have also been shown to lead to enhanced MA-induced neurotoxicity (Fumagalli et al., 1999). Enhancement of VMAT2 function has been suggested to lower dopamine concentrations and related neurotoxic effects and exert a possible neuroprotective effect (Vergo et al., 2007). VMAT2 can therefore be considered therapeutic target for treating MA abuse. Contrastingly, the competitive VMAT2 inhibitor, tetrabenazine has been proposed to have possible therapeutic effects (Howell et al., 1994; Peter and Edwards, 1996). Evidence from animal studies has shown how tetrabenazine opposes the discriminative stimulus properties of MA in rats and decreases the locomotor activity stimulated by MA in mice (Vocci and Appel, 2007).

A few other genes and proteins have also been investigated in MA abusers. The SLC22A3 encodes the organic cation transporter 3 (OCT3) which is a transporter for MA and several other neurotransmitters including dopamine and serotonin (Grundemann et al., 1998; Wu et al., 1998). Decreased expression of OCT3 mRNA was found in the brains of rats that were behaviourally sensitized to the effects of MA (Kitaichi et al., 2003). In a clinical study, SLC22A4 polymorphisms were not associated with MA dependence. However, when test subjects were divided into poly-drug users and MA-only users, genotype and allele frequencies of single nucleotide polymorphism (SNP) 2 and SNP3 and haplotype frequencies

of the two SNPs were found to be significantly different between the groups. This observation suggested that repeated exposure to one drug results in behavioural and neurochemical cross-sensitization to the usage of other drugs (Beyer et al., 2001, Akimoto et al., 1990; Kazahaya et al., 1989). The authors proposed that SLC22A3 polymorphisms may lead to poly-drug use in MA dependent individuals (Aoyama et al., 2006), and this was substantiated by a similar finding in a recent study (Bousman et al., 2009).

A functional polymorphism of the promoter region of the human protein interacting with C-kinase-1 (PICK1) gene has been associated with the susceptibility to spontaneous relapse of MA-induced psychosis (Matsuzawa et al., 2007). Single nucleotide polymorphisms (SNPs) rs713729 was significantly associated with MA abuse and rs713729 and rs2076369 was associated with spontaneous relapse of psychosis (Matsuzawa et al., 2007). PICK-1 is a scaffolding protein that plays a role in the targeting and localization of synaptic membrane proteins (Deken et al., 2001) and the clustering of the DAT on the cell surface (Torres et al., 2001) by the modulation of protein kinase C activity (Blakely and Bauman, 2000; Deken et al., 2001; Robinson, 2002). The PICK-1 gene that has been associated with schizophrenia (Hong et al., 2004; Fujii et al., 2006) was subsequently linked to MA psychosis. A recent study confirmed the implication of a role for the PICK-1 gene in MA dependence (Bousman et al., 2009). It therefore seems that the genetic polymorphisms of the PICK-1 gene results in impaired DAT function and dopamine activity which may give rise to MA-induced psychosis.

Serotonergic system

Various transmitter systems play modulatory roles in dopaminergic circuitry and some of these include serotonin (5-HT), glutamate and gamma-aminobutyric acid (GABA). The 5-HT system is ideally situated for its modulatory role of dopaminergic activity, since the 5-HT neurons from the dorsal and median raphe nuclei innervate dopaminergic neurons of the nigro-striatal and mesolimbic systems (Herve et al., 1987; Phelix and Broderick, 1995). Serotonin has been shown to increase the inhibitory effect of dopamine on dopaminergic VTA neurons (Brodie and Bunney, 1996), resulting in decreased dopaminergic neurotransmission. Di Matteo et al. (2001) have specifically shown that 5-HT2C receptors inhibit mesolimbic dopamine function.

Serotonin plays an important role in drug reinforcement, since mice deficient of 5-HT demonstrate increased self-administration of cocaine when compared to normal mice (Parsons et al., 1998). PET studies have also indicated that global brain 5-HT transporter (SERT) densities were lower in MA abusers (Sekine et al., 2006) and hence lead to the attenuation of dopamine concentration modulation. In addition, agonists of the 5-HT2C receptor and inhibitors of SERT decreases the firing rates of VTA dopaminergic neurons (Prisco et al., 1994; Di Mascio et al., 1998), resulting in reduced dopaminergic concentrations in the NAc (Di Matteo et al., 2000).

The 5-HT2C receptors are located on GABAergic neurons and affects orchestrated by serotonin are proposed to be mediated by increased GABA inhibitory action of VTA dopaminergic neurons (Eberle-Wang et al., 1997; Howell and Kimmel, 2008). The 5-HT6 receptors expressed in the limbic and motor brain areas (Monsma et al., 1993; Ruat et al., 1993) have also been implicated in amphetamine-induced behaviours. For instance, pre-treatment with the novel 5-HT6 receptor antagonist, SB 258510A, dose-dependently enhanced amphetamine's reinforcing effects and increased extracellular dopamine concentrations in the frontal cortex (Frantz et al., 2002). Behavioural and locomotor activities induced by MA that were prevented by DAT inhibitors or the deletion of the DAT gene, coincided with the release of 5-HT via SERT (Torres et al., 2003). The serotonin transporter gene, 5-HTTLPR, polymorphism particularly the S alleles which have reduced transcriptional efficiency and lower 5-HT1A receptor binding have been linked to MA psychosis (Ezaki et al., 2008). The 5-HTT VNTR polymorphism has also been correlated with susceptibility to affective disorders (Ogilvie et al., 1996). However, an association study between the triallelic 5-HTTLPR polymorphism (S, L_G, L_A) and MA psychosis was not significant (Chen et al., 2007).

Activation of other serotonergic receptor subtypes has mixed effects on the central dopaminergic system. For example, stimulation of 5-HT1B receptors reduced GABA inhibitory action in the VTA (Cameron and Williams, 1994) thereby, indirectly stimulating dopamine release. In contrast, antagonism of the 5-HT2A receptor, via the selective 5-HT2A antagonist, SR46349B, decreases amphetamine-induced dopaminergic release in the NAc and striatum (Porras et al., 2002).

Glutamatergic system

It is suggested that glutamatergic tone plays an important modulatory role for dopamine and glutamate concentrations (Howell and Kimmel, 2008). In general, glutamate has an excitatory effect on dopaminergic neurons in the VTA, usually leading to enhanced mesocorticolimbic dopaminergic activity (Jones and Kauer, 1999). Subsequent animal studies have confirmed a role for the glutamatergic system in the rewarding effects of psychostimulants (Pierce et al., 1996; Cornish and Kalivas, 2000; Carlezon and Nestler, 2002; Harris and Aston-Jones, 2003; Kalivas et al., 2003; McFarland et al., 2003). Acute effects of cocaine administration has been found to enhance NAc, VTA and PFC glutamate levels (Smith et al., 1995; Kalivas and Duffy, 1995; Reid and Berger, 1996; Pierce et al., 1996; Reid et al., 1997), while chronic exposure to cocaine results in decreased extracellular NAc glutamate concentrations (Keys et al., 1998; Bell et al., 2000). Moreover, chronic cocaine administration reduces metabotropic glutamate receptor 2 or 3 (mGluR2/3) autoreceptor function in the NAc (Xi et al., 2002a) and this is proposed to be a compensatory mechanism to decrease extracellular glutamate levels (Dackis and O'Brien, 2003). In line with this evidence, the administration of a mGluR2/3 agonist decreases both dopamine and glutamate release in the NAc, striatum and the PFC (Moghaddam and Adams, 1998; Hu et al., 1999; Xi et al., 2002b), while antagonists to the mGluR5 and N-methyl-d-aspartate (NMDA) receptor have been shown to decrease cocaine reward (Pulvirenti et al., 1997; McGeehan and Olive, 2003). Also, mGluR5-deficient mice do not acquire intravenous cocaine self-administration (Karler et al., 1998), thereby supporting an important function of this receptor in cocaine-induced effects.

Interestingly, the administration of N-acetylcysteine (NAC) normalizes glutamate concentrations in cocaine pretreated rats and prevents cocaine-induced drug reinstatement in these animals. Apparently, this is achieved by restoring the cysteine/glutamate exchanger (Xi et al., 2002a; Baker et al., 2003). However, the therapeutic mechanism of action has been questioned, since NAC does not cross the BBB. NAC has a negatively charged carbonyl group which is repelled by the negatively charged surface area of the endothelial cells of the BBB (de Boer and Gaillard, 2006). MA neurotoxicity has been shown to result from dopamine oxidation following MA's release of massive amounts of dopamine and the formation of toxic reactive oxygen species (ROS) (Davidson et al., 2001). As a result, ROS overrides the antioxidant defense mechanism of the BBB, which leads to a state of oxidative stress (Plateel et al., 1995). Disturbances in protective mechanisms of the BBB allow access of toxic subtances into the brain. MA has been shown to cause disturbances of the BBB by

regulation the tight junction expression and Rho-A activation (Bowyer and Ali, 2006; Mahajan et al., 2008). Zhang et al. (2009) has also demonstrated that MA results in a loss of BBB permeability of human brain microvascular endothelial (HBMVEC) cells. Glutathione is an intracellular thiol and acts as an antioxidant which scavenges ROS (Akca et al., 2005), which reduces oxidative stress (Yamamoto and Zhu, 1998) and is critical for maintaining the integrity of the BBB (Agarwal and Shukla, 1999). When glutathione is depleted it leads to a reduction in protein sulfhydryls which is important for membrane function (Agarwal and Shukla, 1999). MA significantly reduces glutathione levels which lead to compromised cell viability (Zhang et al., 2009). NAC has been shown to increase glutathione levels (Chen et al., 2008). Additionally, ROS increases P-glycoprotein (P-gp) (Ziemann et al., 1999; Hirsch-Ernst and Kietzmann, 2000), which is also known as a multidrug resistance protein and located in the BBB transmembrane and hence expressed in endothelial cells (Demeule et al., 2000), which allows the protein to act as an efflux transporter and subsequently limits the uptake of drugs from the blood into the brain. P-gp expression is also increased by glutathione depletion-induced oxidative stress in microvessel endothelial cells of rats (Hong et al., 2006). Similarly, NAC decreases P-gp upregulation which is induced by diethyl maleate (Wu et al., 2009). Since, NAC increases glutathione levels which protects against the oxidative stress induced by MA and decreases P-gp efflux functionality, NAC might gain access to the brain to prevent reinstatement or drug seeking in addicts. Alternatively, the NAC might gain access to the brain, as MA induces an oxidative state leading to altered cell viability, loss of BBB permeability, cell death or apoptosis and hence would allow passage through the BBB.

In a pharmacological study, the administration of modafinil, a non-amphetamine and weak psychostimulant, has been found to enhance striatal glutamate synthesis and striatal glutamate brain concentrations (Touret et al., 1994). Modafinil also directly inhibits rat midbrain dopamine neurons via D2 dopaminergic receptors (Korotkova et al., 2007). Subsequent clinical studies have reported higher prevalence rates for cocaine abstinence with modafinil treatment in patients with cocaine withdrawal symptoms (Dackis et al., 2003; 2005).

NMDA receptors can be activated by either the binding of glutamate to the NR2 subunit (Laube et al., 1997), or the binding of glycine to the NR1 NMDA subunit (Johnson and Ascher, 1987). Glutamatergic transmission from the PFC to the NAc, amygdala and VTA, together with the activation of NMDA receptors in these areas, have been proposed to be instrumental in the development of CPP and behavioural sensitization induced by MA

administration (Wolf, 1998). The glycine transporters (GlyTs) are essential in the regulation of glycine levels at the synapses (Smith et al., 1992). Antagonising the GlyT leads to the inhibition of glycine uptake (Harsing et al., 2003) which enhanced NMDA receptor function (Bergeron et al., 1998; Kinney et al., 2003). It would seem that glycine enhances glutamatergic neurotransmission and therefore glycine administration may be therapeutic. This suggestion may be feasible since the GlyT-1 gene has been suggested to contribute to the vulnerability to MA dependence and psychosis (Morita et al., 2008; Bousman et al., 2009).

Gamma-Aminobutyric acid (GABA)

GABA, an inhibitory amino acid, modulates the release of basal dopamine and glutamate (Dewey et al., 1992) and has therefore been implicated in the effects caused by psychostimulants (Roberts and Brebner, 2000; Cousins et al., 2002). The GABA-A1 and G2 (GABRA1, GABRG2) has been associated with MA abuse and abuse/dependence respectively (Lin et al., 2003; Nishiyama et al., 2005; Bousman et al., 2009). VTA dopamine activity is modulated by GABAergic interneurons (Churchill et al., 1992; White, 1996; Steffensen et al., 1998) and a major portion of NAc afferents are GABAergic. These fibres are thought to regulate dopaminergic input from the VTA to the NAc (Kita and Kitai, 1988).

On the other hand, cocaine and amphetamine both decrease GABAergic activity in the striatum and this effect is mediated by D2 dopamine receptors (Centonze et al., 2002). A number of drugs that have their effects on GABA metabolism have been tested in substance abuse. The idea is to inhibit GABA-transaminase, an enzyme that metabolises GABA, thereby increasing synaptic GABA levels (Dewey et al., 1998; Kushner et al., 1999). Vigabatrin (gamma-vinyl-GABA), an irreversible GABA transaminase inhibitor, has been shown to prevent stimulant-induced dopaminergic increases in the NAc and corticomesolimbic system, leading to an attenuation of their rewarding effects (Ashby et al., 1999; Gerasimov et al., 1999; 2000). Similarly, topiramate, a drug that potentiates GABAergic transmission (Shank et al., 2000), enhanced cocaine abstinence (Kampman et al., 2004).

Norepinephrine (NE)

It is suggested that the reinforcing effects of the central nervous system is not only induced by increases in dopaminergic concentrations alone, since dopamine receptor antagonists do not inhibit the subjective effects caused by cocaine or amphetamine administration (Ohuoha et al., 1997; Brauer and de Wit, 1996; 1997). Amphetamines and cocaine has been shown to release norepinephrine (NE) more potently than they release dopamine and serotonin (Segal and Kuczenski, 1997; Reith et al., 1997; Rothman et al., 2001). These increases in NE have been demonstrated to contribute to the subjective effects induced by stimulants. The locus coeruleus has projections to the VTA and has the ability to influence activity of dopaminergic neurons (Liprando et al., 2004). For example, lesions of the locus coeruleus reduce NAc dopamine levels (Lategan et al., 1990; Grenhoff et al., 1993), while NE depletion in the PFC results in decreased amphetamine-induced CPP (Ventura et al., 2003). Interestingly, dopamine is taken up by both dopaminergic and noradrenergic neurons and hence the inhibition of NE transporters (NET) increases extracellular dopamine (Carboni et al., 1990; Reith et al., 1997; Yamamoto and Novotney, 1998), an effect that may enhance addictive behaviour. It is proposed that cocaine which is an inhibitor of NET (Eshleman et al., 1999) would inhibit dopamine uptake into NE neurons, resulting in increased extracellular dopamine and concomitant reinforcement of cocaine effects (Rothman et al., 2001). These findings therefore caution against the use of drugs that act on NET such as atomoxetine, which is often used in the treatment of attention-deficit hyperactivity disorder (ADHD) (Prince, 2006).

Conclusions

Methamphetamine is a highly addictive substance and the abuse of this drug is increasing at an alarming rate in many countries world wide. MA induces alterations and morphological changes in numerous brain areas and neurotransmitter pathways and results in various neurochemical and behavioural abnormalities. Studies focusing on MA-induced abnormalities can result in a greater understanding of the underlying molecular mechanisms and resultantly address MA-induced changes by using effective therapeutic interventions.

Research done in the field of methamphetamine addiction has shown that the dopaminergic neural system is mainly affected by MA and hence attention has been focused on employing dopaminergic drugs to address and treat abnormalities induced by MA. The most important brain areas recruited by MA involved in the dopaminergic system includes the ventral

tegmental area, prefrontal cortex and the nucleus accumbens which play a role in the initial execution of reward. Additional recruitment of other brain areas is important for the establishment of addictive MA behaviour and neurochemistry which is achieved after multiple or chronic MA exposures.

Methamphetamine has repeatedly been shown to have neurotoxic effects on neurons as a result of accumulation of MA-induced increases in dopamine concentrations. These neurotoxic effects lead to various morphological alterations in affected areas in the brain. Structural brain changes are studied by using multiple imaging techniques and the measurement of brain metabolite levels determine the structural integrity and function of neurons which indicates the measure of neuronal damage inflicted by MA exposure.

Multiple genes and proteins have been implicated in the neurochemical and behavioural effects induced by MA. After the differential effects of cocaine and methamphetamine have been established, pharmacological treatment of MA addiction is still in the initial stages and future studies are needed to further elucidate the effects of MA in order to develop effective treatment strategies for methamphetamine addiction.

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CHAPTER 3

Maternal separation fails to render animals more susceptible to methamphetamine-induced conditioned place preference

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Maternal separation fails to render animals more susceptible to methamphetamine-induced conditioned place preference.

Running title: Maternal separation does not enhance methamphetamine place preference

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Abstract

The maternal separation (MS) paradigm is an animal model that has been successfully used to study the long term effects of child abuse and neglect. Experiments showed that animals subjected to trauma and stress early in life display behavioural, endocrinological and growth factor abnormalities at a later stage in life, results that mirrored clinical conditions. It is apparent that adverse events early in life may affect the development and maturation of the brain negatively. The purpose of the present study was to investigate whether the abnormal brain development occurring in separated animals would also enhance the development of a preference for psychostimulant drug usage. Rats were subjected to maternal deprivation and further exposed to methamphetamine-induced conditioned place preference (CPP) which primarily measures drug reward (ventral striatum), learning and memory. Apomorphine-induced locomotor activity was also assessed to investigate the effects of methamphetamine on the dorsal (primarily locomotor activity) striatal dopaminergic system. We found that 4 consecutive injections of methamphetamine resulted in CPP behaviour 24 hours after the 4th injection. A further 4 injections yielded similar CPP results and this effect lasted for at least 7 days until the third CPP assessment. These animals also had decreased ACTH and corticosterone secretions, but the prolactin levels were increased. Prior exposure to maternal separation did not have any effect on the CPP test. The ACTH and corticosterone secretions were also similarly reduced. However, maternal separation decreased the release of prolactin and this reduction was not evident in the separated group that received methamphetamine. There was no significant difference in the apomorphine-induced locomotor activity of normally reared animals whether they received methamphetamine or saline. Interestingly there was a significant difference in locomotor activity between the two groups of animals that were subjected to maternal deprivation. The separated animals that received methamphetamine displayed markedly reduced locomotor activity upon apomorphine administration when compared to those that were treated with saline. Taken together, we conclude that maternal deprivation differentially influences dorsal and ventral striatal regions implicating dopaminergic mechanisms.

Keywords

Maternal separation; Methamphetamine-induced place preference; Apomorphine-induced locomotor activity; HPA axis; Dopaminergic function; Striatum

Introduction

Early life stress has continually been associated with anxiety-related stress disorders and substance abuse later in adult life. Clinical studies have shown that many anxiety-related disorders for example post-traumatic stress disorder (PTSD), social phobia and panic disorder comorbids greatly with the risk of substance abuse (Penk et al., 1988; Donovan et al., 2001; Lopez et al., 2005). This has lead to various hypotheses of the role of causality, in particular the self-medication hypothesis (Stanton, 1976; Khantzian, 1997) suggesting that substances are taken in order to cope with trauma or stress experienced (Tyssen et al., 1998) or to relieve symptoms of anxiety (Volpicelli et al., 1999). During the adolescent period, in particular, individuals that is in the process of developing coping mechanisms to combat the deleterious consequences of exposure to adverse experiences and social stressors. Failure to develop successful coping strategies has been postulated to result in susceptibility for drug taking (Goeders, 2003). Epidemiological studies have also indicated that early life experiences allows for the identification of populations at risk for abusing drugs in adulthood (Dobkin et al., 1997).

Clinical studies of patients with early trauma and subsequent development of depression and anxiety disorders (Heit et al., 1997; Ladd et al., 2000) show that behavioural abnormalities are frequently coupled to neuroendocrine dysfunction. Similar to humans, animals display behavioural abnormalities at a later stage in life after being subjected to trauma and stress early in life. These changes in adult behaviour have been associated with a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and alterations in neurotrophic factors (Daniels et al., 2004; Uys et al., 2006; Faure et al., 2006). It is therefore evident that alterations in behaviour that appears during adulthood have a strong neurochemical basis.

The mesolimbic pathway is one of major dopaminergic pathways in the brain and is fundamental to the reward system. This pathway projects from the ventral tegmental area (VTA) to the striatum, which consists of both dorsal and ventral regions. In particular, this pathway has been shown to be involved in motivated behaviour and plays a key role in the reinforcing effects of psychostimulants (Koob et al., 1998; Wise, 1998). The mesocortical dopaminergic pathway plays a critical role in the initiation of the progressive process of addiction as it involves certain aspects of learning and memory (Goto and Grace, 2008). It also originates in the VTA where dopaminergic neurons project to the medial prefrontal

cortex. These two dopaminergic pathways are linked via glutamatergic neurotransmission from the medial prefrontal cortex to the nucleus accumbens (McFarland et al., 2003; Kalivas, 2004; Kalivas and Volkow, 2005). The consequences of the administration of psychostimulants may therefore reflect the neurobiological status of these two central dopaminergic systems.

The nigrostriatal dopaminergic pathway is involved in the regulation of locomotor activity and stereotypical or repetitive motor behaviours (Borlongan and Sanberg, 1995). In this case the dopaminergic neurones are located in the substantia nigra and they project to the dorsal striatum and specifically to the area called the caudate putamen (Afifi, 2003). This pathway mediates some of the behavioural effects induced by stimulant drugs (Koob, 1992); therefore the effects of pharmacological manipulation of this pathway can be readily determined by locomotor behavioural assessments.

Prolactin (PRL) is one of the hormones that are primarily involved in the process of lactation. PRL is synthesized and produced in lactrotrope cells that are located in the anterior pituitary gland. The release or inhibition of prolactin is regulated by two major hypothalamic dopaminergic systems; the tuberinfundibular (TIDA) and the tuberohypophysial (THDA) dopaminergic systems. The THDA neurons originate in the periventricular nucleus of the hypothalamus and project to the neurointermediate lobe of the pituitary. TIDA neurons originate in the arcuate nucleus of the hypothalamus and project to the primary capillary plexus in the median eminence. Its content is released in the portal blood system which carries the dopamine to the anterior lobe of the pituitary (Moore and Lookingland, 2000). Measurement of plasma prolactin levels may therefore provide a useful insight into the status of the dopaminergic systems of the hypothalamus (Ben-Jonathan and Hnasko, 2001).

The HPA axis has been implicated in precipitating the deleterious effects of both early life stress as well as substance abuse (Ortiz et al., 1995; Meaney et al., 2002). For example rats subjected to maternal separation exhibit elevated baseline corticosterone levels (Marais et al., 2008), while administration of the corticosterone synthesis inhibitor (ketoconazole) reduced the time required to achieve cocaine self-administration in rats (Campbell and Carroll, 2001). Slotten et al. (2006) found decreased basal corticosterone levels in maternally separated animals when compared to their non-handled counterparts. Discrepancies between these and the present findings can be ascribed to gender differences as well as differential maternal

separation paradigms employed. The HPA axis is also under dopaminergic control since strong immunoreactivity for glucocorticoid receptors were found on hypothalamic dopaminergic cell groups indicating direct connectivity between the two modalities (Härfstrand et al., 1986). The mesocortical dopamine system has been found to be a modulator of stress reactivity including that of the HPA-axis (Sullivan and Dufresne, 2006). Corticosterone release upon stress exposure has also been correlated to mesocortical dopamine release (Sullivan and Gratton, 1998). In addition, amphetamine-induced dopamine release in the ventral striatum was associated with increased cortisol levels and subjects with high cortisol levels were also linked to positive effects of the drug (Oswald et al., 2005). Besides prolactin, the measurement of corticosterone may therefore provide additional information regarding the integrity of the central dopaminergic system.

The present study was done to determine whether animals subjected to maternal deprivation (MS) early in life would display exaggerated responses to methamphetamine treatment and in addition to assess the effects of methamphetamine on dopaminergic systems in rats. Our behavioural measurements included methamphetamine-induced place preference behaviours and apomorphine-induced locomotor activity in the open field. Our neurochemical assessments included the determination of prolactin, ACTH and corticosterone plasma levels.

Materials and Methods

Animals

Twenty seven male Sprague Dawley rats were used in this experiment. Animals were housed at the central research animal facility (AAALAC accredited) of Stellenbosch University. All rats were housed in the same colony room separate from where stress procedures, methamphetamine exposures or dissections were to take place. 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 x 25 x 20 cm Plexiglas cages with corncobs bedding. Temperature was kept constant at 22ºC, humidity at 55% and food and water was available *ad libitum* for the duration of the experiment.

Drugs

R-(-)-Apomorphine hydrochloride was procured from Sigma-Aldrich Chemicals (St. Louis, MO). Methamphetamine hydrochloride was obtained from US Pharmacopeia Convention Inc. (Rockville, USA). Apomorphine and methamphetamine were both dissolved in 0.9 % saline prior to being administered to the rats at a volume of 0.1ml/100g of body weight.

Experimental design

All rat pups were weaned at the age of postnatal day (PND) 21 according to gender and male rats were divided into 4 groups. The groups included:

1) Control saline group (C Sal): animals not subjected to MS and receiving saline injections during conditioned place preference (CPP) (n = 7).
2) Control methamphetamine group (C Meth): animals not subjected to MS and subjected to methamphetamine-induced CPP (n = 7).
3) MS saline group (MS Sal): animals subjected to MS and receiving saline injections during CPP (n = 6).
4) MS methamphetamine group (MS Meth): animals subjected to MS and subjected to methamphetamine-induced CPP (n = 7).

Each of the above groups was tested on PND 37 (after first 4 methamphetamine administrations), PND 43 (after further 4 methamphetamine injections) and 7 days (PND 50) after 8 methamphetamine conditioning sessions to characterise the duration of place preference behaviour. In addition, all of the rats were exposed to apomorphine-induced locomotor activity on PND 38, 44 and 51 after each post conditioning test. Animals were all sacrificed by means of decapitation the day after the last apomorphine-induced locomotor activity in the open field on PND 52.

Maternal separation (MS) paradigm

Male and female rats were paired and their offspring were used. The day of birth was designated as PND 0, where after maternal separation commenced two days later on PND 2 until PND 14 for a 3 hour daily period between 09h00 and 13h00. This protocol is in accordance with the deprivation procedures as employed by Ladd et al. (2000). During the separation period, the pups were removed from the mother and kept in a separate cage and the mother kept in the home cage. The cage containing the pups were then moved to an isolated dedicated room where the pups were kept warm under infrared lights (30-33ºC) in order to control for exposure to hypothermic conditions. Control litters were reared normally without

separations. After maternal deprivation was completed, animals were subjected to normal housing conditions. All rat pups were weaned at the age of 21 days, according to gender. Rats were regularly handled to minimize the effects of handling stress during the behavioural assessments.

Conditioned Place Preference (CPP) paradigm

The CPP test is a Pavlovian classical conditioning test, whereby the drug state is paired with a neutral environment (the CPP two compartment box). Colour and floor texture are differential cues used in the two compartments of the CPP box in order to differentiate between each compartment. These cues aid in the associations made between the drug state and the environment of one of the CPP compartments. In our study, the one compartment was black with a smooth floor, whilst the opposing compartment was black with white vertical stripes and a grid textured floor. The amount of time spent in the drug-paired environment is considered as a measure of the positive reinforcing or rewarding effects of the drug. This phenomenon has been confirmed in human cocaine addicts, whereby drug paraphernalia that is considered as a neutral environmental stimuli, become associated with the effects experienced by cocaine use (Gawin, 1991).

Adolescent male rats were handled from PND 25 until PND 30, where after they were habituated to the test cage the day before the testing (PND 31) for 15 minutes. On PND 32 rats in all groups were subjected to the CCP test. The CPP test consists of 3 stages: pre-conditioning stage, the conditioning stages and the post-conditioning stage.

Pre-conditioning

During the pre-conditioning stage, animals were given free access to both compartments for a period of 15 min of the CPP test box and the amount of time spent in each compartment were recorded and measured. The pre-test phase allows for the determination of the natural preference of the animal for either one of the two compartments of the CPP box.

Conditioning

The conditioning stage involves a pavlovian associative learning method, whereby a neutral stimulus (drug-paired compartment) was paired with the drug state induced by methamphetamine. The animals designated to the methamphetamine groups were intra-peritoneally injected with methamphetamine (1mg/kg, i.p.) 20 min before being confined for

a further 30 min period to the compartment of the test box opposite to the natural preference of the rat. The 50 min period in total was allowed for the drug to take affect and to ensure the formation of associations in the CPP box. The conditioning stage was repeated twice on two separate 4 consecutive day periods (PND 33-36 and PND 39-42). Control rats received equivalent saline injections and were also placed in opposing compartments of the CPP box.

Post-conditioning

On PND 37, 43 and 50 post-conditioning occurred to establish the duration of methamphetamine-induced place preference. During the post-conditioning stage animals were once again given free access to both compartments, and the amount of time spent in each compartment was recorded to allow for analyses at a later stage by using an automated behavioural tracking system (Noldis Ethovision). The CPP score was calculated as the difference between the amount of time spent in the drug-paired compartment before and after the conditioning stages.

Apomorphine-induced locomotor activity in the open field

The non-selective dopamine agonist, apomorphine was used in order to determine whether dopaminergic pathways was activated, and in particular, the striatum of which the nucleus accumbens comprises the ventral area which would lead to the deduction that the dopaminergic pathways to this particular area was activated as well. In addition, try to establish to what extent dopamine transmission induced by methamphetamine has influenced DA receptor numbers or resulted in the desensitization of post-synaptic dopaminergic receptors. Thus, all animals were additionally exposed to 3 separate apomorphine administrations as to determine methamphetamine-induced dopaminergic status over time.

The day after each post-conditioning test on PNDs 38, 44 and 51, rats were subjected to apomorphine-induced locomotor activity in the open field. All rats were exposed to a subcutaneous apomorphine injection (1 mg/kg, s.c.) and placed in the open field after 30 min, to allow for the drug to take affect, in order to record locomotor activity for a 10min period. After each behavioural recording, the open field was cleaned with 70 % ethanol to prevent interference with the next animal's behaviour in the apparatus.

Behavioural analysis of CPP test and locomotor activity

On all days of behavioural testing during the pre-test, post-tests and apomorphine-induced locomotor activity in the open field; behaviour was recorded on video tape in order to allow for later analysis. Video recordings were analysed using Noldus Ethovision (Wageningen, The Netherlands) software where the behaviour of animals were digitally tracked in both the CPP box and the open field.

Determination of plasma neuroendocrine hormones

On PND 52, animals were decapitated and trunk blood collected in pre-cooled EDTA tubes for neurohormonal measurement of adrenocorticotropin hormone (ACTH), corticosterone and prolactin (PRL) concentrations. The blood samples were centrifuged at 4000 rpm for 10 min at 4ºC to separate the plasma, where-after the samples were frozen in liquid nitrogen until assayed. Plasma ACTH concentrations were determined using a solid phase immunoradiometric (IRMA) assay (EURIA-ACTH c.t., Euro-diagnostica, Medeon, Sweden). Plasma corticosterone concentrations were determined using radioimmunoassay (RIA) kits (ImmuChem^{TM} Double Antibody Corticosterone ^{125}I RIA kit, MP Biomedicals, Orangeburg, NY). Plasma prolactin levels were measured using an enzyme immunoassay (EIA) Kit (Rat Prolactin EIA Kit, A05101, SPI-BIO, Bertin Group, Montigny Le Bretonneux, France).

Statistical analysis

The nonparametric analysis of variance (ANOVA), the Kruskal-Wallis test was used to analyse behavioural parameters, ACTH, corticosterone and prolactin data. Statistical significance of this test (H-statistic) was followed by the Mann-Whitney U post hoc test where applicable. Statistical tests were all done with the aid of SPSS (version 15.0). The level of significance was accepted as $p<0.05$. Data is reported as mean ± SEM.

Results

Conditioned place preference behaviour 24hrs after 4 methamphetamine conditioning sessions (CPP score 1) on PND 37

Animals subjected to saline conditioning resulted in spending less time in the compartment allocated for conditioning showing a preference for the opposing side of the place preference box when compared to animals exposed to methamphetamine conditioning ($p = 0.018$) ($H=12.741$, 3 df, $P=0.005$). Similarly, maternally separated rats exposed to saline

conditioning exhibited a preference for the opposing compartment of the CPP box when compared to rats subjected to drug conditioning (p = 0.010). Methamphetamine conditioned rats showed a preference for the drug-paired compartment of the place preference apparatus, while rats also subjected to maternal separation early in life exhibited similar place preference for the drug-paired compartment. Non-MS and MS rats exposed to methamphetamine conditioning exhibited a very large preference in comparison to saline conditioned animals (Figure 1).

Conditioned place preference behaviour after 8 methamphetamine conditioning sessions (CPP score 2)

On PND 43, all animals had been exposed to 8 methamphetamine conditioning sessions, and showed similar preference behaviours as in CPP score 1 behaviours (H=17.232, 3 df, P=0.001). Control (p = 0.003) and maternally deprived (p = 0.010) animals subjected to saline conditioning displayed an opposing preference for the saline-paired compartment. In contrast, animals exposed to methamphetamine conditioning in both control (p = 0.003) and maternally separated rats (p = 0.010) exhibited place preference for the drug-paired compartment. In addition, the maternally separated rats exposed to saline conditioning displayed significantly less preference for the opposing conditioned compartment of the CPP box than when compared to the animals experiencing control rearing conditions (p = 0.046) (Figure 2).

Conditioned place preference behaviour 7 days after last conditioning session (CPP score 3)

In order to characterise the duration of place preference behaviour after 8 methamphetamine administrations a post-test was conducted 7 days after last conditioning session on PND 50. Surprisingly, all animals still displayed similar place preference behaviours as in CPP score 1 and 2 (H=8.905, 3 df, P=0.031). Both control (p = 0.041) and maternally separated rats (p = 0.046) subjected to saline-paired conditioning displayed less preference for the conditioning compartment of the CPP box. Animals exposed to normal rearing (p = 0.041) and to maternal deprivation (p = 0.046) when subjected to methamphetamine conditioning displayed place preference for the drug-paired compartment of the CPP box (Figure 3).

Apomorphine-induced locomotor activity in the open field

Control and maternally separated rats were both exposed to apomorphine-induced locomotor activity after each post-conditioning test on PND 38, 44 and 51 in order to assess dopaminergic function and receptor status. All experimental groups displayed similar

locomotor activity in the open field after apomorphine administration on PNDs 38 (H=4.644, 3 df, P=0.2) and 44 (H=4.105, 3 df, P=0.25). Maternally deprived animals subjected to methamphetamine conditioning displayed a gradual reduction in locomotor activity in the open field and this decrease in locomotion only became significantly different after the third apomorphine-induced open field activity on PND 51 (H=8.952, 3 df, P=0.030). This group differed significantly from the third apomorphine-induced locomotor activity in the animals in both control (p = 0.025) and maternally deprived (p = 0.007) groups subjected to saline-conditioning (Figure 4).

Basal plasma ACTH concentration in rats subjected to CPP

Various significant differences between groups were found regarding ACTH levels in blood plasma (H=14.5, 3 df, P=0.002). All experimental groups showed lower ACTH levels when compared to normally reared animals that received saline injections. The normally reared control rats subjected to methamphetamine conditioning had lower ACTH concentrations when compared to normally reared saline animals (p = 0.007). This group had similar ACTH levels when compared to both groups exposed to maternal separation. Maternally deprived rats that were subjected to saline conditioning had significantly lower ACTH levels when compared to the control saline rats (p = 0.008). Similarly, the maternally separated rats exposed to methamphetamine conditioning showed lower levels compared to control saline rats (p = 0.003) (Figure 5).

Basal plasma corticosterone levels in animals subjected to CPP

Corticosterone concentrations followed a similar pattern as that of ACTH levels in all experimental groups (H=12.729, 3 df, P=0.005). Both groups of animals exposed to methamphetamine and rats subjected to maternal separation and saline conditioning displayed stress corticosterone levels that were comparable. Rats not previously subjected to maternal deprivation but exposed to saline conditioning resulted in higher corticosterone levels when compared to methamphetamine conditioned rats (p = 0.009) and saline conditioned rats previously exposed to maternal deprivation (p = 0.017) and lastly higher corticosterone concentrations than maternally separated animals subjected to methamphetamine conditioning (p = 0.003) (Figure 6).

Basal plasma prolactin concentration in rats subjected to CPP

The control animals exposed to methamphetamine conditioning displayed increased prolactin levels when compared to rats subjected to saline conditioning ($p = 0.018$) ($H=10.575$, 3 df, $P=0.014$). In addition, control rats that received the methamphetamine conditioning also exhibited significantly higher prolactin concentrations when compared to maternally separated rats subjected to saline conditioning ($p = 0.023$). Animals exposed to maternal separation exhibited lower prolactin levels compared to their drug exposed counterparts ($p = 0.043$). Interestingly, the maternally deprived and drug conditioned animals demonstrated increased prolactin concentrations when compared to saline controls ($p = 0.023$) (Figure 7).

Discussion

The major findings of the present study were as follows: (1) animals treated with methamphetamine display conditioned place preference behaviour when compared to their respective controls; (2) the place preference behaviour lasted for at least 7 days; (3) significant reductions in apomorphine-induced locomotor activity were observed only in rats that were exposed to maternal separation and methamphetamine; (4) basal stress hormone levels (ACTH and corticosterone) were similarly reduced in maternally deprived rats and animals treated with methamphetamine and (5) prolactin levels were increased in those animals that were subjected to methamphetamine conditioning.

Addiction usually arises as a result of repeated drug exposures to ensure long lasting chemical and molecular changes induced by repeated drug exposures, hence the increased risk of relapse and craving long after drug cessation (Nestler, 2001). In our study, we found that CPP behaviour resulted 24hrs after 4 consecutive conditioning sessions, in addition to CPP behaviour displayed 7 days after the 8^{th} methamphetamine conditioning session. We administered an additional 4 injections of methamphetamine, since pilot studies done in our laboratory of 4 methamphetamine injections did not result in CPP behaviour. However, Cherng et al. (2007) induced CPP in mice with three, two and even one alternate administration of methamphetamine with saline pairings. These discrepancies between findings can possibly be accounted for by variations in CPP paradigm used as well as mice versus rats used in present study. Similarly, Mizoguchi and colleagues (2004) induced CPP with three cycles of alternate methamphetamine and saline conditioned pairings, here too differences can be ascribed to variations in CPP protocols, dosages used, route of

administration of the drugs, in addition to different animal strains employed. Interestingly, when methamphetamine conditioning takes place without alternate saline pairings it did not result in eliciting reliable methamphetamine induced place preference (Cherng et al., 2007). This latter finding is in contrast to the present findings, since rats exposed to methamphetamine conditioning resulted in significant place preference behaviours. It is not surprising to find prolonged place preference behaviour in animals exposed to methamphetamine conditioning as in the present study. Lin et al. (2007) also demonstrated that six methamphetamine injections resulted in behavioural sensitization and induced conditioned place preference which lasted for 7 days.

We found that animals exposed to early life stress and normally reared controls developed place preference behaviours after repeated methamphetamine exposure. Stress-induced reinstatement is one of the major risk factors resulting in relapse or reinstatement, in addition to the other modalities of a drug dose itself or any other environmental stimuli previously associated with drug taking (Shalev et al., 2002). Numerous studies have shown that early life stress induces definite behavioural and endocrinological effects and that these detriments persist well into adulthood (Matthews et al., 1996a; Ladd et al., 1996; Matthews et al., 1999). A study by Vazquez et al. (2007) indicated that maternal separation resulted in prolonged morphine-induced CPP behaviour when compared to control animals and that these behaviours were accompanied by a decrease in dopamine D2 receptors in the nucleus accumbens core and an even greater decrease in the shell region. Brake et al. (2004) found maternal separation to induce a higher sensitivity to cocaine locomotor activity and display greater dopaminergic response in the nucleus accumbens to a mild stressor and show hyperactivity in a novel setting. The maternally deprived animals also reacted to amphetamine's locomotor effects more strongly when repeatedly stressed in comparison to their handled and non-handled counterparts. These behavioural findings were accompanied by a decrease in DAT levels in the deprived rats which provided evidence for early life stress implicating dopaminergic mechanisms and resulting in a possible neurological vulnerability to abusing drugs (Brake et al., 2004). Contrastingly, we however did not observe a great difference in CPP behaviour between control and maternally separated animals, but maternal deprivation did alter the stress hormones and dopaminergic function. Possible reasons for this discrepancy may reside in differences in animal strain used, as well as variations within the respective maternal separation paradigms. These results illustrate that more research must be done to ascertain the controversial role that early life stress plays in drug addiction and

whether early life stress introduces a vulnerability or resiliency factor for subsequent addictive biochemistry.

Exposure to a novel setting and the resultant locomotor response of the animal has been shown to be a good indicator of rearing differences (Meaney et al., 2002). Maternally separated and non-handled animals initially display either freezing or anxious behaviours in the beginning of an open field exposure after which their locomotor activity becomes significantly more than the handled animals which are usually very active at the beginning after which this activity decreases (Meaney et al., 2002). Higher rates of amphetamine self-administration have also been found in high locomotor responders (Piazza et al., 1989) and they too have increased dopaminergic response to amphetamine in the nucleus accumbens (Hooks et al., 1992). In the present study, animals received an apomorphine injection and were subjected to the open field to assess locomotor activity. No real difference were noted during the first two assessments of apomorphine-induced open field activity between maternally separated and control animals on PNDs 38 and 44. However, after the third apomorphine administration on PND 51 maternally deprived rats previously exposed to methamphetamine conditioning exhibited reduced locomotor activity when compared to the maternally separated rats that received saline conditioning and the normally-reared control animals. Given the fact that animals only showed a significant decrease in apomorphine-induced locomotor activity on PND 51 and not earlier may suggests that a more pronounced effect on locomotor activity might be seen in true adulthood (PND 60+). This finding indicated that early life stress predisposed the animals to subsequent insults such that the added exposures to methamphetamine and apomorphine resulted in significant decreases in locomotor activity. Increased dopaminergic transmission in response to both maternal separation and methamphetamine conditioning may have led to the desensitization of post-synaptic dopamine D2 receptor signalling or even a decrease in D2 receptor number. Such a mechanism may explain the observed decrease in locomotor activity upon apomorphine challenge. A similar result was reported by Matthews et al. (1996b) who found that adult animals previously exposed to repeated maternal deprivation are less responsive to the locomotor effects induced by amphetamine. The reduction in locomotor activity only after the third apomorphine administration in the present study could possibly reflect the gradual adjustment of the central dopaminergic system to the effects of multiple exposures to methamphetamine and apomorphine. Since these drugs are known to induce differential aspects of addiction namely conditioned place preference and behavioural sensitization (Kim

and Jang, 1997; Dias et al., 2006; Bloise et al., 2007), it can be expected that they influence the dopaminergic reward system differently. For instance, it has been shown that methamphetamine primarily acts upon the dopamine transporter (Zahniser and Sorkin, 2004), whilst apomorphine preferentially binds to postsynaptic D2 receptors to exert their effects (Dias et al., 2006). Interestingly, Kaneno et al. (1986) reported that repeated methamphetamine administration enhanced apomorphine stereotyped behaviours but methamphetamine pre-treatment however did not result in changing striatal dopamine, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) contents. In contrast, the sensitivity of striatal dopaminergic metabolism to apomorphine was shown to be reduced after methamphetamine pre-treatment (Kashihara et al., 1987) and that apomorphine lowered striatal dopaminergic metabolism less in methamphetamine treated rats compared to saline treated control rats (Kashihara et al., 1986). As reported in the present study, methamphetamine treatment in maternally separated animals resulted in decreased apomorphine-induced locomotor activity which may rather implicate the role of early life stress in influencing locomotor and hence dorsal striatal dopaminergic mechanisms. It is important to mention that stress too has been shown to play a definite role in the enhancement of the psychomotor stimulating effects of psychostimulant drugs (Kalivas and Stewart, 1991) which is also referred to as stress-induced sensitization. When exposed to stressful situations the resultant increase in glucocorticoids is suggested to play a role in the development of stress-induced sensitization (Marinelli and Piazza, 2002), suggesting that stress cross-sensitize with the effects of psychostimulants. When high levels of glucocorticoids are blocked by metyrapone the psychomotor stimulating effects of cocaine or amphetamine is reduced (Rouge-Pont et al., 1995; Reid et al., 1998). Contrastingly, when stress corticosteroids levels are induced it leads to increased amphetamine psychomotor activity (Deroche et al., 1992). Multiple biochemical role players are involved in behavioural outcomes that caution must be taken when interpreting above findings. However, it would seem that maternal separation plays a significant role in affecting the locomotor response of animals subjected to several methamphetamine exposures.

The stress response system of the brain plays an important role in the early stages of development in determining vulnerability to drug abuse and relapse (Goeders, 1997; Piazza and Le Moal, 1998). Stress activates the HPA-axis which ultimately leads to the secretion of glucocorticoids influencing various areas of the brain where glucocorticoid receptors are expressed. Interestingly, glucocorticoid receptors are expressed on dopaminergic neurons

(Härfstrand et al., 1986) which partially explain the influence of the stress response on drug related effects. For example, stress has been shown to lead to increased glucocorticoid levels causing higher dopamine concentrations especially in mesolimbic pathways. This apparently facilitates enhanced sensitivity to the reinforcing effects of drugs and higher rates of self-administration (Deroche et al., 1997; Piazza and Le Moal, 1998; Le Moal and Koob, 2007). Also, both glucocorticoids and psychostimulants act on the nucleus accumbens shell region (Barrot et al., 2000). In addition, it has been found that corticosterone treatment leads to the development of cocaine sensitization as assessed by VTA regulated locomotor activity (Ortiz et al., 1995). Surprisingly, non-contingent electric footshock or the pre-treatment of corticosterone itself did not lead to the acquisition of methamphetamine self-administration (Moffett and Goeders, 2005). These examples demonstrate that differential hormonal responses are needed to inflict various drug-induced phases of addiction for different psychostimulants even though these drugs have similar behavioural outcomes.

As stress and amphetamine are known to cross-sensitize (Cole et al., 1990; Deroche et al., 1992), it may be possible that stress experienced during the injection of methamphetamine might confound the rewarding effects as tested by the CPP paradigm. Corticosterone has been found to have reinforcing properties, as the administration of corticosterone increases the reinforcing properties of psychostimulants (Deroche et al., 1997), while adrenalectomy reduces psychostimulant-induced reinforcing effects (Goeders and Guerin, 1996; Deroche et al., 1997). In the present study, animals subjected to methamphetamine resulted in lower ACTH and corticosterone levels when compared to rats exposed to saline conditioning. This could possible be ascribed to an exhaustive HPA axis as a result of multiple methamphetamine exposures during the conditioning phase. In addition, Dietz et al. (2007) also found that corticosterone neither resulted in conditioned place preference or aversion when compared to cocaine-induced conditioning. We propose that the added release of corticosterone upon injection of methamphetamine of a stress response system already taxed with large releases of stress hormones, the additional secretion of hormones during injection stress would not have had a significant effect and thus not confound the place preference behaviour induced by methamphetamine.

In the present study, methamphetamine administration led to reduced levels of both ACTH and corticosterone. This finding is in contrast with others that reported methamphetamine to transiently increase plasma corticosterone. This apparent stimulation of corticosterone

secretion was however observed after only a single injection (Asano and Moroji, 1974; Szumlinski et al., 2001). Interestingly, hypoactive HPA response of decreased blood cortisol levels were found during the withdrawal period of cocaine addicts after long-term abuse (Buydens-Branchey et al., 1997). In our experiments, the maternally separated rats also had decreased ACTH and corticosterone concentrations when compared to saline conditioned rats. Previous work done in our laboratory yielded similar results where maternal deprivation alone resulted in reduced ACTH and corticosterone levels (Faure et al., 2006). These findings are therefore in line with clinical observations showing the comorbidity of substance abuse and hypoactive HPA axis responses that occur in individuals suffering from post-traumatic stress disorder (PTSD) (Yehuda et al., 1995) or those that were subjected to childhood abuse (Heim et al., 2001). It is thus not surprising, since maternal separation is an animal model for childhood abuse and neglect that decreased stress hormonal responses were observed in our animals. However, the combination of maternal separation and methamphetamine conditioning did not result in an additive effect but rather in similar levels suggesting that the two interventions may have independent mechanisms of action with respect to the regulation of the HPA axis.

All animals subjected to methamphetamine conditioning had increased prolactin levels when compared to saline conditioned rats. Increased prolactin levels are generally an indication of low dopamine levels or an exhausted hypothalamic-tuberinfundibular dopaminergic system. Alternatively, prolactin release from the anterior pituitary could have been altered (Mantsch et al., 2000). Animals subjected to maternal separation alone had similar prolactin levels when compared to control animals exposed to saline conditioning. This result suggests that maternal separation per se had no effect on hypothalamic dopaminergic neurons.

In summary, our data suggests that maternal separation selectively affects the dorsal striatum and hence had a greater influence on locomotor activity. Maternal deprivation in combination with methamphetamine conditioning resulted in decreased locomotion. This result is in line with work done by others that have shown that adult animals previously exposed to repeated maternal separation do not react on the locomotor effects induced by amphetamine (Matthews et al., 1996b). On the other hand, methamphetamine induced CPP and decreased hypothalamic dopaminergic activity. It was therefore not surprising that prior exposure to maternal separation did not exacerbate the CPP effects mediated by methamphetamine. This was in contrast to what was expected, since the development of substance abuse has been

closely related to stress. Interestingly, exposure to early life stress has been shown to lead to increased locomotor activity and increased dopaminergic turnover in mesolimbic brain areas whereas striatal dopaminergic inputs from the nigrostriatal pathways were less active when exposed to amphetamine challenge (Kehoe et al., 1998). This finding therefore supports our current proposal of a preferential effect of maternal separation on the differential dopaminergic pathways.

Conclusions

Stress during childhood in the form of maternal deprivation did not lead to greater place preference behaviour as was expected. However, maternal separation did introduce a vulnerability factor on apomorphine-induced locomotor activity in the open field and on stress hormone levels and prolactin concentrations. Maternal separation differentially influences dorsal and ventral striatal regions implicating dopaminergic mechanisms.

Acknowledgements

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Figures

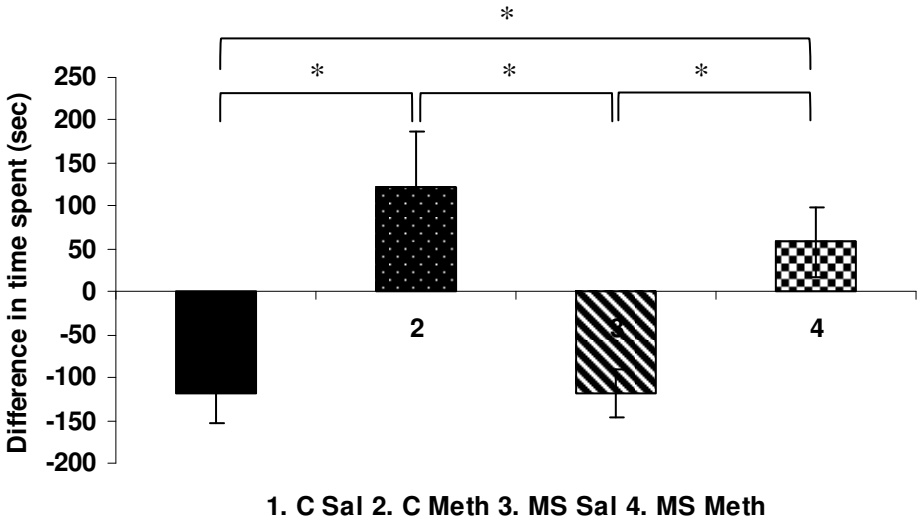

Figure 1: Conditioned place preference (CPP) score 1 (24hrs after first 4 methamphetamine conditioning sessions) of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 7, MS Sal: 6, MS Meth: 7).

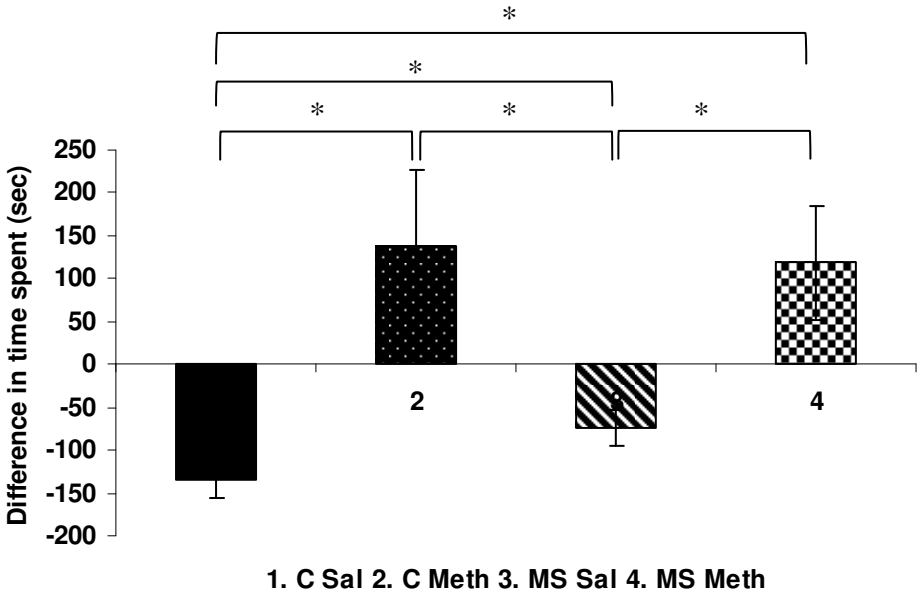

Figure 2: Conditioned place preference (CPP) score 2 (after 8 methamphetamine conditioning sessions) of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 7, MS Sal: 6, MS Meth: 7).

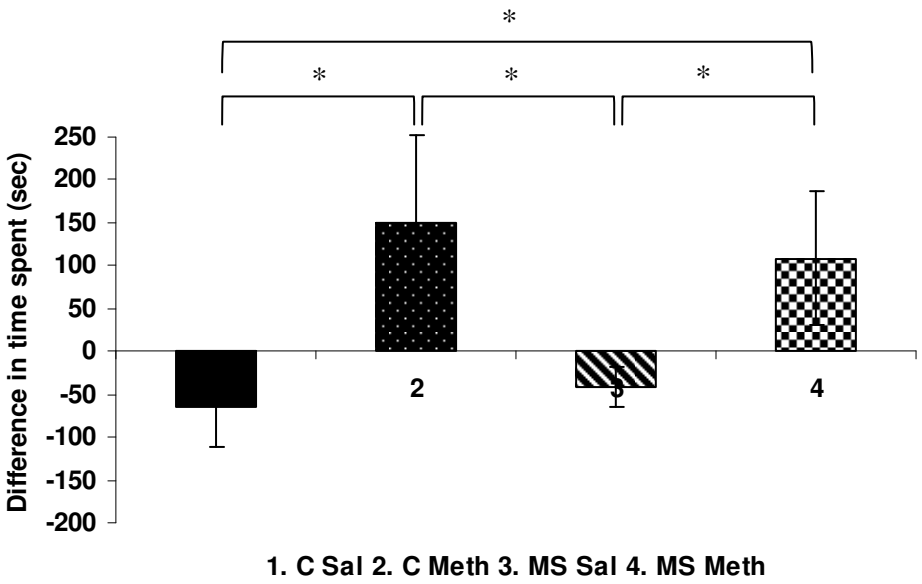

Figure 3: Conditioned place preference (CPP) score 3 (7 days after last conditioning sessions) of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 7, MS Sal: 6, MS Meth: 7).

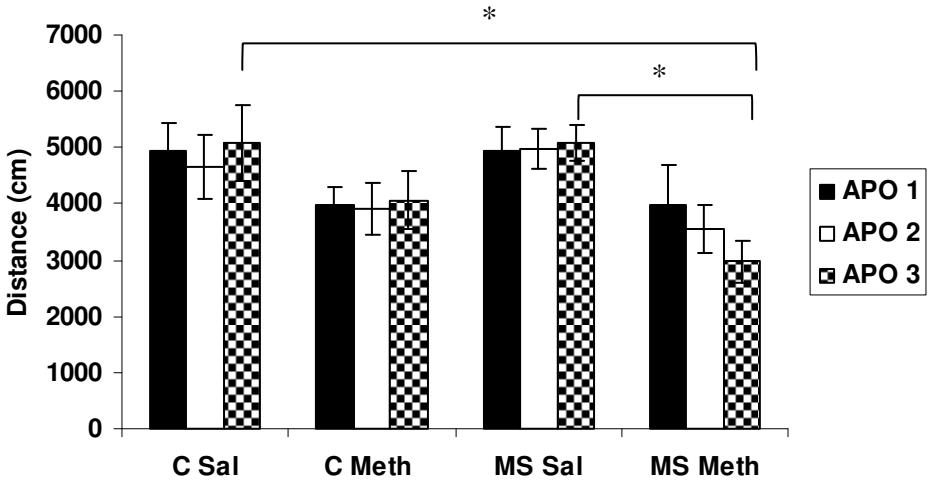

Figure 4: Apomorphine-induced locomotor activity in the open field (Apo 1, Apo 2 and Apo3) the day after each post-conditioning test of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 7, MS Sal: 6, MS Meth: 7).

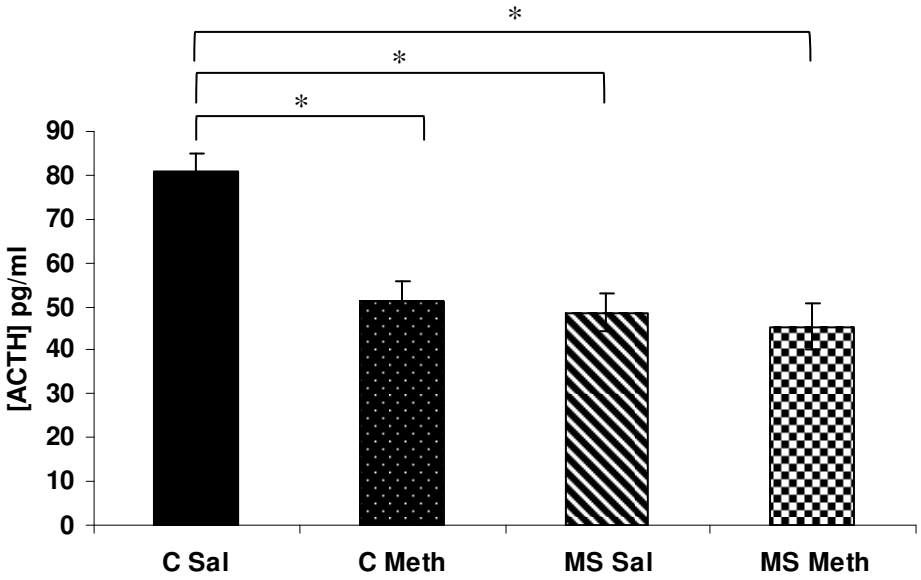

Figure 5: Adrenocorticotrophin (ACTH) concentrations in pg/ml of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 6, MS Sal: 4, MS Meth: 6).

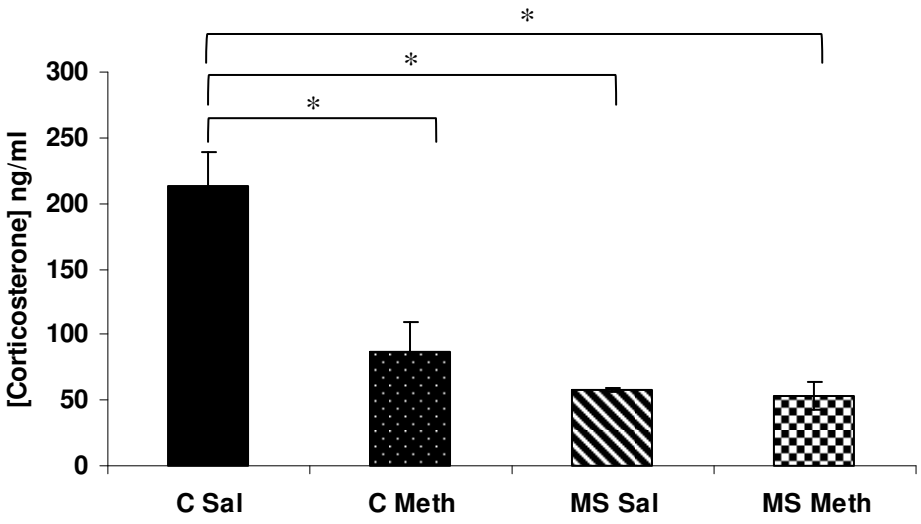

Figure 6: Corticosterone concentrations in ng/ml of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 7, MS Sal: 3, MS Meth: 6).

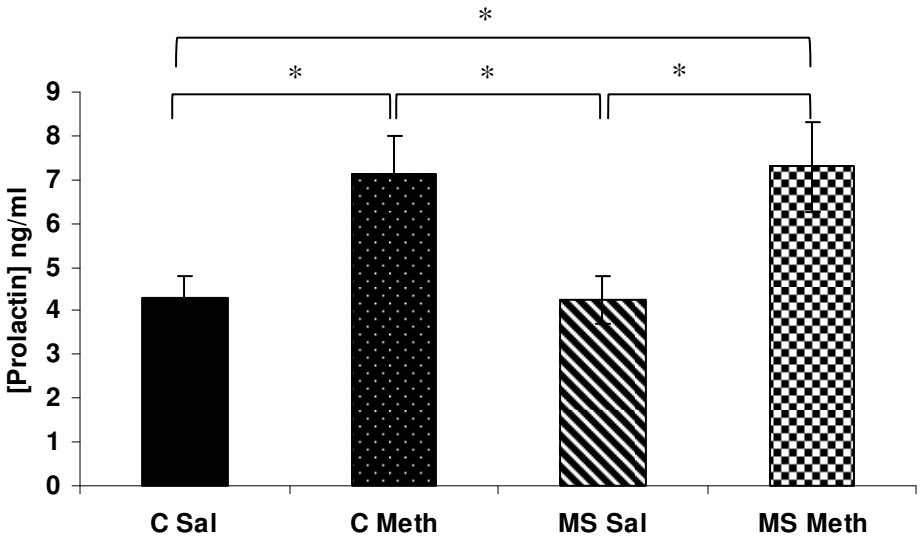

Figure 7: Prolactin (PRL) concentrations in ng/ml of groups subjected to control conditions and maternal separation. Values given as mean ± SEM. * significantly different from controls. $p < 0.05$ (N = C Sal: 7, C Meth: 7, MS Sal: 4, MS Meth: 4).

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CHAPTER 4

Maternal separation differentially alters neurotrophic factors in the dorsal and ventral hippocampus of methamphetamine-treated rats

Maternal separation differentially alters neurotrophic factors in the dorsal and ventral hippocampus of methamphetamine-treated rats

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Abstract

Stress has been shown to lead to increased susceptibility towards abuse of drugs later in life. Early life maternal separation (MS) stress has also been shown to alter the response to drugs of abuse. A link between methamphetamine use and altered neurotrophic factors has also been established. Changes in neurotrophin concentrations may play a role in the underlying molecular mechanisms responsible for drug- or stress-induced behaviours. The purpose of the present study was to investigate the effect of methamphetamine administration and MS on BDNF and NGF levels in both the dorsal and ventral hippocampus and investigate the combined effect of MS stress predisposition to methamphetamine-induced neurotrophic factor concentrations in the hippocampus. Methamphetamine administration and MS resulted in decreased BDNF levels in both the dorsal and ventral hippocampus. Early MS together with later methamphetamine administration also resulted in similar decreases in BDNF concentrations in the dorsal and ventral hippocampus. Neither MS nor methamphetamine altered NGF levels in the dorsal hippocampus. However, MS did decrease NGF levels in the ventral hippocampus. The combination of early MS stress and methamphetamine later in life resulted in significant elevation of NGF levels in the ventral hippocampus. We propose that the ventral hippocampus is specifically vulnerable to the effects of early life stress and that increased neurotrophin protein levels in this brain region, might reflect a compensatory mechanism.

Keywords: Methamphetamine; Neurotrophins; Dorsal Hippocampus; Ventral Hippocampus; Maternal Separation

Introduction

Stress has been shown to lead to increased susceptibility towards abuse of drugs and contributes to relapse in addicted individuals (Sinha, 2001; Gordon, 2002). This has been evidenced for instance by association studies linking psychosocial stressors with an increased risk for developing depression, anxiety or substance abuse in adult life (Kendler et al., 2000). This phenomenon has been clearly demonstrated in animal studies where stressors early in life such as maternal separation (MS) stress have been shown to lead to an altered behavioural response to drugs of abuse. Adult animals subjected to MS early in life exhibited enhanced cocaine self-administration and psychostimulant induced locomotor activity (Brake et al., 2004; Matthews et al., 2003; Meaney et al., 2002; Kikusui et al., 2005).

The rewarding effects of methamphetamine are mainly mediated by its effects on the mesolimbic dopaminergic system (Kalivas and Stewart, 1991). It has been reported that high doses of methamphetamine leads to the excessive release of dopamine, with the eventual depletion of synaptic dopamine levels (Zhang et al., 2001; Broom and Yamamoto, 2005; Xi et al., 2009). Neurotoxic doses of methamphetamine leads to large release of dopamine and the breakdown of the excess synaptic dopamine gives rise to the formation of free radicals and this is thought to partially underlie methamphetamine-induced neurotoxicity and neurodegeneration observed in various regions of the brain (Cubells et al., 1994; Cadet and Brannock, 1998; Fumagalli et al., 1999). Some studies have shown neurotrophic factors to protect against the dopamine depleting effects of neurotoxic doses of methamphetamine. For instance, brain-derived neurotrophic factor (BDNF) prevented death of primary rat cortical neurons subjected to methamphetamine (Matsuzaki et al., 2004), while methamphetamine-induced neuronal death of cerebellum neural cells are prevented by nerve growth factor (NGF) (Zhou et al., 2004). Changes in neurotrophin concentrations may therefore play a part in the underlying molecular mechanisms responsible for drug- or stress-induced behaviours. Clinical studies have demonstrated a link between methamphetamine users and abnormal plasma BDNF concentrations (Kim et al., 2005). In animal studies, methamphetamine administration leads to increased BDNF levels in the hippocampus and neostriatum (Grace et al., 2008). In addition, glial cell line-derived neurotrophic factor (GDNF) mutant mice display enhanced vulnerability towards methamphetamine-seeking behaviour (Yan et al., 2007). Contrastingly, an inducer for GDNF protects against methamphetamine-induced reward and

sensitization (Niwa et al., 2007). These studies therefore suggest that the exact role of neurotrophins in methamphetamine-related effects, remain controversial.

Neurotrophins which play a fundamental role in synaptic plasticity and neuroprotection are also affected by stress (Smith et al., 1995; Thoenen, 1995; Duman et al., 1997). Maternal separation has been associated with long-term alterations in neurotrophic levels in various brain areas such as the hippocampus (Cirulli et al., 1998; Cirulli, 2001; Roceri et al., 2004; Sale et al., 2004; Branchi et al., 2006; Faure et al., 2006). Exposure to brief as well as longer periods of separation stress has been shown to increase nerve growth factor (NGF) levels in the hippocampus, cerebral cortex and hypothalamus (Cirulli et al., 1998; 2000). Similarly, rats subjected to repeated maternal separation during the first two weeks of postnatal life resulted in increased BDNF mRNA levels in the hippocampus and frontal cortex (Roceri et al., 2004). These findings of elevated neurotrophin levels possibly represent an adaptive response to maternal separation and may therefore form part of the overall compensatory strategy of the brain to combat the onslaughts of stress.

Previously, we too have reported increased neurotrophin levels in the hippocampus of rats that were subjected to maternal separation and additional bouts of swim stress (Faure et al., 2006). However, early life stress in combination with exposure to repeated subsequent stress appear to affect the hippocampus differentially with increases in the dorsal hippocampus and decreases in the ventral hippocampus in neurotrophin levels being reported (Marais et al., 2008). Therefore, in view of both maternal separation and methamphetamine causing changes in hippocampal neurotrophin levels, the present study determined what the effect of a combination of the two interventions would be on BDNF and NGF levels in the dorsal and ventral hippocampus.

Materials and Methods

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 where stress procedures, methamphetamine treatment or where dissections of brain tissue 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 x 25 x 20 cm Plexiglas cages with corncobs as bedding. Temperature was kept constant at 22ºC, humidity at 55% and food and water was available *ad libitum* for the duration of the experiment.

Drugs

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

Experimental design

All rat pups were weaned at the age of postnatal day (PND) 21 according to gender. Male rats were subjected to a total of 8 methamphetamine administrations on PND 33 -36 and PND 39 - 42. The animals were divided into 4 groups:

1) C Sal group: animals not subjected to maternal separation (MS) and receiving saline treatment (n = 9).
2) C Meth group: animals not subjected to MS and receiving methamphetamine treatment (n = 7).
3) MS Sal group: animals subjected to MS and subjected to saline treatment (n = 12).
4) MS Meth group: animals subjected to MS and subjected to methamphetamine treatment (n = 12).

The rational for subjecting rats to methamphetamine during their adolescent period in life (PND 33 – 36 and PND 39 - 42), is that in humans, adolescents typically have an increased sensation seeking drive for drugs of abuse and hence presents with an important risk to susceptibility for taking drugs (Laviola et al., 1999; Spear, 2000). All animals were decapitated on PND 52 for brain tissue collection.

Maternal separation

Male and female rats were paired and their offspring used for experimental purposes. The day of birth was designated as PND 0, where after maternal separation commenced two days later

on PND 2 until PND 14 for a 3 hour daily period. The separation always occurred between 09h00 and 13h00. This protocol is in accordance with the deprivation procedures as employed by Ladd et al. (2000). During the separation period, the pups were removed from the mother and kept in a separate cage and the mother kept in the home cage. The cage containing the pups were then moved to a dedicated room where the pups were kept warm under infrared lights (30-33ºC) in order to control for the development of hypothermic conditions. Control litters were reared normally without separations. After maternal separation was completed, animals were left undisturbed under normal housing conditions.

Measurement of neurotrophic factors (BDNF and NGF) in the hippocampus

On PND 52, animals were decapitated and the brains were rapidly removed and both the left and right hippocampi were dissected into dorsal and ventral parts on an ice cold glass plate. The tissue was rapidly frozen and stored in liquid nitrogen for measurement of BDNF and NGF concentrations at a later stage.

Dorsal and ventral hippocampus brain tissue samples were weighed and suspended in 300 µl lysis buffer (137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 % Nonidet P-40 (NP 40), 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin and 0.5 mM sodium vanadate). The samples were then sonicated for 40 s in the lysis buffer, vortexed and then centrifuged at 14 000 rpm at 4 ºC for 20 min and the supernatants were aliquoted into test tubes. BDNF and NGF concentrations were determined using the Emax ImmunoAssay system (Promega, Madison, USA). ELISA kits were completed following the manufacturer's instructions. Determinations were made in duplicate. Results were expressed in pg/mg wet weight (ww).

Statistical analysis

BDNF and NGF results were analyzed using the Kruskal-Wallis test. Statistical significance of the H-statistic was followed by the Mann-Whitney U post hoc test. Statistical tests were all done with the aid of SPSS (version 17.0). The level of significance was accepted as $p<0.05$. Data is reported as mean ± SEM.

Results

Brain-derived neurotrophic factor concentrations in the hippocampus

There were significant differences in the BDNF levels in the dorsal hippocampus of the four experimental groups (H = 15.164, 3df, P = 0.002). The BDNF concentration in animals subjected to methamphetamine was significantly less than animals treated with saline (p = 0.001). Animals previously subjected to maternal separation also showed significantly reduced BDNF levels compared to non-separated controls (p = 0.006). Separated rats treated with methamphetamine had significantly decreased BDNF levels (p = 0.002), but this reduction was similar to that observed in animals subjected to the respective treatments alone. The combination of maternal separation and methamphetamine exposure did not result in a further decrease in BDNF levels.

Similar to the dorsal hippocampus, analysis of the BDNF levels in the ventral hippocampus also revealed significant differences between the four groups (H = 13.564, 3df, P = 0.004). Methamphetamine treatment alone (p = 0.034), maternal separation (p = 0.001) and maternal separation plus methamphetamine (p = 0.004) all caused a significant reduction in BDNF levels in the ventral hippocampus when compared to normally reared saline-treated controls.

Nerve growth factor concentrations in the hippocampus

There were no significant differences in the NGF levels in the dorsal hippocampus of the various experimental groups (H = 4.538, 3df, P = 0.209). However, NGF levels in the ventral hippocampus were affected by the treatment procedures (H = 8.726, 3df, P = 0.033). While methamphetamine treatment per se did not result in significant changes, maternal separation alone caused a significant reduction in NGF levels (p = 0.026). Interestingly, maternally separated rats subjected to methamphetamine treatment had significantly higher NGF levels in the ventral hippocampus when compared to the separation group (p = 0.005).

Discussion

Several studies have implicated a role for neurotrophins in both maternal separation and methamphetamine treatment. Here we investigated the effects of a combination of these modalities on hippocampal neurotrophin levels. The main findings were; (1) methamphetamine administration and maternal separation independently resulted in decreased BDNF levels in both the dorsal and ventral hippocampus; (2) early separation together with

later methamphetamine treatment yielded similar decreases in BDNF concentrations in the dorsal and ventral hippocampus; (3) neither maternal separation nor methamphetamine altered NGF levels in the dorsal hippocampus; (4) separation per se reduced NGF levels in the ventral hippocampus; (5) and finally the combination of early separation stress and methamphetamine later in life resulted in NGF levels in the ventral hippocampus comparable to controls.

The effect of maternal separation on neurotrophins concentrations in the brain remains unclear with both increased and decreased levels being reported. One of the major reasons for this inconsistency stems from discrepancies in methodology. For instance a brief 45 min session of maternal separation on postnatal day (PND) 2 resulted in increased NGF expression levels in the dentate gyrus and hilus of the hippocampus (Cirulli et al., 1998). Similarly, a longer separation period of 1 hour or 3 hours respectively also resulted in elevated NGF expression levels even when measured at different ages (PND 9 and PND 16). Interestingly, this study demonstrated a dose-dependent like effect with longer periods of maternal separation generating greater increases in NGF (Cirulli et al., 2000). Not only has elevated NGF levels been documented. A daily interval of 180 min of maternal separation between PNDs 2 – 14 resulted in increased BDNF levels on PND 17. However, this effect was not long lasting as the elevated BDNF levels returned to normal at the end of puberty (Roceri et al., 2004). In general, elevations in neurotrophic factor concentrations in the brain have been proposed to be part of the compensatory mechanisms in the brain that are triggered to protect this vital organ against the negative effects of stress and glucocorticoid exposure during the developmental period (Faure et al., 2006; 2007; Marais et al., 2008).

In contrast, a number of studies have demonstrated reductions in neurotrophin levels following paradigms of maternal separation. In the present study, we found a decrease in NGF levels in the ventral hippocampus, a finding that was in accordance with a reduction in BDNF concentration in the hippocampus as reported by Lippmann et al. (2007) and Jaworska et al. (2008), but opposite to Cirulli et al. (2000) that found increased NGF levels in the hippocampus after maternal separation. An explanation for this discrepancy in the findings may be ascribed to the fact that neurotrophin concentrations were measured in young rats in the Cirulli study, while our measurements were performed on tissue collected from adult rats. Other studies employing separation paradigms supported a decrease in neurotrophic levels in the hippocampus. A previous study conducted in our lab, found reduced NGF and

neurotropin-3 (NT-3) levels in the ventral hippocampus of adult rats after being subjected to 180 min of separation between PND 2-14 (Marais et al., 2008). Using a comparable separation paradigm, Lippmann et al. (2007), found decreased BDNF levels in both the hippocampus and striatum. Similarly, an acute 24 hour separation period on PND 9 produced decreased BDNF expression in adulthood (Roceri et al., 2002). Experiments using different animal species and models reported analogous data with maternally separated male gerbils (PNDs 4-20 for 4 hours daily) reflecting decreased BDNF levels (Jaworska et al., 2008), while individually isolated pups (1 hour per day from PND 2-9) yielded decreased expression of NGF mRNA in both juvenile (PND 28) and adults (PND 90). The juvenile animals also had decreased GDNF mRNA levels in their hippocampi (Kawano et al., 2008).

These studies suggest that the maldevelopment of the brain imposed by maternal separation causes the brain to enter a state of prolonged stress. Initially, the brain responds by increasing the release of neurotrophins in order to ensure that processes such as neurogenesis, synaptogenesis and network development are not significantly compromised. However this continuous demand may eventually lead to exhaustion and hence the hyposecretion of neurotrophins at a later stage in life. In conjunction with the above-mentioned process is the contribution of the hypothalamic-pituitary-adrenal axis to the neuropathology associated with maternal separation. Rats subjected to maternal separation during their first two weeks of life display increased plasma ACTH and corticosterone responses to stress at adulthood (Plotsky and Meaney, 1993). Elevated corticosterone concentrations are toxic to neurons (McEwen, 2007), and therefore studies showing dysregulated HPA axis activity, further reflect the stressful nature under which the brain may have to function. However, the animals subjected to maternal separation, resulted in decreased ACTH and corticosterone concentrations. This result might be explained by an exhaustive HPA axis system and subsequent reduced stress hormone levels as consequence of repeated stress exposures and hence chronic stimulation. Alternatively, the HPA axis might be under an enhanced negative feedback inhibition.

In the current study, methamphetamine administration, like maternal separation, resulted in changes in neurotrophin levels in the brain. BDNF protein levels were markedly decreased in both the dorsal and ventral hippocampus; however methamphetamine treatment did not alter NGF levels in either the dorsal and ventral hippocampus substantially. Our BDNF data is in agreement with the findings of Angelucci et al. (2007) who observed decreased BDNF levels in the occipital cortex and hypothalamus of adult rats chronically treated with

methamphetamine. However in their study they also documented reduced NGF levels in these brain areas, as well as the hippocampus. A possible reason for the difference in the NGF finding may be due to their measurement of neurotrophin concentrations the day after the last methamphetamine administration, while our data reflect growth factor levels after a period of withdrawal from chronic drug treatment. Our finding of reduced BDNF levels is in line with suggestions that amphetamine-induced neurotoxicity leads to a decrease in neurotrophin synthesis, thereby compromising the protection of the brain afforded by neurotrophins against the damaging effects of amphetamine (Matsuzaki et al., 2004; Angelucci et al., 2007). Interestingly these observations of decreased neurotrophin levels after withdrawal is in contrast with the results of a clinical study that reported increased plasma BDNF levels after more than 30 months amphetamine use followed by a month of abstinence. This increased BDNF levels was suggested to compensate for amphetamine-induced neurotoxicity (Kim et al., 2005). The discrepancy in findings may possibly be explained by the fact that plasma neurotrophin concentrations is considered a global reflection of whole brain extracellular neurotrophin concentrations and cannot be compared to specific regional brain tissue neurotrophin levels as measured in the hippocampus. In addition, Filip et al. (2006) found increased BDNF expression levels in the hippocampus of adult rats after a withdrawal period. The increased neurotrophin expression levels (Meredith et al., 2002; Filip et al., 2006) has been proposed to be a compensatory mechanism for decreased protein neurotrophin concentrations (Angelucci et al., 2007) as was found in the present study.

Interestingly, the present study demonstrated that maternal separation resulted in increased NGF levels in the ventral hippocampus of methamphetamine exposed rats. Dorsal and ventral regions of the hippocampus have been shown to play different roles in information processing and emotional processes. The dorsal hippocampus plays a preferential role in learning and memory related processes, while the ventral hippocampus plays a preferential role in anxiety-related responses (Bannerman et al., 2004). It would seem that maternal separation has a definite effect on NGF levels specifically in the ventral hippocampus, which emphasises the preferential role of the ventral hippocampus in stress-related processes. Similar findings in our laboratory in which repetitive stressors that consisted of a combination of maternal separation and two forced swim re-stress session later in life, also resulted in increased neurotrophin concentrations in the hippocampus (Faure et al., 2006). Increased neurotrophin levels in response to both early life stress and methamphetamine treatment would seem to

reflect the brain's protective response against ventral hippocampal neuronal stress induced by both stress and drug exposure.

Conclusion

Maternal separation and methamphetamine treatment independently resulted in decreased neurotrophin concentrations in the hippocampus. However, combining maternal separation and methamphetamine treatment resulted in increased NGF levels in the ventral hippocampus. These observations suggest that neurotrophins play an important role in protecting neuron structure and function against the detrimental effects of repetitive exposure to stressful events. However the nature of this protective role remains elusive and more investigations are required to unravel molecular mechanisms and consequences following alterations in neurotrophin expression.

Acknowledgements

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Figures

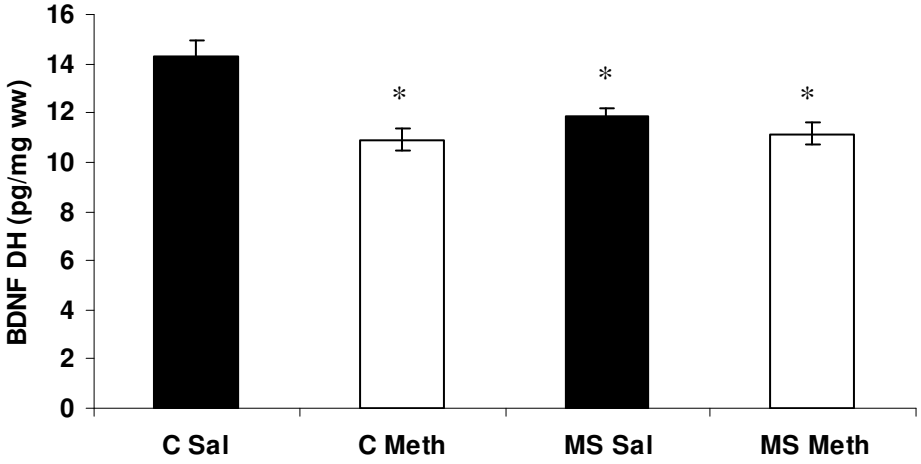

Figure 1: Brain-derived neurotrophic factor (BDNF) concentration (pg/mg ww) in the dorsal hippocampus of groups subjected to maternal separation and control conditions. Values given as mean ± SEM. * significantly different from C Sal group ($p<0.05$).

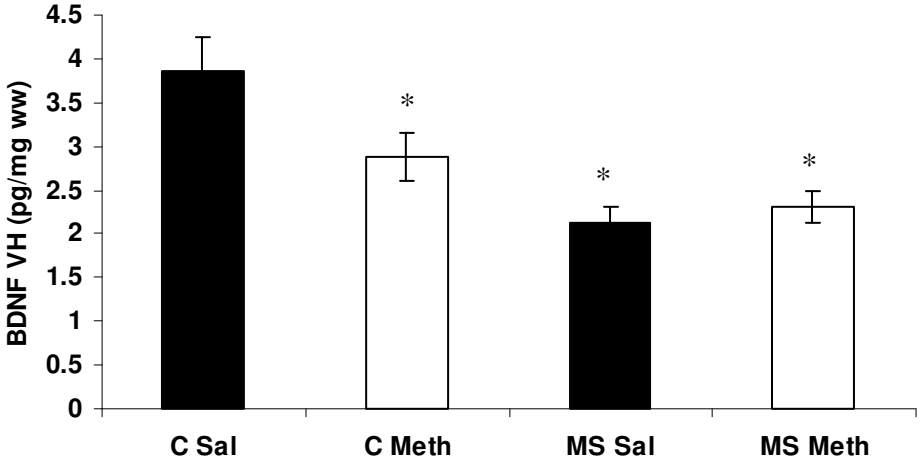

Figure 2: Brain-derived neurotrophic factor (BDNF) concentration (pg/mg ww) in the ventral hippocampus of groups subjected to maternal separation and control conditions. Values given as mean ± SEM. * significantly different from C Sal group ($p<0.05$).

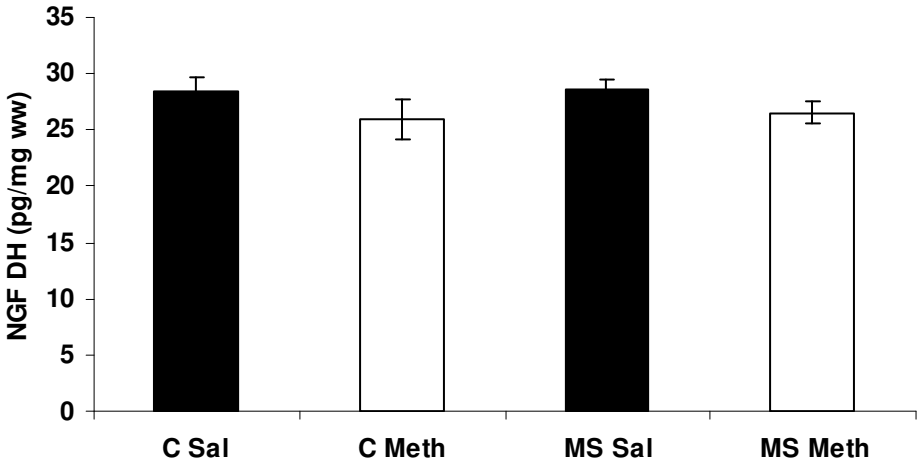

Figure 3: Nerve growth factor (NGF) concentration (pg/mg ww) in the dorsal hippocampus of groups subjected to maternal separation and control conditions. Values given as mean ± SEM.

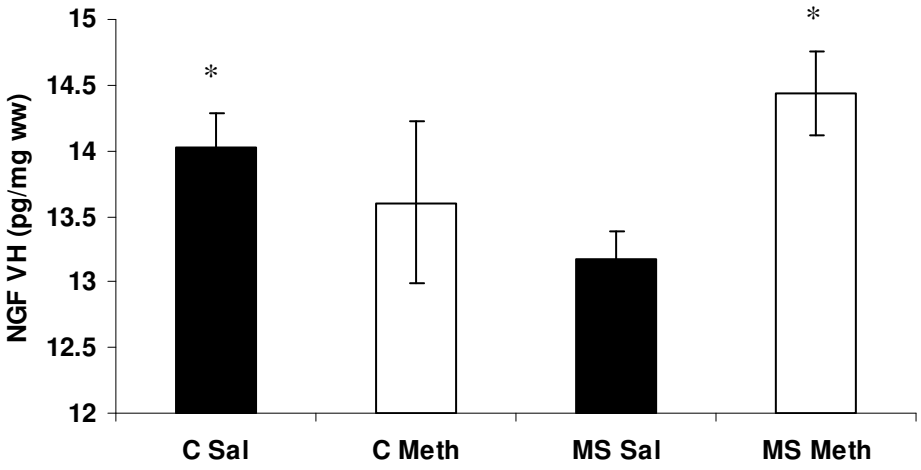

Figure 4: Nerve growth factor (NGF) concentration (pg/mg ww) in the ventral hippocampus of groups subjected to maternal separation and control conditions. Values given as mean ± SEM. * significantly different from MS Sal group ($p<0.05$).

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CHAPTER 5

The effects of lobeline and naltrexone on methamphetamine-induced place preference behaviour and striatal dopamine and serotonin levels of non-stressed and maternally separated rats

The effects of lobeline and naltrexone on methamphetamine-induced place preference behaviour and striatal dopamine and serotonin levels of non-stressed and maternally separated rats

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Abstract

Exposure to early stress may increase an individual's vulnerability to MA dependence. There are relatively few data on the optimal treatment of such dependence, but the opioid and VMAT2 may be target systems insofar as they play pivotal roles in addictive neurochemistry. Here we investigated the effects of naltrexone (opioid antagonist) and lobeline (VMAT2 inhibitor) on MA-induced place preference behaviour in rodents subjected to early stress (maternal separation) and controls, including the effects on dopaminergic and serotonergic levels in the striatum. We found; (1) maternal separation attenuated methamphetamine-induced place preference; (2) two weeks treatment with either lobeline or naltrexone did not alter methamphetamine-induced place preference behaviour; (3) lobeline and naltrexone treatment had differential effects on serotonin and dopamine concentrations in the striatum. Early life stress attenuates MA-induced behavioural effects, however influences neurochemical pathways which may underlie a predisposition towards drug dependence.

Keywords

Maternal separation; Striatum; Methamphetamine; Lobeline; Naltrexone; Conditioned Place Preference; Dopamine; Serotonin

Introduction

Stress has been demonstrated to make an individual more vulnerable to drug abuse (Gordon, 2002). For example, adults with a history of child adversity have an increased risk for substance abuse (Dinwiddie et al., 1992; Gutierres et al., 1994). Stressful life events, like MS stress, have been shown to increase the probability of abusing drugs at a later stage in life (Sinha, 2001). Early MS led to increased use of cocaine in adult rodents (Matthews and Robbins, 2003), as well as to alterations in locomotor response to psychostimulants (Meaney et al., 2002). Various animal studies have also shown that the opioid system is affected by early life stress, whereby morphine decreases separation-induced distress vocalizations, whilst opioid antagonists potentiate separation distress (Herman and Panksepp, 1978; Panksepp et al., 1978; Kehoe and Blass, 1986). These findings suggest that MS alters neurochemical role players (Meaney et al., 2002) that are critical in the development of addictive processes.

Methamphetamine (MA) is a potent psychostimulant and the abuse of this illicit substance is escalating worldwide (United Nations Office on Drug and Crime, 2003). Few research outputs exist on the optimal pharmacological treatment of MA abuse and dependence (Vocci and Ling, 2005; Vocci et al., 2005). After the differential mechanism of action of both cocaine and MA was established, more specific pharmacological clinical trails began for the treatment of MA abuse. Currently, only a few treatments have shown some promise in successfully treating certain aspects of MA addiction symptomology.

The rewarding effects of MA are mediated by its effects on the mesolimbic dopaminergic system (Kalivas and Stewart, 1991). MA's primary mechanism of action is to increase the release of dopamine into the synapse via its action on vesicular monoamine transporters (VMAT2) (Sulzer and Rayport, 1990; Pifl et al., 1995; Wilhelm et al., 2004; Sulzer et al., 2005). Other neurotransmitter systems may also modulate dopaminergic activity in the brain by interacting with its circuitry. For instance, serotonin (5-HT) modulates dopaminergic neurotransmission by inhibiting dopamine activity in the VTA (Brodie and Bunney, 1996). The binding of 5-HT to serotonin type 2C receptors (5-HT2C) (Di Matteo et al., 2001) results in decreased firing of these neurons and consequently a reduction in dopaminergic neurotransmission.

The endogenous opioid system was also found to mediate the neurochemical and behavioural effects of amphetamines. The administration of MA has been demonstrated to lead to increased endogenous opioid concentrations (Olive et al., 2001) and the combined administration of morphine (a μ-opioid receptor agonist) and amphetamine resulted in additive rewarding effects (Hubner et al., 1987). In addition, mesolimbic dopamine activity has been shown to be indirectly regulated by μ-opioid receptors in the ventral tegmental area (VTA) (Johnson and North, 1992; Bergevin et al., 2002) via a mechanism that included GABAergic inhibition (Spanagel and Weiss, 1999).

Lobeline, an alkaloidal constituent of Indian tobacco (*Lobelia inflata*), acts both as an agonist and antagonist at nicotinic receptors (Teng et al., 1997; Stolerman et al., 1995; Lecca et al., 2000; Terry et al., 1998; Miller et al., 2000). However, lobeline can also alter presynaptic dopamine storage and release by binding to the dihydrotetrabenazine sites on VMAT2, thereby inhibiting dopamine storage (Scherman et al., 1986; Kilbourn et al., 1995; Liu et al., 1996). Furthermore, lobeline does not reverse dopamine transporter (DAT) function and has no inhibitory action on monoamine oxidase (MAO) activity. These effects of lobeline may therefore reverse the pharmacological effects of MA (Dwoskin and Crooks, 2002). Taken together, lobeline reduces the available vesicular dopaminergic pool and so increases the cytosolic pool. Lobeline pretreatment has been shown to inhibit amphetamine-induced enhanced locomotor activity and discriminative stimulus properties (Miller et al., 2001) and reduces MA self-administration in rats (Harrod et al., 2001).

Naltrexone, a nonselective opioid antagonist, is a promising pharmacotherapy for MA addiction, since endogenous opioids have been implicated in the rewarding effects of psychostimulants and are disrupted by early adversity. Blockade of opioid receptors have been shown to cause a reduction in amphetamine-induced extracellular dopamine levels in the nucleus accumbens and striatum (Hooks et al., 1992; Spanagel et al., 1992; Schad et al., 1995; Schad et al., 1996) via the increase in GABAergic activity in the VTA (Johnson and North, 1992; Bergevin et al., 2002). Naltrexone also attenuates the induction and expression of MA-induced behavioural sensitization (Chiu et al., 2005) and reduces the locomotor activity induced by amphetamines (Winslow and Miczek, 1988; Balcells-Olivero and Vezina, 1997). In addition, naltrexone diminishes amphetamine- and cocaine-induced reinstatement of drug seeking behaviour in rats (Brebner et al., 2005).

Although it is clear that early adversity is a vulnerability factor for MA, perhaps via disruptions in opioid, nicotinic, DA, and serotonin systems, little work has examined the ability of agents acting on these systems (e.g. opioid, nicotoninic) to reverse behavioural and neurotransmitter effects of MA (e.g. DA, 5-HT). The purpose of the present study was to investigate the effect of both naltrexone (opioid) and lobeline (nicotinic) in maternally separated and control rats. We were interested in examining both behavioural effects (i.e. effects on MA-induced place preference) and neurotransmitter effects (i.e. effects on dopaminergic and serotonergic levels in the striatum). Unlike, various earlier studies that administer treatment prior to MA exposure, the present study employed a post-MA treatment regime. The rational behind this treatment strategy was that the independent effects of the treatment on drug abuse are more evident and that in real life treatment almost always follows the abuse of drugs and hence makes the post-abuse treatment approach more relevant.

Materials and Methods

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 where stress procedures, methamphetamine and treatment exposures or where 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 x 25 x 20 cm Plexiglas cages with corncobs as bedding. Temperature was kept constant at 22ºC, humidity at 55% and food and water was available *ad libitum* for the duration of the experiment.

Drugs

Methamphetamine hydrochloride was obtained from US Pharmacopeia Convention Inc. (Rockville, USA). α-Lobeline and naltrexone hydrochloride was procured from Sigma-Aldrich Chemicals (St. Louis, MO). Methamphetamine was dissolved in 0.9% saline and administered at a dose of 1mg/kg. Lobeline was dissolved in distilled water and naltrexone dissolved in 0.9 % saline and both administered at a dose of 10mg/kg.

Maternal separation paradigm

Male and female rats were paired and their offspring used for experimental purposes. The day of birth was designated as PND 0, where after maternal separation commenced two days later on PND 2 until PND 14 for a 3 hour daily period between 09h00 and 13h00. This protocol is in accordance with the deprivation procedures as employed by Ladd et al. (2000). During the separation period, the pups were removed from the mother and kept in a separate cage and the mother kept in the home cage. The cage containing the pups were then moved to an isolated dedicated room where the pups were kept warm under infrared lights (30-33ºC) in order to control for exposure to hypothermic conditions. Control litters were reared normally without separations. After maternal deprivation was completed, animals were subjected to normal housing conditions.

Experimental design

All rat pups were weaned at the age of postnatal day (PND) 21 according to gender as only male rats were used for the experiments. Rats were regularly handled (PND 25 -30) to minimize the effects of handling stress during the behavioural assessments and treatment procedures. Two weeks of treatment commenced from PND 37 until PND 50. The rats in the lobeline treatment groups were divided into 4 groups. These were:

1) Cont Sal L group: animals not subjected to MS and receiving saline injections during CPP and subjected to 2 weeks of lobeline treatment (n = 10).
2) MS Sal L group: animals subjected to MS and subjected to saline injections during CPP and receiving 2 weeks of lobeline treatment (n = 9).
3) Cont MA L group: animals not subjected to MS and receiving methamphetamine-induced CPP and subjected to 2 weeks of lobeline treatment (n = 9).
4) MS MA L group: animals subjected to MS and subjected to methamphetamine-induced CPP and 2 weeks of lobeline treament (n = 10).

Male rats in the naltrexone treatment groups were divided into 4 similar groups. The groups were:

1) Cont Sal N group: animals not subjected to MS and receiving saline injections during CPP and subjected to 2 weeks of naltrexone treatment (n = 13).

2) MS Sal N group: animals subjected to MS and subjected to saline injections during CPP and receiving 2 weeks of naltrexone treatment (n = 14).
3) Cont MA N group: animals not subjected to MS and receiving methamphetamine-induced CPP and subjected to 2 weeks of naltrexone treatment (n = 10).
4) MS MA N group: animals subjected to MS and subjected to methamphetamine-induced CPP and 2 weeks of naltrexone treatment (n = 14).

Male rats in the saline and no treatment groups were divided into 3 groups. Here only rats exposed to methamphetamine CPP were subjected to saline 2 weeks treatment to assess if the treatment has any effects on methamphetamine exposure. The groups were:

1) Cont MA group: animals not subjected to MS and receiving methamphetamine-induced CPP and subjected to no treatment (n = 8).
2) Cont MA S group: animals not subjected to MS and subjected to methamphetamine-induced CPP and 2 weeks of saline treatment (n = 9).
3) MS MA S group: animals subjected to MS and receiving methamphetamine-induced CPP and subjected to 2 weeks of saline treatment (n = 8).

Each of the above groups was tested at 24hrs (CPP1) on PND 37 and 2 weeks after treatment or no treatment (CPP2) on PND 51 after first 4 methamphetamine conditioning sessions (PND 33 - 36) to determine if the treatment had any effect on place preference behaviour. The rational for subjecting rats to methamphetamine during their adolescent period in life (PND 33 – 36), is that in humans, adolescents typically have an increased sensation seeking drive for drugs of abuse and hence presents with an important risk to susceptibility for taking drugs at this phase (Laviola et al., 1999; Spear, 2000). Animals were all sacrificed by means of decapitation the day after the 2 week CPP test (CPP2) on PND 52.

Conditioned Place Preference (CPP) paradigm

The CPP test is a Pavlovian classical conditioning test, whereby the drug state is paired with a neutral environment (the CPP two compartment box). The amount of time spent in the drug-paired environment is considered as a measure of the positive reinforcing or rewarding effects of the drug. This phenomenon has been confirmed in human cocaine addicts, whereby drug paraphernalia that is considered as a neutral environmental stimuli, become associated with the effects experienced by cocaine use (Gawin, 1991). The two compartment box used had

differential cues unique to each compartment in order for the rats to associate the cues in the environment with the rewarding effects of the drug. The cues differed in term of wall colour and floor texture. The one compartment had black walls and a smooth floor, whilst the adjacent compartment had white and black stripped walls and a grid-textured floor.

Adolescent male rats were habituated to the test cage the day before the testing (PND 31). On PND 32 rats in all groups were subjected to the CCP test. The CPP test consists of 3 stages: pre-conditioning stage, the conditioning stages and the post-conditioning stage.

Pre-conditioning

During the pre-conditioning stage on PND 32, animals were given free access to both compartments for a period of 15 min of the CPP test box and the amount of time spent in each compartment was recorded. The pre-test phase allows for the determination of the natural preference of the animal for either one of the two compartments of the CPP box.

Conditioning

The conditioning stage involves a pavlovian associative learning method, whereby a neutral stimulus (drug-paired compartment) was paired with the drug state induced by methamphetamine. On PND 33 - 36, the animals designated to the methamphetamine groups were intra-peritoneally injected with methamphetamine (1mg/kg, i.p.) 20 min before being confined for a further 30 min period to the compartment of the test box opposite to the natural preference of the rat. The 50 min period in total was allowed for the drug to take affect and to ensure the formation of associations in the CPP box. Control rats received equivalent saline injections and were also placed in opposing compartments of the CPP box.

Post-conditioning

On PND 37, the first post-conditioning test was done to confirm the presence of place preference. On PND 51 a second CPP test was done to determine the effect of the different drug treatments on methamphetamine-induced place preference. During the post-conditioning stage animals were once again given free access to both compartments, and the amount of time spent in each compartment was recorded for analyses at a later stage. The CPP score was calculated as the difference between the amount of time spent in the drug-paired compartment before and after the conditioning stages.

Behavioural analysis of the CPP test

On all days of behavioural testing during the pre-test (PND 32) and post-tests (PND 37 and 51); behaviour was recorded on video tape. Video recordings were analyzed using Noldus Ethovision software (Wageningen, The Netherlands).

Determination of dopamine and serotonin tissue concentrations

On PND 52, animals were decapitated and the brains were rapidly removed and both the left and right striata were dissected on an ice cold glass plate and rapidly frozen and stored in liquid nitrogen for measurement of dopamine and serotonin striatal concentrations at a later stage. The striatal tissues were first weighed and placed in ice cooled solution containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA and 1 mM EGTA (1 ml/50mg wet weight brain tissue). Samples were homogenized and vortexed for 30 seconds followed by centrifugation (15, 000 g for 15 min at 4 °C) and the supernatant divided into aliquots and stored at -80 °C until assayed. Striatal aliquots were again centrifuged at 12, 000 g for 10min at 4 °C before commencing with the dopamine and serotonin assays. Striatal tissue dopamine and serotonin concentrations were determined using commercial available ELISA kits (IBL, Hamburg, Germany). ELISA kits were completed following the manufacturer's instructions. Determinations were made in duplicate. Results were expressed in ng/ml.

Statistical analysis

CPP behavioural parameters, dopamine and serotonin results were analyzed using non-parametric ANOVA, the Kruskal-Wallis test. Statistical significance of the H-statistic was followed by the Mann-Whitney U post hoc test. Statistical tests were all done with the aid of SPSS (version 17.0). The level of significance was accepted as $p<0.05$. Data is reported as mean ± SEM.

Results

Effect of lobeline treatment, MA and MS on CPP behaviour

Two weeks lobeline treatment between first (CPP1) and second (CPP2) post conditioning tests did not result in decreased place preference in any of the experimental groups. Animals subjected to methamphetamine demonstrated place preference for the drug paired compartment of the CPP box ($p = 0.004$) ($H = 13.764$, 6df, $P = 0.032$). As expected, rats

exposed to saline conditioning did not result in place preference behaviour. Surprisingly, animals exposed to both maternal separation and methamphetamine resulted in attenuated CPP behaviour ($p = 0.021$) (Figure 1).

Effect of lobeline treatment on striatal serotonin and dopamine levels

Experimental groups exposed to lobeline treatment demonstrated an increased serotonin levels when compared to the groups receiving saline for two weeks respectively ($p < 0.05$) ($H = 36.583$, 6df, $P = 0.001$) (Figure 2). Similarly those exposed to lobeline exposed groups had decreased dopaminergic levels in the striatum when compared to the control methamphetamine CPP (Cont MA) group ($p < 0.05$) ($H = 18.634$, 6df, $P = 0.005$) (Figure 3).

Effect of naltrexone treatment, MA and MS on CPP behaviour

Two weeks naltrexone treatment did not attenuate place preference behaviour between the first (CPP1) and second (CPP2) post conditioning tests. Methamphetamine exposed rats displayed place preference behaviour when compared to groups subjected to saline conditioning ($p < 0.05$), except the group exposed to both maternal separation and methamphetamine conditioning and two weeks saline treatment (MS MA S) ($H = 26.843$, 6df, $P = 0.001$) (Figure 4).

Effect of naltrexone treatment on striatal serotonin and dopamine levels

Naltrexone treatment resulted in decreased striatal serotonin levels, although MS rats resulted in increased serotonin levels in both saline ($p = 0.006$) and MA CPP ($p = 0.051$) ($H = 37.034$, 6df, $P = 0.001$). Saline treatment and no treatment groups also resulted in increased striatal serotonin concentrations when compared to the group subjected to maternal separation, saline CPP and two weeks of naltrexone treatment and the group subjected to non-stressed conditions, methamphetamine CPP and two weeks of naltrexone treatment (MS Sal N and Cont MA N) ($p < 0.05$) (Figure 5).

Dopamine concentrations in the striatum were increased in the groups exposed to naltrexone treatment as well as in the saline and no treatment groups when compared to the group exposed to maternal separation, methamphetamine CPP and naltrexone treatment (MS MA N) ($p < 0.05$) ($H = 35.468$, 6df, $P = 0.001$) (Figure 6).

Discussion

The main findings of the present study are as follows; (1) maternal separation attenuated methamphetamine-induced place preference behaviour; (2) two weeks treatment with either lobeline or naltrexone did not reduce methamphetamine-induced place preference behaviour; (3) lobeline and naltrexone treatment had differential effects on serotonin and dopamine concentrations in the striatum.

The CPP paradigm is a widely accepted method of investigating the rewarding or reinforcing effects of psychostimulants. A study investigating the effects of methamphetamine CPP in both a single- and dual-cue CPP box, found that methamphetamine resulted in CPP behaviour and this effect was more pronounced in the dual-cue CPP box (Shimosato and Ohkuma, 2000). These findings are similar to our data, where the same dose of methamphetamine was used (1 mg/kg) and a dual-cue CPP box which also resulted in increased CPP scores. However, our study differed in the method of drug administration whereby Shimosato and Ohkuma (2000) made use of alternate pairing of methamphetamine and saline during the conditioning phase of the CPP test. Our results are also in agreement with the findings of Kim and Jang (1997) who also found methamphetamine induced place preference, although in mice. Nevertheless, the effects of MA on place preference may differ over development; Adriana and Laviola (2002) demonstrated that adolescents display decreased CPP behaviour when compared to adults. One of the differences between the present study and that of Adriana and Laviola (2002) was that we did not administer MA again in adulthood to assess CPP behaviour afterwards, rather assessed the duration of place preference behaviour after adolescent MA administration. This allowed us to show that CPP lasted for at least two weeks after MA administration in our experimental paradigm.

Environmental stressors may affect behavioural responses to psychostimulants. Socially isolated rats display enhanced locomotor activity when exposed to a novel environment and this response is even greater after amphetamine administration. Animals also exhibit sensitization toward the locomotor effects induced by repeated amphetamine administration (Smith et al., 1997). Moreover, socially reared rats have been shown to be more sensitive to CPP (Schenk et al., 1986), while isolated rats were found to be more sensitive to the locomotor activating effects of cocaine. In addition, this latter group of animals shows increased acquisition and maintenance of cocaine self-administration compared to rats reared

in groups (Schenck et al., 1987; Boyle et al., 1991; Phillips et al., 1994; Zhang et al., 2005; Kosten et al., 2000; 2004).

Similarly, other stressful events such as repeated maternal separation have been shown to affect the response to amphetamine administration (Matthews et al., 1996). MS animals were found less responsive to amphetamine-induced locomotor effects and displayed enhanced acquisition of cocaine self-administration (Matthews et al., 1999). Maternal separation, in the present study, resulted in attenuated methamphetamine-induced place preference behaviour during adolescence and this effect lasted until adulthood. This finding was in contrast to what was expected, i.e. an exaggerated place preference response. Previous studies have shown MS animals to have a greater preference for cocaine self-administration and drug-induced locomotor activity when compared to handled rats (Meaney et al., 2002; Brake et al., 2004; Moffett et al., 2006). Animals subjected to morphine-induced CPP have also shown greater place preference behaviour, however the study employed another MS method and measured CPP only in adulthood (Michaels and Holtzman, 2008). However, MS in the present study could possibly have altered neurochemical pathways involved in addiction in a way that later administration of MA failed to induce definite behavioural effects. In the present study, dopamine concentrations in the striatum of MS rats did not result in pronounced differences between control groups, since the rewarding effects of MA are mediated by increased dopamine neurotransmission (Kalivas and Stewart, 1991; Pifl et al., 1995; Wilhelm et al., 2004; Sulzer et al., 2005) and the finding of decreased MA-induced CPP behaviour could possible be explained by the lack of neurochemical changes in the present study. Addictive behaviour is complex and hence the present result remains difficult to interpret, since contrasting MS findings exist in the current literature.

Early life stress induces long lasting neurochemical changes in the brain. Rats previously exposed to MS have decreased DAT levels in the nucleus accumbens and the caudate putamen (Meaney et al., 2002; Brake et al., 2004). The decreased transporter levels are proposed to render maternally separated rats hypersensitive to the effects of cocaine since a lower dose of cocaine is needed to affect extracellular dopamine concentrations. This is further supported by the finding that adult rats subjected to MS present with an enhanced mesolimbic dopaminergic response to amphetamine (Hall et al., 1999). Also, repeated MS during the first two weeks of life has been found to enhance stress-induced sensitization to amphetamine and alter the resultant nucleus accumbens dopamine and behavioural responses

to stress and cocaine (Brake et al., 2004). Infants and juvenile rats subjected to repeated isolation stress also respond to amphetamine with increased striatal dopamine levels (Kehoe et al., 1996, 1998; Kosten et al., 2003). Cross-sensitization between stress and drugs is suggested to occur with repeated exposures to stress enhancing the behavioural response to drugs of abuse (Robinson and Becker, 1986; Sorg and Kalivas, 1991; Stewart and Badiani, 1993). Furthermore, it is suggested that early life events may alter mesolimbic dopaminergic brain pathways in such a way that the response to stress and psychostimulants later in life might be permanently changed (Antelman et al., 1980; Sorg and Kalivas, 1991; Kalivas and Stewart, 1991; Leyton and Stewart, 1990; Meaney et al., 2002). Similarly, the present study, maternally separated rats subjected to methamphetamine-induced CPP later in life resulted in slightly higher dopamine levels in adulthood when compared to control animals, although the difference did not reach statistical significance. This can possibly be explained by the fact that baseline tissue content levels were measured two weeks after MA exposure and thus not in response to MA. Another possible explanation might be the lower serotonin levels found when compared to dopamine concentrations, which plays a modulatory role in dopamine levels. Serotonin has been shown to increase the inhibitory effect of dopamine on dopaminergic VTA neurons (Brodie and Bunney, 1996), resulting in decreased dopaminergic neurotransmission.

Lobeline has been demonstrated to inhibit the neurochemical and behavioural effects of amphetamines in rodents (Miller et al., 2001; Harrod et al., 2001; Harrod et al., 2003) and attenuates the stimulant and rewarding properties of MA via its non-competitive interaction with VMAT2. Lobeline pre-treatment attenuated MA-induced hyperactivity in mice and also decreased the discriminative stimulus properties of MA in rats (Miller et al., 2001). The attenuation of discriminative stimulus effects in animals is proposed to lower subjective effects of stimulants in humans (Schuster and Johanson, 1988; Holtzman, 1990). Lobeline has also been shown to decrease MA self-administration in rats (Harrod et al., 2001) and to reduce MA-induced stereotypy in adolescent mice (Tatsuta et al., 2006). Conversely, two weeks of lobeline treatment failed to attenuate MA-induced CPP behaviour in the present study. This finding may possibly be explained by the fact that although the CPP paradigm is used to measure the rewarding effects of psychostimulants, the reward is measured based on associative learning between the rewarding effects of the drug and the animals' surrounding environment. It would seem that once learning association has taken place, lobeline was unable to eradicate its effects.

Only one previous study from the literature could be found that employed post-MA lobeline treatment. Lobeline was administered 5 and 7 hours after neurotoxic MA administrations (4 x 10mg/kg injections over 8 hrs) and examined striatal dopamine and serotonin content. Lobeline treatment resulted in attenuating MA neurotoxic effects and led to increased dopamine and serotonin striatal concentrations, 7 days after the last MA administration (Eyerman and Yamamoto, 2005). Similarly, in the present study two weeks of lobeline treatment in a non-neurotoxic MA paradigm (one 1mg/kg administration per day over 4 days) resulted in increased striatal serotonin levels, however with no significant change in dopamine concentrations. The finding of increase serotonin levels is in line with evidence that lobeline interacts with and inhibits the serotonergic transporter (SERT) and observations that lobeline increases serotonin release from rat hippocampal slices (Lendvai et al., 1996). Animals exposed to lobeline post-treatment failed to significantly differ in dopamine levels when compared to animals from the saline treatment groups. Similarly, acute, intermittent and continuous systemic lobeline administration (1.0 – 30 mg/kg) did not deplete striatal dopamine or DOPAC content, however inhibited amphetamine evoked dopamine release from superfused striatal slices (Miller et al., 2001). This finding is in line with in vivo microdialysis studies where lobeline treatment did not change extracellular dopamine or DOPAC levels in rat striatum (Benwell and Balfour, 1998). This effect of lobeline was also demonstrated in experiments using a cell system co-expressing DAT and VMAT2 that showed lobeline decreased the dopamine releasing effects of MA (Wilhelm et al., 2008). Interestingly, VMAT2 knockout mice had diminished amphetamine-induced CPP when compared to wild-type mice (Takahashi et al., 1997). The lack of change in dopamine levels in lobeline treated animals may possibly explain why lobeline post treatment had no effect on MA-induced CPP behaviour in the present study.

A number of drugs acting on opioid receptors have been suggested as therapeutic agents in addiction. Naloxone and naltrexone have been found to induce dysphoria in humans (Hollister et al., 1981; Grevert and Golstein, 1977) and resulted in conditioned aversive responses in animals and blocks cocaine-induced CPP (Iwamoto, 1985; Parker and Rennie, 1992; Skoubis and Maidment, 2003). Naltridole (a non-selective δ-opioid receptor antagonist) and naltriben (a selective δ_2 – opioid receptor antagonist) were reported to attenuate cocaine- and MA-induced CPP (Suzuki et al., 1994; Menkens et al., 1992; Van Ree et al., 1999). In contrast to these findings, our experiments, two weeks of naltrexone treatment failed to alter CPP

behaviour of MA-treated rats. This finding may possibly be ascribed to the fact that naltrexone pretreatment before MA administration is needed to block the cue-induced reinstatement of drug seeking behaviour. Differences in treatment dosages may also contribute to the discrepancy in data as well as differences in species and strain of animals used. It may be that a higher dosage of the opioid receptor antagonist is required to decrease MA-induced CPP, since the amount of dopamine is released from nerve terminals is greater when compared to cocaine's mechanism of action (Suzuki et al., 1994; Di Chiara and Imperato, 1988).

It remains difficult to explain our findings especially in view of the overwhelming evidence demonstrating the important role of opioids in the rewarding effects of psychostimulants (Miller et al., 2001; Harrod et al., 2001; Pettit et al., 1984; Dworkin et al., 1988). The inhibition of the opioid system via the blocking of the opioid receptors is presumed to lead to an attenuation of the rewarding effects of drugs of abuse, hence making the finding of no difference after two weeks naltrexone post-MA treatment of the present study difficult to interpret. However, other studies similarly have found that naltrexone treatment did not have a significant effect on cocaine use in rats and MA intake in rhesus monkeys (Harrigan and Downs, 1978; Ettenberg et al., 1982; Hemby et al., 1996). In line with this evidence, the non-selective opioid antagonist, quadazocine, also failed to affect cocaine reinforcing responding in rhesus monkeys (Winger et al., 1992). Interestingly, chronic naltrexone treatment (10 mg/kg/day for 12 days) which was followed by a period of no treatment resulted in the facilitation of cocaine self-administration by supposedly enhancing the reinforcing effects of cocaine (Ramsey and Van Ree, 1990). In another study, rhesus monkeys trained to self-administer cocaine were subjected to naltridole treatment for 10 consecutive days. While a decrease in cocaine intake was initially observed, in some of the monkeys the response rate for cocaine returned to baseline levels during the last days of the treatment (Negus et al., 1995). Prolonged MA treatment generally results in the desensitization of μ-opioid receptors (Chiu et al., 2006). Since, naltrexone preferentially inhibits the μ-opioid receptor (Uwai et al., 2004), blocking already desensitized receptors may not necessarily cause a decrease in CPP behaviour as seen in our study.

Generally it is believed that opioid receptor agonists increase the release of dopamine from VTA terminals by suppressing inhibitory GABA neurons (Di Chiara and North, 1992; Leone et al., 1991; Rada et al., 1991). The administration of an opioid receptor antagonist would

therefore result in increased inhibition of VTA neurons by GABA leading to decreased dopamine being released. This mechanism has been proposed for the action of naloxone and naltrexone in their attenuation of amphetamine-induced effects (Hooks et al., 1992; Schad et al., 1995; Yu et al., 2001). In the present study, naltrexone treatment did not have an expected effect on either CPP behaviour or dopaminergic levels in the striatum. This can possibly be explained by the overall decrease in serotonin levels in animals subjected to naltrexone treatment. The reduction in serotonergic levels has been shown to lead to a decreased inhibitory control over dopaminergic concentrations in the VTA (Brodie and Bunney, 1996). Such an explanation is plausible as an increase in dopaminergic levels in animals exposed to naltrexone treatment was observed. In the group subjected to maternal separation, MA-induced CPP and two weeks naltrexone treatment (MS MA N) decreased dopamine levels were obtained. Despite this decrease, the animals in this group continued to display CPP behaviour after naltrexone treatment.

Conclusions

Most studies investigating the effects of lobeline or naltrexone on MA consumption employs a pre-MA treatment strategy. The clinical relevancy of these studies can be questioned, since in real life clinicians are first presented with a drug addict where-after treatment would commence. In the present study, post-MA treatment results in no significant behavioural changes, however does affect neurochemical role players of drug addiction. Interestingly, early life stress attenuated behavioural effects induced by MA. Addictive behaviour and neurochemical changes are complex and the treatment used to address these drug induced changes is of need of further investigation to find promising pharmacological interventions for MA addiction.

Acknowledgements

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Figures

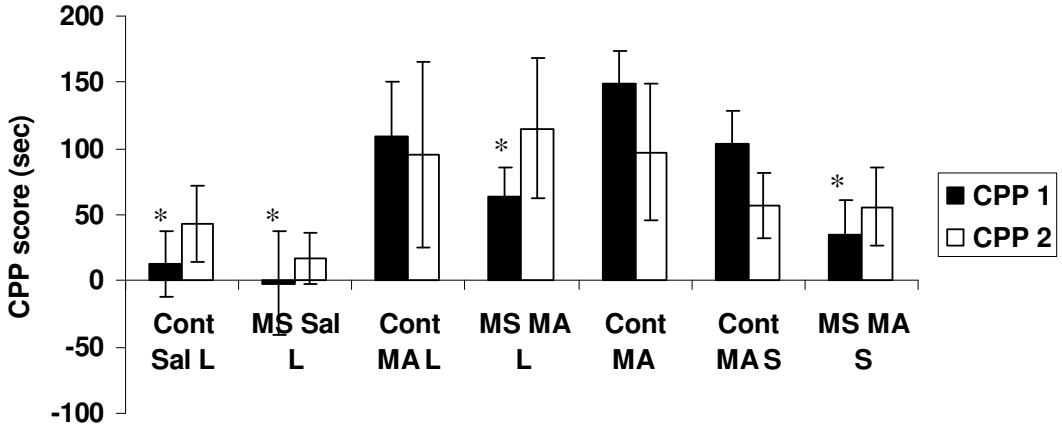

Figure 1: Conditioned Place Preference (CPP) of groups subjected to control conditions or maternal separation. Additionally, groups were either subjected to two weeks of lobeline / saline / no treatment conditions. The first CPP test occurred after MA administration (CPP1) and the second CPP test occurred after the two weeks of lobeline / saline / no treatment (CPP2). Values given as mean ± SEM. * significantly different from the Cont MA group (CPP1) ($p<0.05$)

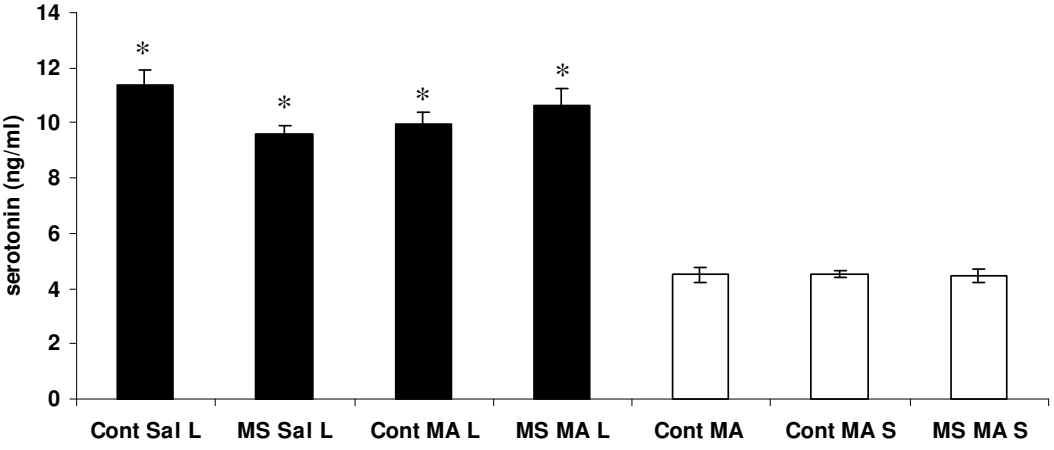

Figure 2: Serotonin concentrations (ng/ml) in the striatum of groups subjected to two weeks lobeline / saline / no treatment conditions. Values given as mean ± SEM. * significantly different from the Cont MA, Cont MA S and MS MA S groups ($p < 0.05$)

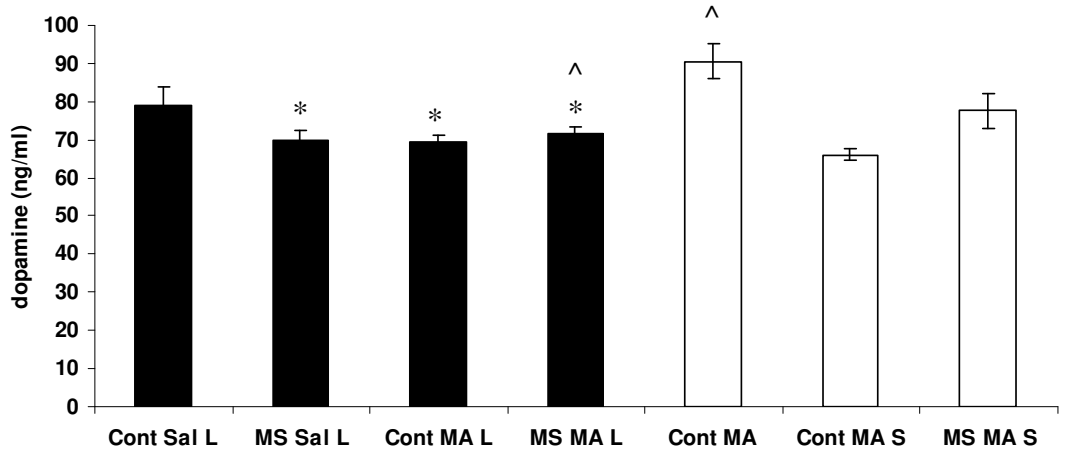

Figure 3: Dopamine concentrations (ng/ml) in the striatum of groups subjected to two weeks lobeline / saline / no treatment conditions. Values given as mean ± SEM. * significantly different from the Cont MA group ($p<0.05$); ^ significantly different from the Cont MA S group ($p<0.05$)

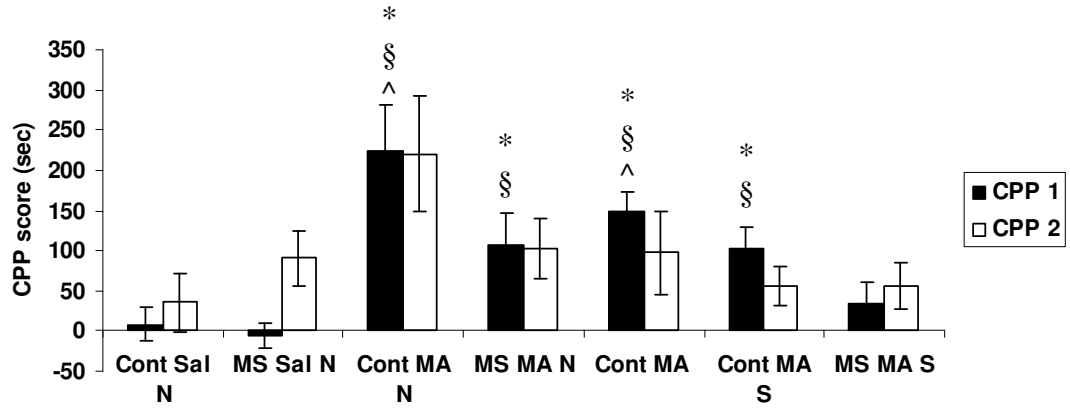

Figure 4: Conditioned Place Preference (CPP) of groups subjected to control conditions or maternal separation. Additionally, groups were either subjected to two weeks of naltrexone / saline / no treatment conditions. The first CPP test occurred after MA administration (CPP1) and the second CPP test occurred after the two weeks of naltrexone / saline / no treatment (CPP2). Values given as mean ± SEM. * significantly different from the Cont Sal N group ($p<0.05$) (CPP1); § significantly different from the MS Sal N group ($p<0.05$) (CPP1); ^ significantly different from the MS MA S group ($p<0.05$) (CPP1)

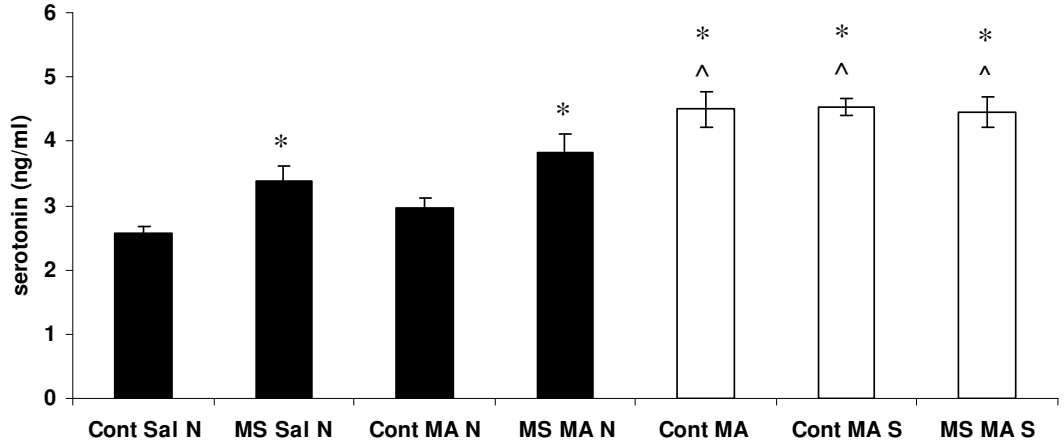

Figure 5: Serotonin concentrations (ng/ml) in the striatum of groups subjected to two weeks naltrexone / saline / no treatment conditions. Values given as mean ± SEM. * significantly different from Cont Sal N group ($p<0.05$); ^ significantly different from MS Sal N and Cont MA N groups ($p<0.05$)

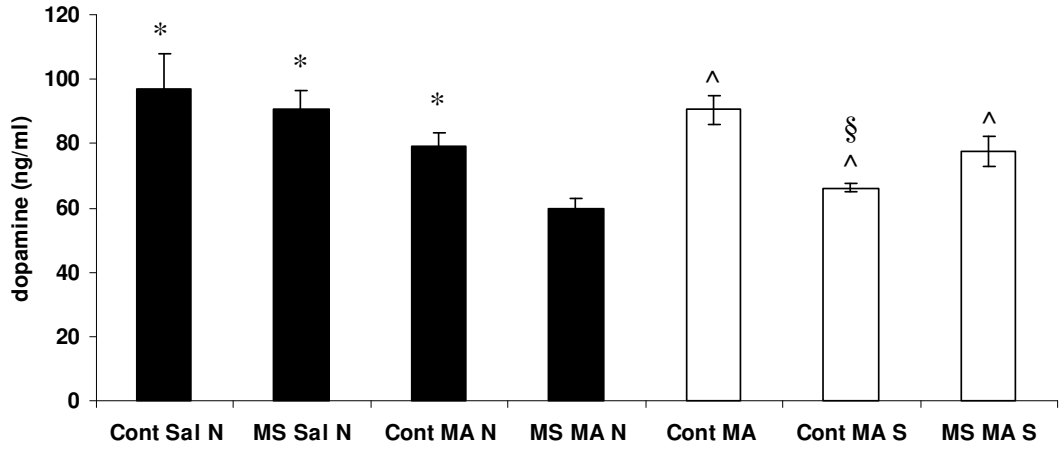

Figure 6: Dopamine concentrations (ng/ml) in the striatum of groups subjected to two weeks naltrexone / saline / no treatment conditions. Values given as mean ± SEM. * significantly different from MS MA N and Cont MA S groups ($p<0.05$); ^ significantly different from the MS MA N group ($p<0.05$); § significantly different from the Cont MA group ($p<0.05$)

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CHAPTER 6

Proteomic analysis reveals differentially expressed proteins in the rat frontal cortex after methamphetamine treatment

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Proteomic analysis reveals differentially expressed proteins in the rat frontal cortex after methamphetamine treatment

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Running head: Proteomics: methamphetamine results in differentially expressed rat cortical proteins

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Abstract

Methamphetamine (MA) is an addictive psycho-stimulant and the illicit use of the drug is escalating. In the present study, we examined protein expression profiles in the rat frontal cortex exposed to a total of 8 MA injections (1 mg/kg, intraperitoneal) using 2-DE based proteomics. We investigated protein changes occurring in both the cytosolic fraction and the membrane fraction. 2-DE analysis resulted in 62 cytosolic and 44 membrane protein spots that were differentially regulated in the frontal cortex of rats exposed to MA when compared to control animals. Of these spots, 47 cytosolic and 42 membrane proteins were identified respectively, using ESI-Quad-TOF, which included ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1), β-synuclein, 78 kDa glucose-regulated protein (GRP 78), γ-enolase, dihydropyrimidase-related protein 2 (DRP 2), complexin 2 and synapsin II. These proteins are associated with protein degradation, redox regulation, energy metabolism, cellular growth, cytoskeletal modifications, and synaptic function. Proteomic research may be useful in exploring the complex underlying molecular mechanisms of MA dependence.

Keywords

Methamphetamine; Frontal cortex; Cytosolic proteins; Membrane proteins; Proteomics; Neuronal plasticity

Introduction

Methamphetamine (MA) is a highly addictive psycho-stimulant and the illicit use of this drug is continuing to escalate in many countries world wide. MA initially elicits its rewarding effects and subsequent detrimental effects by increasing extracellular dopamine concentrations in the mesolimbic dopaminergic system and later proceeds to alter the function of various other brain pathways (Everitt and Wolf, 2002; Di Chiara et al., 2004; Pierce and Kumaresan, 2006). MA achieves this by three unique mechanisms of action. Firstly, MA enters neurons via dopaminergic transporters and reverses their transporter function (Zahniser and Sorkin, 2004; Wilhelm et al., 2006). Secondly, MA reverses the function of the vesicular monoamine transporter 2 (VMAT2), which normally is responsible for intracellular vesicular storage of dopamine, serotonin, noradrenaline and histamine in the central nervous system (Erickson et al., 1992; Weihe and Eiden, 2000).

Effects of MA are exerted by influencing mainly the mesocorticolimbic dopamine system which projects from the ventral tegmental area (VTA) to the nucleus accumbens, olfactory tubercle, frontal cortex and amygdala (Kalivas and Stewart, 1991; Volkow et al., 2003). Above the ventral striatum (or nucleus accumbens) is the dorsal striatum, and this brain area has been implicated in the locomotor response to psychostimulants (Hamamura et al., 1991; Nestler, 2001). There is growing interest in the role of frontal cortex in addiction given its contribution to decision making and inhibitory control (Royall, 2002). Glutamatergic projections from the prefrontal cortex to the nucleus accumbens have been found to be pivotal in drug seeking behaviour (Kalivas et al., 2005). Thus, investigation of protein changes in frontal cortex, which plays an important role in long term addictive processes, may result in a better understanding of the neurobiology of methamphetamine addiction.

Proteomics is a high-throughput technique used to detect global protein changes in complex biological systems. It is particularly useful to study multifactorial disease processes, since this technique can analyze the final end products of genes (i.e. proteins in their final state), it thus provides functional insight. By employing proteomic methodology the differential expression of proteins that contribute to a disease state can be identified. This method therefore allows for discovery driven rather than hypothesis driven investigations (Lewis et al., 2000; Lee, 2001). In turn the information gained by these experiments can be used for hypothesis formulation and subsequent verification (Liao et al., 2005). The proteomics approach has

proven useful in the investigation of the molecular basis of MA dependence (Liao et al., 2005; Yang et al., 2008; Li et al., 2008).

The present study was designed to generate a more comprehensive picture of differentially expressed cytosolic and membrane proteins in the frontal cortex that are altered by MA treatment. To achieve this we used a 2-dimensional gel electrophoresis (2-DE) based proteomics approach in combination with image analysis software and mass spectrometry. This approach differs from western blotting techniques, allowing identification of changes in the expression of multiple proteins rather than researcher-dependent selection of individual proteins.

Materials and Methods

Animals

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 where methamphetamine exposures or 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 x 25 x 20 cm Plexiglas cages with corncobs as bedding. Temperature was kept constant at 22ºC, humidity at 55% and food and water was available *ad libitum* for the duration of the experiment. Sprague Dawley, male rats (*Rattus norvegicus*) were used in this experiment.

Drugs

Methamphetamine hydrochloride was obtained from US Pharmacopeia Convention Inc. (Rockville, USA). R-(-)-Apomorphine hydrochloride was procured from Sigma-Aldrich Chemicals (St. Louis, MO). Apomorphine and methamphetamine were both dissolved in 0.9 % saline prior to being administered to the rats at a dose of 1mg/kg.

Experimental design

Male rats in the experimental group were subjected to 8 methamphetamine intra-peritoneal (i.p.) injections in total, each administered on PND 33-36 and PND 39-42 and 3 apomorphine subcutaneous (s.c.) injections in total, each administered on PND 38, 44 and 51. The rational for subjecting 33 day old rats to methamphetamine is that this period in a rats' life corresponds to adolescent period in humans. It is known that most addicts start experimenting with drugs during their adolescence. Apomorphine was administered as an assessment of apomorphine-induced locomotor activity to further evaluate possible hyperactivity induced by methamphetamine administration (Faure et al., 2009). An apomorphine control group was not included and this could impose a possible limitation when interpreting results, since proteins expressed could be due to apomorphine treatment or interaction with methamphetamine. However, control rats received volume controlled 0.9% saline injections (1ml/kg) whilst experimental animals were subjected to methamphetamine injections which were dissolved in 0.9% saline and administered at a dose of 1mg/kg. A low dose of MA was specifically used as such doses are useful in elucidating protein expression changes that may be related to neuroadaptive processes in the brain (Yamada et al., 2005). A non-neurotoxic dose of 1mg/kg was repeatedly (8 days) administered to allow for changes to occur in drug-dependent genes and resultantly measure protein expression changes (Yamada et al., 2005; Iwazaki et al., 2006). Animals were all sacrificed by means of decapitation on PND 52. The brains were removed and frontal cortex was dissected on a cold glass plate, and rapidly frozen and stored in liquid nitrogen for later analysis.

Two-dimensional gel electrophoresis

The frontal cortex is a large enough brain area that pooling of samples was not deemed necessary as they render sufficient protein for the subsequent experiments. The frontal cortex of 3 brain samples from each group (experimental and control) were used and run in triplicate for both the cytosolic and membrane protein fractions respectively. Tissue samples were first 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 protein extracts. Most abundant protein changes take place in the cytosolic and membrane regions of neurons subjected to methamphetamine and hence the rational to focus and investigate the protein expression changes in these two fractions. The cytosolic and membrane protein extracts were further used to determine the total protein concentration by using a Bradford method to adhere to the specifications of the ReadyPrep 2-D Cleanup Kit (Bio-Rad). These stipulate that 1-500

µg of protein in a final volume of not more than 100µl per 1.5ml microcentrifuge tube is allowed for efficiency of the cleanup procedure and result in low conductivity samples necessary for good iso-electric focusing (IEF) and 2D gel electrophoresis (2-DE). After the cleanup samples were dissolved in 2D Sample Buffer (Bio-Rad). A RC DC Protein Assay (Bio-Rad) was performed to quantify protein concentrations after the cleanup procedure, which contrary to the Bradford, is not affected by interference of reducing agents in the 2D Sample Buffer. The first dimension of gel electrophoresis was done using an immobilized pH gradient gel (IPG strip, pH 5-8, 11 cm) in a Protean IEF Cell (Bio-Rad). Gels were rehydrated with cytosolic and membrane samples respectively from the experimental and control groups containing 150 µg of total protein in a total volume of 200 µl per IPG strip for 12 h and focused for 7h until the IEF Cell reached 40 000 volt hours (Vh) at 20 ºC. The strips were equilibrated twice after IEF for a 15 min period each in equilibration buffer I followed by equilibration buffer II and embedded onto Criterion Precast Gels (4-12% Bis-Tris, Bio-Rad). The second dimension gel electrophoresis was carried out at 200V for a period of approximately 55 min. After electrophoresis, the gels were fixed in a 40% methanol and 7% acetic acid solution (v/v) for 1 h and stained overnight at room temperature with Brilliant Blue G-Colloidal dye solution (Sigma-Aldrich). The gels were then subjected to a quick destain (1 min, 40% methanol and 10% acetic acid) solution followed by destain in which a 25% methanol solution was used for a 2h period and a further 30 min in newly replaced destain. The gels were either scanned immediately or stored in 25% ammonium sulfate at room temperature until day of scanning. Prior to scanning gels were first washed in bi-distilled water to remove ammonium sulfate and then placed in destain to scan.

Gel image analysis and digestion of differentially expressed protein spots

Gel images were obtained by scanning the 2D gels on a densitometer (GS-800 scanner, Bio-Rad) using the Quantity One - 4:5.2 (Basic) software program (Bio-Rad). The scanned images were analyzed using PD Quest Advanced version 8.0.1 software program, to identify differentially expressed ($p \leq 0.05$; t-test) protein spots. Protein spots of interest were manually excised from the gels and placed into a 96-well microtiter plate for in gel digestion. The spots were washed twice with 50% acetonitrile in 100 mM ammonium bicarbonate and rinsed with acetonitrile and air dried for a 10min period after which samples were reduced with 10mM dithiothreitol in 100mM ammonium bicarbonate for 30 min and alkalyted with 55mM iodoacetamide in a 100mM ammonium bicarbonate solution. Spots were then rinsed with

acetonitrile and then 100mM ammonium bicarbonate and another further three washes with acetonitrile. A 25µl aliquot of 6 ng/µl trypsin was added to each sample and incubated at 37 ºC for 4.5 h. The resultant tryptic peptides were extracted twice, first by using 30µl of an aqueous solution containing 2% acetonitrile and 1% formic acid and secondly using 15µl of an aqueous solution containing 51% acetonitrile and 0.5% formic acid. The two extractions were combined and placed in a second cooled 96-well plate. If immediate analysis was not possible at this stage, the extractions were stored at -80 ºC before mass spectrometry analysis.

Mass spectrometry identification of cytosolic and membrane extracted peptides

An in-line NanoAcquity LC and autosampler system was used to resolve extracted tryptic peptides. LC solvents were supplied by Mallinckrodt Baker, Inc. A 4.9µl aliquot of each sample was injected onto a nanoACQUITY UPLC^{TM} trapping column 10kpsi Symmetry C18 180 µm x 20mm 5µm (Waters) equilibrated in 3% aqueous acetonitrile containing 0.1% formic acid and the column flushed with 1% aqueous acetonitrile/0.1% formic acid at 15µLmin^{-1} for 1 minute. The peptides were then eluted onto a nanoACQUITY UPLC BEH C18 Column, 1.7 µm, 100µm x 100mm, 10K psi (Waters) at 1.2µ 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 ran over 20min. 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 was used to analyze the eluted peptides. 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 on a subsequent acquisition was <10ppm. 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. Data dependent acquisition (DDA) mode over the mass/charge (m/z) range of 50-2000 the instrument was operated in. During the DDA analysis, the three most intense peptides eluting from the column both MS and tandem mass spectrometry (CID) was performed. The un-interpreted MS/MS data were processed using the Waters ProteinLynx Global Server v2.3 software package (smoothed, background subtracted, centered and deisotoped) then mass corrected against the doubly charged GFP peptide. Peak list file (pkl) was created and subjected t o Mascot (www. Matrixscience.com) using MS/MS Ion Search and SwissProt database to identify differentially expressed proteins. Search parameters included up to one missed tryptic cleavage, fixed modifications of Carbamido-

methyl (C) and variable modifications of Oxidation (M), Phospho (ST) and Phospho (Y) and mass tolerance 0.5 Da. A positive identification was defined when the MOWSE scores (>37) was significant ($p<0.05$).

Results

Proteins from both the cytosolic and membrane protein fractions of the rat frontal cortex were separated by 2-DE. Fig. 1 (I) demonstrates a typical composite of triplicate 2D gels of cytosolic proteins present in the rat frontal cortex. The gels of both cytosolic and membrane fractions from the MA exposed group of animals were compared to those of the control group. Analyses by PD Quest software showed 62 cytosolic and 44 membrane protein spots to be differentially expressed in the frontal cortex of MA exposed rats ($p<0.05$; Student's t-test). These differentially expressed proteins were then excised from the gel and submitted to mass spectrometry for identification purposes. Following mass spectrometric analyses 47 of the 62 cytosolic and 42 of the 44 membrane proteins were identified with confidence ($p<0.05$; Student's t-test). To illustrate Spot 6609 was identified as dihydropyrimidase-related protein 2 (DRP-2) and its expression was significantly reduced in the MA treatment group (Fig. 1 (II)). A summary of the identified proteins is presented in Table I and Table II.

Discussion

Using 2-DE based proteomics, the present study revealed that a number of proteins are differentially expressed; 47 in the cytosolic fraction and 42 in the membrane fraction following MA treatment. As evident from the data, most of these proteins are associated with important biological functions such as protein degradation, redox regulation, energy metabolism, cellular growth, cytoskeletal modifications, and synaptic signaling. To discuss the biological significance of each of these proteins is beyond the scope of this manuscript. However the implication of the changes in the expression of some of the cytosolic and membrane proteins will be discussed.

In the present study, ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) expression was increased in the cytosolic cortical fraction and β-synuclein was increased in the

membrane cortical fraction. UCH-L1 is involved in the ubiquitin proteosome pathway and this protein in particular has been shown to be altered in various methamphetamine studies (Liao et al., 2005; Iwazaki et al., 2006). UCH-L1 is a neuronal protease and plays a key role in the proteolytic degradation of misfolded or damaged proteins via the 26S proteosomal pathway (Lowe et al., 1990; Castegna et al., 2002; Fornai et al., 2004a; Fornai et al., 2004b; Fornai et al., 2006; Carolan et al., 2006). The increased expression of UCH-L1 is in line with a previous study where a single non-toxic dose of MA (1mg/kg) also resulted in increased UCH-L1 expression. However, the present study administered eight MA injections and this might have produced a greater effect on toxic cascades. β-synuclein, which is the non-amyloidogenic homolog of α-synuclein, has been shown to inhibit aggregation of intracellular proteins. The finding of increased β-synuclein levels may therefore reflect a reactive response to the neurotoxic effects of MA.

MA produces a massive increase in cytosolic dopamine levels via its action on both VMAT2 and MAO. Dopamine and its resultant metabolites are known to generate reactive oxygen species (ROS), which ultimately result in neurotoxicity and cell death (Slivka and Cohen, 1985; Gibb et al., 1990). The neurotoxic effects of MA include destruction of dopaminergic axons and terminals in the striatum, while the dopaminergic somas in the midbrain areas are kept intact (Ricaurte et al., 1982; McCann and Ricaurte, 2004). Increased 78 kDa glucose-regulated protein (GRP78) mRNA levels after MA administration have been suggested to compensate for MA-induced oxidative stress (Cadet et al., 1994; Jayanthi et al., 1998; Cadet and Brannock, 1998), since GRP78 protect cells against apoptosis (Morris et al., 1997). In the present study, GRP78 was increased in the cytosolic fraction and decreased in the membrane fraction. It would seem that in our treated rats GRP78 may also have a compensatory effect against MA-induced oxidative stress. This protection might be enhanced by the observed increased levels of peroxiredoxin-2 in both cytosolic and membrane fractions in these animals. Peroxiredoxins are enzymes that exert their protective antioxidant effects in cells through their peroxidase activity, whereby the levels of hydrogen peroxide, peroxynitrite and other organic hydroperoxides are reduced and detoxified (Poole and Ellis, 1996; Bryk et al., 2000; Peshenko and Shichi, 2001; Hofmann et al., 2002).

Differential expression of various proteins involved in energy metabolism was observed in the present study. For example enolase, an enzyme that catalyses the formation of

phosphoenolpyruvate from 2-phosphoglycerate (Harris et al., 1976), was down regulated in the cytosolic fraction. In addition, MA-sensitized animals also had differentially modulated amygdala proteins involved in ATP production and some of these included fructose-biphosphate aldolase C and D-3-phosphoglycerate dehydrogenase (Iwazaki et al., 2008), which were also identified in our study. Clinical studies have noted cerebral glucose hypometabolism in human MA abusers (Kim et al., 2005). In addition, a significant correlation was found between MA-induced decreases in ATP levels and dopamine depletions in the striatum (Chan et al., 1994). Our findings indicate that MA treatment may be associated with reduced glucose metabolism, possibly leading to neuron malfunction.

In addition to the role of α-enolase in glycolysis, the enolases have also been shown to act as neurotrophic factors. Gamma-enolase has been shown to be responsible for increased survival of cortical neurons and neuron specific enolase has a functional role in the plasma membrane of neuronal cells, where it demonstrates neurotrophic properties (Hattori et al., 1994). In our study the increase in gamma-enolase levels in the cytosolic fraction may indicate the presence of compensatory mechanisms protecting against the deleterious effects of MA.

The three-dimensional structure of neurons is maintained by microtubules and actin filaments, which are primarily scaffolding proteins (Vale et al., 1992; Liao et al. 2005). The cytoskeletal network is stabilized by F-actin capping proteins which binds to the fast growing ends of actin filaments and hence prevents the loss of actin subunits from these ends (Kilimann and Isenberg, 1982). As mentioned earlier, MA results in the loss of dopaminergic terminals while the cell bodies are spared (McCann and Ricaurte, 2004), and as expected, Liao et al. (2005) found decreased F-actin capping protein beta subunit in striatal tissue after a neurotoxic dose of MA. This has been suggested to lead to the disruption of cytoskeletal architecture and ultimately to interrupted neuronal transport in the axonal terminals. Similarly, Kobeissy et al. (2008) reported decreased concentrations of the F-actin capping protein after an acute MA neurotoxic dose supporting previous findings that indicated that the cytoskeletal structure is susceptible to MA (Warren et al., 2005; Warren et al., 2007). Decreased expression of actin beta was found even after a single dose of MA (Iwazaki et al., 2006). Therefore after repeated exposures one would expect structural modifications to be more severe and to lead to chronic alterations in specific brain structures and function. In the present study, decreased actin (cytoplasmic 1 and 2) was identified in the membrane cortical fraction together with

abnormalities in tubulin levels, in both membrane and cytosolic cortical fractions. This supports suggestions that MA leads to structural alterations in the frontal cortex.

Dihydropyrimidase-related protein, also referred to as collapsing response mediator protein 2 (DRP-2 / CRMP-2 / DPYL-2), is an extracellular guidance cue and hence involved in axonal outgrowth mediated by extracellular cues (Hamajima et al., 1996). Modifications in this protein has been reported in the striatum of animals subjected to chronic use of MA (Iwazaki et al., 2007), as well as causing altered neuronal plasticity in the amygdala after acute treatment of MA (Iwazaki et al., 2008). In the present study, DRP-2 and DRP-3 levels were both decreased in the cytosolic cortical fraction, and DRP-2 and DRP-5 levels were increased in the membrane cortical fraction. It is difficult to reconcile these data, but one possibility is that the membrane fraction DRP-2 may be more involved in signalling processes after MA exposure, while at the cytosolic level DRP-2 may be fulfilling a cytoskeletal role and so is down-regulated in response to MA effects on the cytoskeletal structure. DRP-2 has previously been associated with postsynaptic densities in the rat brain (Roberts and Sheng, 2000) and mitochondrial septin (M-septin) has been found to induce mitochondrial translocation of CRAM, which is a CRMP-associated molecule that belongs to the same unc-33 gene family (Inatome et al., 2000; Takahashi et al., 2003). This infers that DRP-2 could very well be present or associated with the membrane and cytosolic fractions and play a role in translocation and could possibly be a different form of the same protein.

Phosphatidylethanolamine-binding protein 1 (PEBP1), also referred to as hippocampal cholinergic neurostimulating peptide (HCNP), is a calpain substrate (Chen et al., 2006) and a raf-kinase inhibitor, and is functional as a neuronal protein involved in cell signaling (Kobeissy et al., 2008). The HCNP has been found work with nerge growth factor or independently to enhance choline acetyltransferase production which aids in cholinergic development of medial septal nuc1ei (Butterfield et al., 2006). PEPB1 would subsequently be involved with neuroplastic changes in brain circuitry involved in addiction. Previously, PEBP1 expression levels have been reported to be altered by MA (Iwazaki et al., 2007; Iwazaki et al., 2008; Kobeissy et al., 2008). PEBP1 has been proposed to be an inhibitor of the proteosome (Hengst et al., 2001; Chen et al., 2006) and PEBP1 up regulation in both the cytosolic and membrane cortical protein fraction in the present study may suggest a protective role against proteolytic degradation activated by MA.

Synapsins are also involved in signaling processes. They regulate neurotransmitter release and synaptogenesis (Chin et al., 1995; Ferreira et al., 1995), neuronal development and maintenance (Ferreira and Rapoport, 2002), the coating of vesicular surfaces, the anchoring of synaptic vesicles to the presynaptic membrane and mediate axonal outgrowth (Ferreira et al., 1998; Sudhof, 2004; Fdez and Hilfiker, 2006). In addictive brain circuitry, synapsins would hence play a huge role in neuoroplasticity induced by repeated psychostimulant use. Disruption of synapsin in knockout models or via antibody injections has demonstrated that synapsin down regulation leads to the reduction in the number of synaptic vesicles distal to the active zone in excitatory neurons (Li et al., 1995; Rosahl et al., 1995; Pieribone et al., 1995). Glutamate has been shown to be associated with synapsin II (Vawter et al., 2002; Villanueva et al., 2006) and may play a key role in the effects of drugs of abuse on cortico-accumbens glutamatergic projections (Kalivas et al., 2005). Increased synapsin II in the membrane cortical fraction in the present study is consistent with previous work emphasizing the importance of glutamatergic signaling from the frontal cortex to the ventral striatum in response to MA administration (Iwazaki et al., 2007).

Various presynaptic proteins play a role at nerve terminals for synaptic vesicle exocytosis of neurotransmitters into the synaptic cleft to occur. In the present study two proteins involved in exocytosis, i.e. SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) activities were identified. They are the complexin and septin proteins. Complexins binds with high affinity to the SNARE complexes (Chen et al., 2002) and function by stabilizing the SNARE complexes in a highly fusogenic state (Reim et al., 2001; Xue et al., 2007). Complexins accomplishes this by binding to the groove between syntaxin and synaptobrevin in an anti-parallel fashion (Marz and Hanson, 2002) or by acting as a prefusion clamp that arrests SNARE complexes to prevent fusion with the presynaptic membrane. Altered complexin expression levels leads to deficits in evoked synaptic transmitter release (Reim et al., 2001; Huntwork and Littleton, 2007) and hence implicates psychostimulant-induced neuroplasticity. After MA exposure, complexin 2 was increased in the cytosolic cortical fraction, and complexin 1 and complexin 2 were increased in the membrane cortical fraction in the present study. This is in line with enhanced neurotransmission of dopamine in various brain areas in response to MA.

Conclusion

In summary, the present study investigated the effects of methamphetamine using a systems biology approach. We have shown methamphetamine to impact neurons at numerous levels. For instance MA increased the expression of proteins related to degenerative processes, interfered with normal neurotransmission and subsequent intracellular signaling, disrupted cyto-architecture, and altered energy metabolism. Additional work is needed to compare the effects of MA on neurobiological systems in different regions of the brain, as these may differ (Liao et al, 2005; Yang et al, 2008; Li et al, 2008). Further research is also needed to determine precisely how alterations in the expression of proteins in such systems serve to mediate the particular effects of MA on brain and on behavior.

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Tabel I: Differentially expressed cytosolic proteins of the rat frontal cortex exposed to MA (* Proteins identified in more than one experiment – Biological replicates).

Protein name	MASCOT score	% Sequence Coverage	Swiss Prot Accession nr	Mr (Da)	pI	Ratio Exp:Con
Protein Fate / Redox Regulation						
Ubiquitin carboxyl-terminal hydrolase isozyme L1	155	12	**Q00981**	25165	5.14	1.43
Peroxiredoxin-2	138	17	**P35704**	21941	5.34	1.43
78 kDa glucose-regulated protein	425	17	**P06761**	72473	5.07	1.56
Prohibitin	241	21	**P67779**	29859	5.57	2.01
26S protease regulatory subunit 6A	60	2	**Q63569**	49415	5.13	0.18
Protein disulfide-isomerase A3	129	6	**P11598**	57044	5.88	0.63
Proteasome subunit alpha type-1	396	35	**P18420**	29784	6.15	0.81
Proteasome subunit alpha type-2	194	13	**P17220**	26024	6.92	0.59
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	136	9	**P22062**	24683	7.14	0.59
Energy Metabolism						
Rab GDP dissociation inhibitor alpha	240	14	**P50398**	51074	5	0.14
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	137	21	**P49432**	39299	6.2	1.48
Alpha-enolase *	521	24	**P04764**	47440	6.16	0.78
Fructose-bisphosphate aldolase C	334	21	**P09117**	39658	6.67	3.16
Creatine kinase B-type	112	5	**P07335**	42983	5.39	1.68
Rab GTPase-binding effector protein 1	168	3	**O35550**	99766	4.95	2.86
Lactoylglutathione lyase	107	9	**Q6P7Q4**	20977	5.12	1.23
Rho GDP-dissociation inhibitor 1	52	15	**Q5XI73**	23450	5.12	1.35
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	159	6	**Q99NA5**	40044	6.47	0.69
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	109	4	**P08461**	67637	8.76	0.45
D-3-phosphoglycerate dehydrogenase	455	17	**O08651**	57256	6.28	0.39
Triosephosphate isomerase	321	32	**P48500**	27345	6.89	0.39
Aconitate hydratase, mitochondrial	962	27	**Q9ER34**	86121	7.87	4.96
Carbonic anhydrase 1	167	14	**B0BNN3**	28339	6.86	1.84
V-type proton ATPase subunit B, brain isoform	188	7	**P62815**	56857	5.57	0.44
Protein Synthesis / Neurotrophic						
Elongation factor Tu, mitochondrial	167	5	**P85834**	49890	7.23	0.72
Gamma-enolase	743	33	**P07323**	47510	5.03	1.3
Adenylate kinase isoenzyme 1 *	234	19	**P39069**	21570	7.66	0.64

Structural / Cytoskeletal						
Tubulin beta-5 chain *	677	41	**P69897**	50095	4.78	4.01
Tubulin beta-2A chain	69	4	**P85108**	50274	4.78	0.61
Fascin (Fragments)	69	5	**P85845**	22110	5.86	0.46
Transgelin-3	64	14	**P37805**	22657	6.84	1.26
Microtubule-associated protein 1A	154	1	**P34926**	300831	4.87	1.84
Synaptic Signalling						
Phosphatidylethanolamine-binding protein 1	156	14	**P31044**	20902	5.48	1.43
Dihydropyrimidinase-related protein 2 *	369	18	**P47942**	62638	5.95	0.78
Dihydropyrimidinase-related protein 3	389	14	**Q62952**	62327	6.04	0.57
Dual specificity mitogen-activated protein kinase kinase 1	113	5	**Q01986**	43779	6.18	1.68
Guanine nucleotide exchange factor MSS4	127	22	**Q08326**	14261	5.19	1.99
Guanine nucleotide-binding protein subunit beta-1	100	6	**P54311**	38151	5.6	0.44
Proto-oncogene C-crk	232	17	**Q63768**	33881	5.39	0.77
Voltage-dependent anion-selective channel protein 2	204	17	**P81155**	32353	7.44	0.59
Complexin-2 *	101	19	**P84087**	15499	5.06	1.6
Protein kinase C and casein kinase substrate in neurons protein 1	144	7	**Q9Z0W5**	50760	5.15	0.14
Guanine deaminase	365	19	**Q9WTT6**	51554	5.56	0.5
Other						
Heat shock cognate 71 kDa protein	718	19	**P63018**	71055	5.37	1.74
Stress-induced-phosphoprotein 1	573	27	**O35814**	63158	6.4	0.53
T-complex protein 1 subunit beta	308	12	**Q5XIM9**	57764	6.01	0.74
Protein LZIC	51	4	**Q5PQN7**	21438	4.89	1.24

Tabel II: Differentially expressed membrane proteins of the rat frontal cortex exposed to MA (* Proteins identified in more than one experiment – Biological replicates).

Protein name	MASCOT score	% Sequence Coverage	Swiss Prot Accession nr	Mr (Da)	pI	Ratio Exp:Con
Protein Fate / Redox Regulation						
Peroxiredoxin-2	85	24	**P35704**	21941	5.34	1.39
78 kDa glucose-regulated protein	784	28	**P06761**	72473	5.07	0.52
Energy Metabolism						
Dihydrolipoyllysine-residue acetyltransferase	328	12	**P08461**	67637	8.76	0.72
Isocitrate dehydrogenase [NAD] subunit alpha *	372	22	**Q99NA5**	40044	6.47	0.77
Triosephosphate isomerase	457	42	**P48500**	27345	6.89	1.24
Dihydrolipoyl dehydrogenase	123	12	**Q6P6R2**	54574	7.96	2.05
Phosphoglycerate kinase 1	303	22	**P16617**	44909	8.02	0.56
NADH dehydrogenase [ubiquinone] flavoprotein 2	295	25	**P19234**	27703	6.23	1.37
ATP synthase subunit beta *	158	10	**P10719**	56318	5.19	0.44
Pyruvate dehydrogenase E1 component subunit alpha *	230	11	**P26284**	43883	6.2	0.59
Aconitate hydratase	852	25	**Q9ER34**	86121	7.87	2.09
Dihydrolipoyllysine-residue succinyltransferase	167	10	**Q01205**	49236	8.89	0.63
Pyruvate kinase isozymes M1/M2	131	10	**P11980**	58294	6.63	1.82
Protein Synthesis / Neurotrophic						
Beta-synuclein	74	9	**Q63754**	14495	4.48	1.33
Structural / Cytoskeletal						
Tubulin beta-5 chain	641	47	**P69897**	50095	4.78	1.78
Tubulin alpha-1B chain	326	33	**Q6P9V9**	50804	4.94	0.47
Tubulin beta-2C chain	185	20	**Q6P9T8**	50225	4.79	0.44
Actin, cytoplasmic 1 *	561	38	**P60711**	42052	5.29	0.27
Actin, cytoplasmic 2 *	561	38	**P63259**	42108	5.31	0.27
Neuronal-specific septin-3	236	17	**Q9WU34**	40914	6.74	0.59
Septin-5	151	10	**Q9JJM9**	43281	6.34	0.59
Annexin A3	512	30	**P14669**	36569	5.96	0.77
T-complex protein 1 subunit epsilon	393	16	**Q68FQ0**	59955	5.51	0.54
Synaptic Signalling						
Guanine nucleotide-binding protein subunit beta-1 *	453	24	**P54311**	38151	5.6	0.54
Guanine nucleotide-binding protein subunit beta-2 *	355	19	**P54313**	38048	5.6	0.54
Guanine nucleotide-binding protein subunit beta-3 *	183	13	**P52287**	38125	5.51	0.54
Dihydropyrimidinase-related protein 2	353	20	**P47942**	62638	5.95	1.95

Dihydropyrimidinase-related protein 5	533	20	**Q9JHU0**	62071	6.6	1.78
Phosphatidylethanolamine-binding protein 1	383	45	**P31044**	20902	5.48	1.35
Calmodulin	107	18	**P62161**	16827	4.09	2.01
Synapsin-2	125	9	**Q63537**	63702	8.73	2.81
Myc box-dependent-interacting protein 1	243	15	**O08839**	64721	4.95	0.52
Complexin-2 *	198	35	**P84087**	15499	5.06	1.21
Protein kinase C and casein kinase substrate in neurons protein 1	255	27	**Q9Z0W5**	50760	5.15	0.67
Complexin-1 *	51	8	**P63041**	15169	4.93	1.6
Endophilin-A1	350	23	**O35179**	40045	5.26	0.27
Endophilin-A2	216	10	**O35964**	41694	5.45	0.27
Voltage-dependent anion-selective channel protein 1	250	18	**Q9Z2L0**	30851	8.62	0.79
Other						
Mu-crystallin homolog *	229	26	**Q9QYU4**	33704	5.34	2.02
Isovaleryl-CoA dehydrogenase	267	13	**P12007**	46862	8.03	0.59
Heat shock 70 kDa protein 1A/1B	327	11	**Q07439**	70427	5.61	1.32
60 kDa heat shock protein	219	13	**P63039**	61088	5.91	0.54

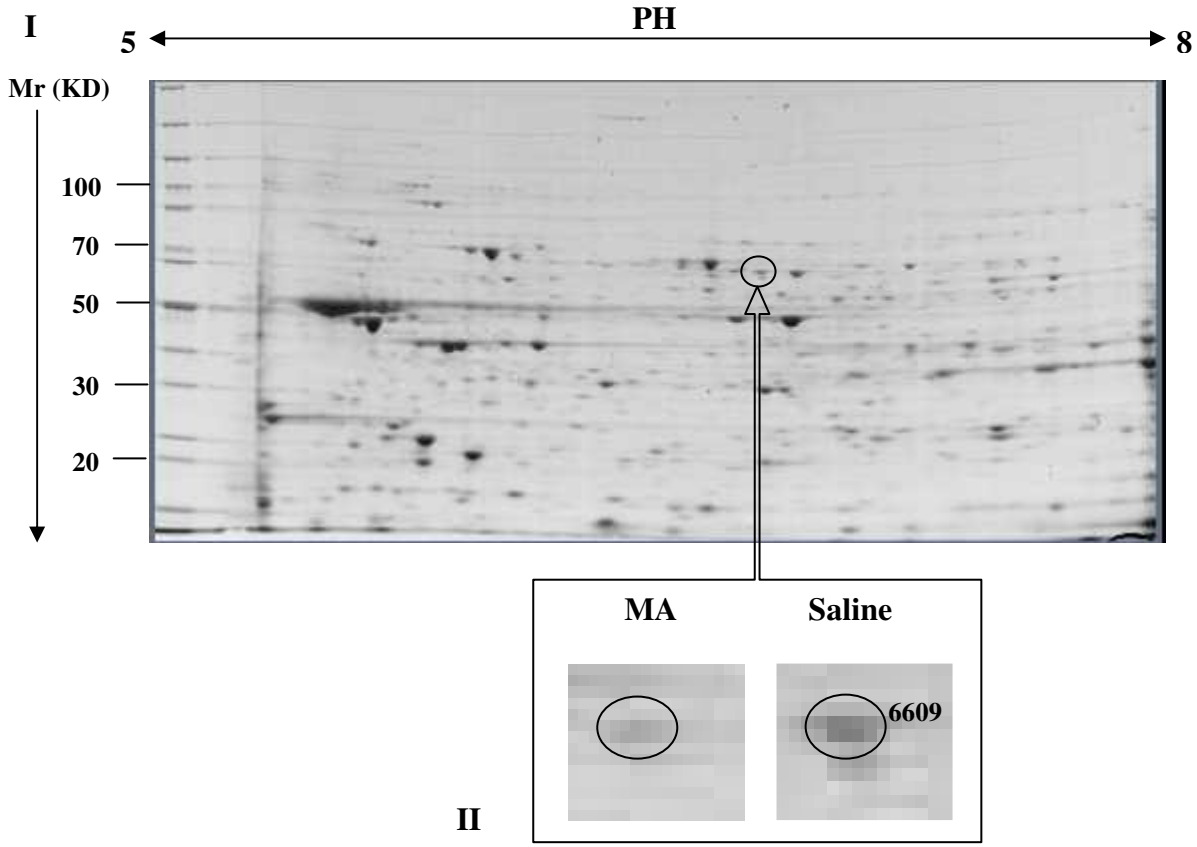

Figure 1. (I) A 2D gel image of expressed proteins in the rat frontal cortex of the cytosolic protein fraction. (II) An enhanced image of spot 6609 identified as dihydropyrimidase-related protein 2 in both MA exposed and control groups. The expression of spot 6609 was decreased in the MA exposed group in the cytosolic cortical protein fraction.

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CHAPTER 7

Methamphetamine differentially affects cytosolic proteins in the nucleus accumbens shell and core of maternally separated and non-stressed control rats: a proteomic quantification approach.

Methamphetamine differentially affects cytosolic proteins in the nucleus accumbens shell and core of maternally separated and non-stressed control rats: a proteomic quantification approach

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Abstract

The nucleus accumbens core and shell sub-regions are differentially affected by both stressors and methamphetamine (MA). Early adversity may predispose individuals to subsequent MA use. This study aimed to better characterize and quantify MA-induced protein role players in both the shell and core of the nucleus accumbens of animals exposed to maternal separation. Proteomics was used to identify proteins involved in the mechanisms and effects induced by stress and methamphetamine combined with the isobaric tagging of peptides (iTRAQ) which enables simultaneous identification and quantification of peptides by employing tandem mass spectrometry (MS/MS). Processes affected by the two interventions were similar in the respective subregions. However, significant differences were observed when the individual proteins were identified between shell and core sub-regions. Another key finding was the involvement of additional proteins when rats were exposed to both early life maternal separation and MA treatment. We found 28 cytosolic accumbal shell and 25 accumbal core proteins which were quantitatively significantly expressed between experimental groups investigated. Some of these proteins included actin cytoplasmic 2, thymosin beta-4, creatine kinase B-type, proSAAS, 14-3-3 protein gamma, myristoylated alanine-rich C-kinase substrate (MARCKS), Arpp-21, ubiquitin carboxyl-terminal hydrolase isozyme L1, alpha-synuclein, brain acid soluble protein 1 (BASP1) and myotrophin. These proteins are associated with cytoskeletal modifications, energy metabolism, intracellular signaling, protein degradation and cellular growth. The quantification of differentially expressed proteins may be useful in determining the protein role players involved in the molecular mechanisms of early life stress and MA dependence.

Keywords

Maternal separation; Nucleus accumbens shell; Nucleus accumbens core; Methamphetamine; Cytosolic proteins; iTRAQ; Proteomics

Introduction

Stress has been shown to affect the core and shell subregions of the nucleus accumbens differently. Whereby, emotionally stressed rats that witnessed other rats receive electric footshocks; have increased extracellular dopamine concentrations in the shell and not the accumbens core (Wu et al., 1999). Since maternal separation (MS) has been shown to alter the animal's response to drugs of abuse (e.g. enhanced cocaine self-administration and psychostimulant induced locomotor activity (Brake et al., 2004; Matthews et al., 2003; Meaney et al., 2002; Kikusui et al., 2005)), we hypothesized that this form of early life stress would also differentially affect the shell and core sub-regions of the nucleus accumbens.

Methamphetamine (MA) has also been shown to differentially affect the nucleus accumbens core and shell sub-regions. Microdialysis studies in rodents have indicated that drugs of abuse increases dopamine neurotransmission preferentially in the nucleus accumbens shell (Imperato and Di Chiara, 1986; Imperato et al., 1986; Carboni et al., 1989). This finding was confirmed by subsequent brain imaging studies in humans (Drevets et al., 2001; Leyton et al., 2002; Boileau et al., 2003). Similarly, reinstatement of cocaine seeking behavior in the rat involved the activation of dopamine receptors in the accumbal shell rather than the core region (Schmidt et al., 2006). The involvement of the shell has been consistently found and is involved in emotional and motivational activities while the core region is involved in somatomotor functions (Alheid and Heimer, 1988; Heimer et al., 1991). However, not all data are consistent - neurotoxic doses of MA selectively caused damage to the dopaminergic input to the core, while those to the shell appeared unaffected (Broening et al., 1997). Secondly, we hypothesize that MA would also affect the shell and core sub-region of MS rats.

Proteomics is a powerful technique to identify the changes in expression of a multitude of proteins following an intervention. This experimental approach has previously been used to unravel the mechanisms and effects induced by methamphetamine usage (Liao et al., 2005; Iwazaki et al., 2006; 2007; 2008; Li et al., 2008; Yang et al., 2008). Various mass spectrometry techniques allow unbiased identification of target proteins and assess the post-transcriptional modifications of proteins. Isobaric tagging of peptides (iTRAQ) enables simultaneous identification and quantification of peptides using tandem mass spectrometry (MS/MS) (Thompson et al., 2003). A major advantage of using iTRAQ labeling is the

simultaneous proteomic analysis of more than one experimental sample. In the present study the parallel proteomic analysis of multiple experimental samples was done.

Since the molecular components mediating stress and MA-induced effects in the shell and core sub-regions are not well established, the aim of our study was to characterize and quantify protein role players in these conditions by using iTRAQ methodology on both the shell and core of the nucleus accumbens of MA-treated animals previously exposed to maternal separation and non-stressed rearing conditions. We hypothesize that different functional proteins would be involved in MS and MA-mediated effects in the shell and core sub-regions.

Materials and Methods

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 where stress procedures, MA injections or where 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 x 25 x 20 cm Plexiglas cages with corncobs as bedding. Temperature was kept constant at 22ºC, humidity at 55% and food and water was available *ad libitum* for the duration of the experiment.

Drugs

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

Maternal separation paradigm

Male and female rats were paired and their offspring used for experimental purposes. The day of birth was designated as postnatal day (PND) 0, where after maternal separation

commenced two days later on PND 2 until PND 14. Rat pups were separated from their mothers for a 3 hour daily period between 09h00 and 13h00. This protocol is in accordance with the deprivation procedures as employed by Ladd et al. (2000). The pups were moved to a new cage, while the mother remained in the home cage. The cage containing the pups were then moved to an isolated dedicated room where the pups were kept warm under infrared lights (30-33ºC), thereby preventing exposure to hypothermic conditions. Control litters were reared normally without separations. After maternal deprivation was completed, animals were subjected to normal housing conditions.

Experimental design

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

1) Control Saline (CS) group: animals not subjected to MS and receiving 4 saline injections.
2) MS Saline (MS) group: animals subjected to MS and subjected to 4 saline injections.
3) Control MA (CM) group: animals not subjected to MS and receiving 4 methamphetamine injections.
4) MS MA (MM) group: animals subjected to MS and subjected to methamphetamine injections.

Methamphetamine administrations occurred on PND 33 – 36. The rational for subjecting rats to methamphetamine during their adolescent period in life (PND 33 – 36), is that in humans, adolescents typically have an increased sensation seeking drive for drugs of abuse and hence presents with an important risk to susceptibility for taking drugs at this stage of life (Laviola et al., 1999; Spear, 2000). Animals were all 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 (Paxinos and Watson, 1986) and immediately frozen and stored in liquid nitrogen for later analysis.

Fractionation of striatal shell and core tissue

Nine shell and nine core accumbal tissue samples were pooled respectively for each experimental group 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 protein extracts. The most abundant protein changes often take place in the cytosolic region of cells, hence the rationale to focus and investigate the protein expression changes in this cellular fraction. The cytosolic protein extracts were subsequently used to determine the total protein concentration. This was achieved by using a Bradford method adhering to the specifications of the ReadyPrep 2-D Cleanup Kit (Bio-Rad). After the completion of the Cleanup kit, samples were re-suspended in ammonium bicarbonate. The samples were further subjected to a roto-evaporator (Eppendorf) to reduce the sample volume to form a tight pellet for further analysis.

Sample preparation and tryptic digestion

Each sample was re-suspended in 50µl 1% PPS (pyridinium propyl sulfonate) silent surfactant according to the PPS silent detergent protocol and the insoluble matter removed by centrifugation. Protein concentration was again determined, this time a nano-drop spectrophotomer was used. The samples were combined using equal amounts of protein from each to obtain the final group protein. The combined samples formed groups 1 to 8 respectively. Equal aliquots (100µg) were taken from each group and digested with trypsin according to a slightly modified PPS protocol with trypsin added in a 1:10 ratio. The resultant digestion was evaluated using both mass spectrometric and liquid chromatography data. Mass spectral analysis showed that digestion occurred and the liquid chromatogram indicated that the digest ran to completion.

Isobaric tag for relative and absolute quantitation (iTRAQ) labeling

Peptides from the 8 groups (50µg) were labeled using 8 – plex iTRAQ labeling. The iTRAQ labeling reaction was performed according to the ABI silent protocol substituting isopropanol for ethanol. 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 with MS and MS/MS. The data indicated that all 8 samples were modified with iTRAQ tags.

Peptide clean-up

The mixture of labeled peptides was separated by re-suspending the sample in strong cation exchange (SCX) equilibration buffer (5mM KH_2PO_4) (Sigma), 25% acetonitrile (ACN)

(ROMIL) and applied to a pre-equilibrated SCX SPE device (Supelco SupelClean). The peptides were eluted from the device with 300 µL elution buffer (1M HCO_2NH_4/25% acetonitrile (Sigma). Mass spectra indicated some peptide in the flow through and wash as expected and peptide was detected in the eluate from the SPE device. The sample volume was reduced to 25µL using a roto-evaporator (Eppendorf). The samples for MS analysis were desalted using ZipTip C18 SPE devices.

Liquid chromatography

Peptides were separated on a Dionex Ultimate 3000 nano-LC with a C18 Pepmap column (75µm x 15cm, LCPackings). 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. Peptide were eluted 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 200nL/min. The eluted peptides were spotted onto a MALDI source plate using a Probot (LCPackings) with continuous matrix addition at 600nL/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 12s 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 shows that internal calibration was obtained in 99.6% (1014/1018) 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 16kV. The spectra were processed using the PepMix4 internal calibration points. MS/MS spectra were recorded in positive mode with 1kV deceleration voltage and a total of 600 laser shots/spectrum with the laser set to 5000 arbitrary units.

Data Analysis

Mass spectral data was analyzed using Peak Explorer software and data analysis was performed using ProteinPilot. The parameters were set as follows: Analysis type, quantitation 8-plex iTRAQ with standard correction factors; Enzyme, trypsin; Cys modification, MMTS; Database, *Ratus ratus*; Search effort, thorough; Modifications, biological modifications; Quantitation, 113 relative (Shell cytosolic fraction of the control saline (CS) group) and 117 relative (Core cytosolic fraction of the control saline (CS) group).

Results

Using the *Ratus ratus* database, 126 proteins (95% confidence) were identified. The average mass deviation was calculated as -0.080 Da. The iTRAQ quantitation was performed using ProteinPilot with default settings and relative abundance expressed in terms of reporter signal 113 (Shell fraction) and 117 (Core fraction). Analysis of the peptide report showed that 1129 ions 48.36% (546/1129) could be auto quantified, 26.48% (299/1129) were auto quantified but shared sequence data with other proteins and 24.09% (277/1129) could be auto quantified with low confidence. In total 98.93% (1117/1129) of all ions could be quantified. Fragmentation data showed that 0.97% of the ions fragmented did not contain an iTRAQ label and the experimental groups / samples examined were tagged with isobaric iTRAQ tags ranging from 113 to 121 (Table 1). The MS/MS to sequence conversion ratio was 46.4% with 720 distinct peptides 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 quantitationally significantly different from control groups in both shell (Table 2) and nucleus accumbens core (Table 3) fractions, are reported.

We found 28 cytosolic shell and 25 core proteins which we quantitatively significantly expressed between the experimental groups investigated. The cytosolic shell proteins of animals subjected to MA mostly showed significant changes in proteins involved in cytoskeletal or structural functions. Similarly, cytosolic shell proteins of animals exposed to MS resulted in cytoskeletal modifications in addition to alterations in energy metabolism, protein fate and redox regulatory proteins. The combination of MS and later MA treatment resulted in quantitative protein changes in almost all protein functional groups (Table 2). The cytosolic core proteins of MA exposed rats resulted in mainly cytoskeletal and energy

metabolism protein changes. Animals subjected to MS early in life resulted in less protein changes in the nucleus accumbens core. However, similarly to protein changes in the shell sub-region, the major core quantitative protein changes occurred in the group subjected to both MS followed by MA treatment (Table 3).

Discussion

In the present study we found that processes affected by the two interventions were similar in the respective subregions. However, the combination of early life stress exposure and later methamphetamine treatment resulted in greater differential expression of proteins in both the shell and core sub-region when compared to individual stressors. To our knowledge this is the first study to quantify the differential expression of cytosolic nucleus accumbal shell and core proteins of animals subjected to MS followed by MA treatment. This study is of relevance since the shell and core sub-regions of the nucleus accumbens have been shown to respond to drugs of abuse and stress in disparate ways. The proteins which were quantitatively significantly expressed between the experimental groups investigated are functionally associated with cytoskeletal modifications, energy metabolism, intracellular signaling, protein degradation and cellular growth.

Cytoskeletal proteins like 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. Cytoskeletal alterations have been shown to occur after methamphetamine administration in various brain areas including the striatum, hippocampus, prefrontal cortex, cingulated 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). In the present study, a number of cytoskeletal proteins were differentially expressed following methamphetamine treatment which included actin cytoplasmic 2, tubulin alpha-1B chain and beta-2A chain and microtubule-associated protein 2. In both the shell and core the expression of these proteins were mostly decreased. This data shows that maternal separation and methamphetamine administration result in changes in cytoskeletal structure that may affect neurotransmission

and synapse function. These effects may subsequently contribute to methamphetamine-induced neuroplasticity in the brain.

In support, thymosin beta-4 concentrations were found to be increased in the accumbens core of animals exposed to both early separation and methamphetamine treatment. Thymosin beta-4 forms a 1:1 complex with actin and inhibits actin polymerization (Safer et al., 1990). 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 Carlier, 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 advocating adjustments in the expression of proteins related to plasticity, under conditions of stress and toxicity.

Clinical studies have reported cerebral glucose hypometabolism in human methamphetamine abusers (Kim et al., 2005). Repeated methamphetamine use has also been shown to result in 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 was found to cause striatal ATP reductions which paralleled methamphetamine-induced dopamine depletion (Chan et al., 1994). Similarly, 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 sub-regions. Some of these proteins included glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase isozyme M1/M2 and V-type proton ATPase subunit E1. The core was affected more so than the shell region in the methamphetamine treated group, while a greater number of proteins involved in metabolism were reduced in the shell following maternal separation in combination with later methamphetamine treatment. Overall, methamphetamine led to dysregulation of various proteins involved in energy metabolism and this effect seemed to be greater in animals with prior exposure to stress, particularly in the accumbens shell.

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. For example, in the shell sub-region, ProSAAS and 14-3-3 protein gamma respectively showed increased and decreased expression after MS and methamphetamine treatment, respectively. 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 function 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 14-3-3 protein gamma was significantly decreased after methamphetamine treatment in the present study. This protein is known to regulate signal transduction pathways which is involved in proliferation, differentiation and survival (Jin et al., 2004; Bridges and Moorehead, 2005; Aitken, 2006; Chen et al., 2006; Ajjappala et al., 2009). With regards to methamphetamine-induced proteins that give rise to addictive behavioral pathology, 14-3-3 protein gamma activates tyrosine and tryptophan hydrolases, protein kinase C (PKC) and Raf-1 in the mitogen activated protein kinase (MAPK) signal transduction pathway (Aitken et al., 1995). PKC has also been implicated to play a role in methamphetamine-induced behavioral sensitization in the nucleus accumbens (Narita et al., 2004). Similarly, Yang et al. (2008) found decreased 14-3-3 protein gamma expression levels in the hippocampus of methamphetamine-treated rats. However, in contrast to our findings, increased 14-3-3 protein gamma phosphoproteins of the nucleus accumbens was found in cocaine self-administering rhesus monkeys (Tannu et al., 2008).

Signal transduction and neurotransmission proteins in the accumbal core, myristoylated alanine-rich C-kinase substrate (MARCKS), Arpp-21 and alpha-synuclein were found to be quantitatively expressed. 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., 1996; Arbuzova et al., 1998; Ohmori et al., 2000; Arbuzova et al., 2002). It is proposed that MARCKS mediate 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). The increase in MARCKS after methamphetamine treatment in the present study may therefore play an important role in methamphetamine mediated signal transduction processes.

Arpp-21 is a neuronal phosphoprotein which occurs in high concentrations in the limbic striatum, including 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 possibly act as a third messenger in the intracellular cascade involving adenylate cyclase (AC). One of the first messengers activating AC includes dopamine binding to dopaminergic 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 likely to participate in the mediation of methamphetamine-induced intracellular signal transduction. Also, stimulation of D1 or D2 receptors in the shell and not the core has been associated with the reinstatement of cocaine-seeking behavior (Schmidt et al., 2006). The increased in Arpp-21 levels in the core as observed in the present study, demonstrated that signaling processes in the two sub-divisions of the nucleus accumbens remains unclear and that additional studies are needed to determine the exact role of shell and core drug-mediated proteins in addiction.

Methamphetamine neurotoxicity has been found to lead to the formation of inclusion bodies in nigral and striatal neurons (Lotharius and Brundin, 2002) in both the soma and terminal endings (Fornai et al., 2004a; Brenz Verca et al., 2003). 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). Methamphetamine causes an increase in α-synuclein in

both the soma and terminal endings of nigral (Fornai et al., 2004a) and striatal neurons (Brenz Verca et al., 2003). 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 might confer a compensatory mechanism to protect neurons against oxidative damage induced by methamphetamine (Li et al., 2008). This is in line with findings of the present study which found increased alpha-synuclein levels in the accumbens core after methamphetamine treatment in conjunction with early maternal separation.

A number of proteins involved in protein synthesis or neurotrophic functions were identified to be differentially expressed in the shell and core. Increased expressions of brain acid soluble protein 1 (BASP1) were seen after maternal separation in the shell and when exposed to further methamphetamine treatment in 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). A similar increase in myotrophin levels were observed after methamphetamine treatment in the core. This protein has been shown to regulate protein synthesis (Taoka et al., 1992; 1994; Fujigasaki et al., 1996), as well as controlling the expression of catecholaminergic enzymes. Overexpression of myotrophin results in increased tyrosine hydrolase, 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 of action by which methamphetamine elicits its reinforcing effects (Koob et al., 1998). In agreement with our finding, 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 might represent a compensatory mechanism to protect neurons against the damaging effects induced by methamphetamine and early life stress. On the other hand, decreased gamma-enolase, beta-synuclein and prosaposin which have all been shown to possess neurotrophic qualities (Hattori et al., 1994; Misasi et al., 2001; Hashimoto et al., 2001; 2004; Sorice et al., 2008), is mostly affected by the combination treatment of early maternal separation and later methamphetamine treatment. The reduction in the

concentrations of the latter proteins may therefore reflect continued detriment of maternal separation combined with methamphetamine treatment on the central nervous system and neuronal functionality.

Conclusions

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. Collectively, processes affected by the two interventions were mostly similar in the respective subregions. However, significant differences were observed when the individual functional proteins were identified between shell and core sub-regions. Another main finding was the involvement of a certain population of proteins in methamphetamine use and additional proteins are recruited when the same animals are also exposed to early life maternal separation. It would therefore be interesting to ascertain whether these additional proteins in fact render individuals more susceptible to later drug abuse. Interpretation of the data as generated in this study, remains challenging and further research is required if the intricacies of addictive behavior is to be completely understood. Subsequent studies will aim to confirm quantitative protein results obtained by using Western Blotting or ELISA methodology, as only a few samples were pooled to represent each experimental group.

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Table 1. iTRAQ isobaric tags attached to corresponding experimental groups examined.

iTRAQ sample group number	Experimental group	Nucleus Accumbens Cytosolic fraction	iTRAQ isobaric tag
1	Control Saline (CS)	Shell	113
2	Control MA (CM)	Shell	114
3	MS Saline (MS)	Shell	115
4	MS MA (MM)	Shell	116
5	Control Saline (CS)	Core	117
6	Control MA (CM)	Core	118
7	MS Saline (MS)	Core	119
8	MS MA (MM)	Core	121

Table 2. iTRAQ Ratios for nucleus accumbens shell cytosolic proteins in methamphetamine and maternally separated samples.

Accession nr	Protein Name	MS:CS	P val MS:CS	CM:CS	P val CM:CS	MM:CS	P val MM:CS
Cytoskeletal / Structural							
sp|P63259	Actin, cytoplasmic 2	0.409	**0.001**	0.536	**0.001**	0.315	**0.001**
sp|Q6P9V9	Tubulin alpha-1B chain	0.262	**0.001**	0.483	**0.001**	0.213	**0.001**
sp|P19332	Microtubule-associated protein tau	1.269	**0.052 ***	1.000	1.000	1.219	0.343
sp|P85108	Tubulin beta-2A chain	0.406	**0.011**	0.544	**0.002**	0.300	**0.002**
sp|Q62658	Peptidyl-prolyl cis-trans isomerase FKBP1A	1.417	**0.001 ***	0.914	0.496	1.195	0.125
sp|P15146	Microtubule-associated protein 2	0.565	**0.007**	0.557	**0.001**	0.450	**0.001**
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**	0.772	**0.001**	0.663	**0.001**
sp|P04797	Glyceraldehyde-3-phosphate dehydrogenase	0.450	**0.001**	0.645	0.058	0.395	**0.001**
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**	0.753	0.264	0.417	**0.001**
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**	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**	0.829	0.182	0.805	**0.003**
sp|P62898	Cytochrome c, somatic	1.516	**0.039 ***	1.310	0.440	1.673	**0.008 ***
sp|P35704	Peroxiredoxin-2	0.733	**0.014**	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 ***
sp|P07323	Gamma-enolase	0.500	**0.001**	0.607	**0.007**	0.447	**0.002**
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**	1.355	**0.031 ***	0.829	**0.029**
sp|P63055	Purkinje cell protein 4	1.370	**0.001 ***	1.073	0.574	1.341	**0.007 ***
sp|P63018	Heat shock cognate 71 kDa protein	0.377	**0.013**	0.729	**0.004**	0.400	**0.002**
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**

BOLD ratios *, significantly increased compared to CS ($p<0.05$); BOLD ratios, significantly decreased compared to CS ($p<0.05$)

Table 3. iTRAQ Ratios for nucleus accumbens core cytosolic proteins in methamphetamine and maternally separated samples.

Accession nr	Protein Name	MS:CS	P val MS:CS	CM:CS	P val CM:CS	MM:CS	P val MM:CS
Cytoskeletal / Structural							
sp|P63259	Actin, cytoplasmic 2	0.835	**0.001**	0.672	0.001	0.803	**0.001**
sp|Q6P9V9	Tubulin alpha-1B chain	0.773	**0.008**	0.623	**0.003**	0.754	**0.016**
sp|P19332	Microtubule-associated protein tau	1.082	0.417	1.171	**0.016 ***	1.067	0.479
sp|P85108	Tubulin beta-2A chain	0.800	0.059	0.662	**0.001**	0.796	**0.002**
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**
Energy Metabolism							
sp|P07335	Creatine kinase B-type	0.980	0.588	0.859	**0.001**	0.870	**0.001**
sp|P04797	Glyceraldehyde-3-phosphate dehydrogenase	0.891	0.318	0.716	**0.010**	0.832	**0.012**
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 ***	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 ***	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 ***	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 ***
sp|P07323	Gamma-enolase	0.908	0.089	0.758	**0.003**	0.851	**0.050**
tr|Q6P7A4	Prosaposin	0.995	0.987	1.289	0.414	0.813	**0.054**
sp|P62775	Myotrophin	1.014	0.799	1.114	**0.006 ***	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 ***	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 ***	0.959	0.638	1.023	0.731

BOLD ratios *, significantly increased compared to CS ($p<0.05$); BOLD ratios, significantly decreased compared to CS ($p<0.05$)

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CHAPTER 8

General Conclusions

The main aim of the study was to establish whether early life adversity would render individuals more vulnerable to later drug usage. We adopted maternal separation as our animal model of early life adversity and treated these animals with methamphetamine during adolescence. A conditioned place preference (CPP) paradigm was subsequently used to determine the rewarding effects of methamphetamine. To obtain an understanding of the underlying molecular mechanisms of methamphetamine-induced behaviour, we also measured neurochemical changes on a neuroendocrine, neurotrophic, neurotransmitter and proteome level.

Statistical tests performed on CPP behaviour, locomotor activity and the neurochemical measures were all analysed by non-parametric statistical tests, since the number of animals per group (n) in each study investigated were small and a small biological sample size usually does not conform to a symmetrical / normal distribution of the date points. The median and quartiles are usually reported when making use of non-parametrical tests, since data is not normally distributed. However, for comparative purposes with previous studies in the literature, the mean and SEM were reported throughout the present study.

On a behavioural level, we found contrary to expectation, that maternal separation did not affect rewarding behaviour as measured by the CPP test, but instead decreased apomorphine-induced locomotor behaviour in methamphetamine-treated rats. This result suggested that maternal separation preferentially affected the behavioural repertoire of the dorsal striatum rather than that of the ventral striatum. Maternal separation therefore exhibited a differential impact on the central dopaminergic system. This conclusion was further substantiated by increased concentrations of plasma prolactin levels in maternally separated animals subjected to methamphetamine treatment, suggesting a reduction in dopamine inhibition.

Further characterization of the methamphetamine-induced CPP model indicated that place preference behaviour lasted for at least 2 weeks after the last methamphetamine administration. Neuroendocrine abnormalities of the HPA axis were evaluated by measuring plasma ACTH and corticosterone levels. A general down regulation of neuroendocrine

activity was observed in animals subjected to maternal separation or methamphetamine treatment, as well as those subjected to the combination of the two interventions. This result was in line with previous findings of hypoactivity of the HPA axis (Yehuda et al., 1995; Buydens-Branchey et al., 1997; Faure et al., 2006; Heim et al., 2001). However the combination group did not display an exaggerated effect, suggesting that the interventions may have independent mechanisms of action with respect to the regulation of the HPA axis.

Altered neurotrophin concentrations in the dorsal and ventral hippocampus were found in maternally separated and methamphetamine treated animals independently. Maternal separation resulted in increased NGF levels in the ventral hippocampus of methamphetamine treated rats. This observation suggested that the ventral hippocampus may specifically be vulnerable to the effects of early life stress, which is in agreement with the preferential role the ventral hippocampus plays in anxiety-related responses (Bannerman et al., 2004). The increased neurotrophin protein concentrations may further reflect a compensatory response of the brain to stress and drug exposure.

Two pharmacological interventions (Lobeline and Naltrexone) were used to probe the contributions of the cholinergic and opioid systems in place preference behaviour. We employed a post-methamphetamine treatment strategy which was unlike many previous studies that rather investigated the effects of drug treatment on methamphetamine consumption using a pre-drug treatment strategy. The clinical relevancy of these earlier experiments can therefore be questioned. Lobeline and naltrexone post-methamphetamine treatment failed to reverse the methamphetamine-induced place preference on a behavioural level. However, on a biochemical level administration of the drugs did lead to alterations in striatal dopamine and serotonin levels. Surprisingly, in this particularly study, early maternal separation resulted in a reduction in methamphetamine-induced place preference behaviour. In chapter 3, this particular finding of decreased CPP behaviour was not significantly different from animals exposed to MA without previous maternal separation. This could possibly be ascribed to differential MA exposure paradigms used. Since maternal separation altered neurotransmitter levels, these changes may partially indicate neurochemical mechanisms of resiliency that early adversity confer on the brain against the effects of methamphetamine. Our findings therefore underline the fact that early life stress in combination with later drug usage is a complex interplay between environment and neurochemical changes that is in need of further investigation.

We employed proteomic techniques to generate a global overview of protein changes in the frontal cortex of methamphetamine-treated animals, in an attempt to obtain a better understanding of how this brain area may contribute to addictive behaviour. Using 2-DE based proteomics we investigated the protein changes in both the cytosolic and membrane fractions of methamphetamine-treated rats. We identified 47 cytosolic and 42 membrane proteins using ESI-Quad-TOF. Examples of the identified proteins included UCH-L1, β-synuclein, GRP 78, γ-enolase, DRP 2, complexin 2 and synapsin II. These proteins are associated with protein degradation, redox regulation, energy metabolism, cellular growth, cytoskeletal modification and synaptic function. In this study we demonstrated how methamphetamine affected neurons at numerous levels. However further research is needed to determine how these alterations in protein expression translate into the mediation of methamphetamine's effect on the brain and behaviour.

Finally, we conducted a quantitational proteomic study investigating effects of methamphetamine on the nucleus accumbens shell and core subregions. To our knowledge, this is the first study using proteomics to quantify the differential expression of proteins in the shell and core after methamphetamine treatment and determining the effects of maternal separation on methamphetamine-induced protein expression. Using isobaric tagging for relative and absolute quantitation (iTRAQ) followed by LC MS/MS we found 28 cytosolic shell and 25 core proteins to be differentially expressed. Some of these proteins included actin cytosolic 2, thymosin beta-4, creatine kinase B-type, proSAAS, 14-3-3 protein gamma, MARCKS, Arpp-21, α-synuclein, BASP1 and myotrophin. These proteins are associated with cytoskeletal modifications, altered energy metabolism, degenerative processes, interruptions with normal neurotransmission and intracellular signaling. Although these processes were similarly affected in the shell and core regions of the nucleus accumbens, we were able to demonstrate a remarkable difference in the specific proteins in these two brain areas. The present study also showed that on a proteome level, more proteins are quantitatively expressed in rats that were exposed to maternal separation followed by methamphetamine treatment. We therefore proposed that early adverse events may predispose animals to the addictive effects of stimulant drugs. However, future studies are needed to establish how differentially expressed proteins in the nucleus accumbens shell and core impact on the function of other brain areas to mediate the particular effects of early life stress and subsequent methamphetamine abuse.

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