

Inhibitory Regulation of the Prefrontal Cortex in an Animal Model of Methamphetamine Behavioural Sensitization: Implications for the Maintenance of Chronic Psychoses

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Statement of Authentication and Ethical Accordance

This thesis is submitted to Macquarie University in fulfilment of the requirements for the degree of Combined Doctor of Philosophy with the Master of Clinical Neuropsychology.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

All animal research carried out in this thesis was approved by the Macquarie University Animal Ethics Committee (2010/045, 2012/047) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th Edition (National Health and Medical Research Council, 2013).

This thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

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We, the undersigned, acknowledge that this work represents that of Travis Ashley Wearne, and where appropriate, accurate information regarding co-author contribution is supplied.

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List of Publications

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- Sauer, M., Mirzaei, M., **Wearne, T.**, Haynes, P.A., Goodchild, A.K. and Cornish, J.L. (2012, September). Biological changes in the hippocampus following chronic methamphetamine use: a proteomic approach. *Australian Neuroscience Society Meeting*, Melbourne, Australia
- Wearne, T.A.**, Mirzaei, M., Goodchild, A.K., Haynes, P.A. and Cornish, J.L. (2013, February). A proteomic analysis of the prefrontal cortex in an animal model of methamphetamine-induced behavioural sensitization. *Australian Neuroscience Society Meeting*, Melbourne, Australia.
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- Wearne, T.A.**, Mirzaei, M., Goodchild, A.K., Haynes, P.A. and Cornish, J.L. (2012, November). A proteomic analysis of the prefrontal cortex in an animal model of methamphetamine-induced behavioural sensitization. *Proteomic Symposium-Proteomic and Beyond*, Macquarie University, NSW, Australia.
- Franklin, J.F., Sauer, M., **Wearne, T.**, Clemens, K.J., Homewood, J., Haynes, P.A. and Cornish, J.L. (2012, September). Extended exposure to caffeine and sucrose has differential effects on locomotor response and the proteome of adult and adolescent sprague dawley rats. *ComBio 2012*, Adelaide South Australia.
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Table 3. Bivariate relationships between mRNA expression for upregulated GABAergic genes and interneuronal markers in the OFC of METH sensitized rats

Chapter Six

Table 1. Changes to GABAergic network in the PFC across the PFC and METH sensitization: enzymes, transporters, synaptic GABA_A receptors, extrasynaptic GABA_A receptors, metabotropic GABA_B receptors, calcium binding proteins and neuropeptides.

Abbreviations

µg	microgram
µL	microlitre
µm	micrometre
5-HT	5-hydroxytryptamine, serotonin
ACN	acetonitrile
ADHD	Attention Deficit Hyperactivity Disorder
AGAL	Australian Government Analytical Laboratories
AIHW	Australian Institute of Health and Welfare
ARA	animal research authority
ATP	adenosine triphosphate
BACS	Brief Assessment of Cognition in Schizophrenia
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
cm	centimetre
CNS	central nervous system
COMT	catechol-o-methyltransferase
Ct	cycle threshold
°C	degree centigrade
D1	dopamine D1 receptor
D2	dopamine D2 receptor
DA	dopamine
DAOA	d-amino acid oxidase activator
DISC	disrupted in schizophrenia
DLPFC	dorsolateral prefrontal cortex
DSM-V	Diagnostic and Statistical Manual – 4 th Edition
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FDR	false discovery rate
g	gram
GABA	gamma aminobutyric acid
GABA _A	gamma aminobutyric acid receptor A
GABA _B	gamma aminobutyric acid receptor B
GABA _T	gamma aminobutyric acid transaminase
GAD	glutamate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAT	gamma aminobutyric acid transporter
GPM	global proteome machine
h	hour
H ₂ O	water
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HSP	heat shock protein
Hz	hertz
IPA	ingenuity pathway analysis

IPKB	ingenuity pathways knowledge base
i.p.	intraperitoneal
IPSC	inhibitory postsynaptic current
kDa	kilo Dalton
kg	kilogram
L	litre
LC-MS	liquid chromatography with mass spectrometry
LDT	laterodorsal tegmentum
LTD	long-term depression
m	metre
METH	methamphetamine, methyl-alpha-methylphenethylamine
mg	milligram
min	minute
MAO-A	monoamine oxidase-A
mPFC	medial prefrontal cortex
mRNA	messenger RNA
NA	noradrenaline
NAc	nucleus accumbens
NADH	nicotinamide adenine dinucleotide
NH ₄ HCO ₃	ammonium bicarbonate
NSAF	normalized spectral abundance factor
OCD	obsessive compulsive disorder
OFC	orbitofrontal cortex
P2P	Phenyl-2-propane
PAGE	polyacrylamide gel electrophoresis
PANNS	Positive and Negative Syndrome Scale
PBS	phosphate buffered saline
PBT	phosphate buffered saline with 0.1% Tween 20
PCR	polymerase chain reaction
PFC	prefrontal cortex
PPP	phosphoprotein phosphatase
PRL	prelimbic cortex
qPCR	quantitative polymerase chain reaction
SAMHSA	Substance Abuse and Mental Health Services Admin
SDS	sodium dodecyl sulfate
sec	second
SEM	standard error of the mean
SD	Sprague-Dawley rat
s.d.	standard deviation
SDS	sodium dodecyl sulfate
SNP	single-nucleotide polymorphism
TCA	tricarboxylic acid
TH	tyrosine hydroxylase
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
VGAT	vesicular GABA transporter
VMAT2	vesicular monoamine transporter 2
vs	versus
VTA	ventral tegmental area
WWII	World War II

Abstract

Methamphetamine is a potent psychostimulant that can induce psychosis among recreational and chronic users, with some users developing a persistent psychotic syndrome that is indistinguishable to schizophrenia with respect to positive, negative, and cognitive symptomatology. Given that methamphetamine psychosis and schizophrenia are characterised by a persistent vulnerability to psychotic relapse, previous studies have placed these similar symptoms in the context of behavioural sensitization, a phenomenon whereby repeat exposure to a stimulus results in a progressively increased behavioural response to that stimulus following a period of abstinence. As such, examination of the neurobiological changes that mediate sensitization to methamphetamine could enable further understanding of the mechanisms responsible for different aspects of psychotic disease states.

Inhibitory γ -aminobutyric acid (GABA)-mediated neurotransmission plays an important role in regulating the prefrontal cortex (PFC), with dysfunctional inhibitory control of the PFC believed to underlie certain symptoms reported across psychotic disorders. While GABAergic dysfunction in schizophrenia has been well described, however, research surrounding PFC GABA-mediated neurotransmission following methamphetamine sensitization has received considerably less attention. The current thesis therefore contributes to this existing body of empirical work by providing a multi-method evaluation of the proteomic, genomic and cellular changes associated with the GABAergic system across global and localised regions of the PFC following sensitization to methamphetamine.

The aim of Chapter 2 was to investigate changes to protein expression in the PFC following behavioural sensitization to methamphetamine using label free proteomics with mass spectrometry. Proteomic analysis revealed 96 proteins that were differentially expressed in the PFC of methamphetamine rats, with 20% of these proteins previously implicated in the

neurobiology of schizophrenia. Specifically, methamphetamine sensitization downregulated the expression of GAD₆₇, parvalbumin and neuroligin2, and upregulated the expression of gephyrin, key proteins in the regulation of inhibitory neurotransmission, placing such biological changes as potential mediators in the maintenance of vulnerability to psychosis.

In light of the results from Chapter 2, the aim of Chapter 3 was to determine whether methamphetamine sensitization altered GABAergic mRNA expression of the PFC. Using quantitative polymerase chain reaction (qPCR), the mRNA expression of GABA transporters (GAT₁ and GAT₃), GABA_A receptor subunits (α 3 and β 1), together with the GABA_B1 receptor, were upregulated in the PFC of sensitized rats compared with saline controls. These findings indicate that GABAergic mRNA expression is significantly altered at the pre and postsynaptic level following sensitization to methamphetamine in the PFC, which could have significant consequences on GABA-mediated neurotransmission.

Given that the PFC is a heterogeneous structure, with anatomically and functionally distinct subregions, the aim of chapter 4 was to extend the results of Chapter 3 and examine GABAergic mRNA expression across subregions of the PFC following methamphetamine sensitization, specifically the prelimbic (PRL) and orbitofrontal (OFC) cortices. GAD₆₇, GAD₆₅, GAT₁, GAT₃, VGAT, GABA_T, GABA_A β 2, GABA_B1 mRNA expressions were upregulated in the PRL while ionotropic GABA_A receptor subunits α 1, α 3, α 5 and β 2 together with GABA_B2 were specifically upregulated in the OFC. These findings suggest that alterations to GABAergic mRNA expression following sensitization to methamphetamine are biologically dissociated between the OFC and the PRL, with GABAergic gene expression differentially expressed in a brain-region and GABA-specific manner.

Finally, in Chapter 5, the effect of methamphetamine sensitization on GABAergic interneuronal cell types across the PRL and OFC, as examined through calcium binding proteins and neuropeptides, was assessed through the use of qPCR. Results indicated that

calbindin, calretinin, somatostatin, cholecystokinin, and vasoactive intestinal peptide mRNA expressions were upregulated in the PRL while parvalbumin, somatostatin, cholecystokinin and vasoactive intestinal peptide mRNA expressions were specifically upregulated in the OFC. These findings suggest that the OFC and PRL are associated with distinct inhibitory cellular profiles following sensitization to methamphetamine, which could have significant consequences on inhibitory neurotransmission and neuronal synchronisation. In order to determine whether the previously identified GABAergic changes co-localized within specific cell types, the last analysis investigated whether GABAergic mRNA expression correlated with interneuronal mRNA expression. Altered GABA neurotransmission within parvalbumin-containing neurons was indicated by the correlation of parvalbumin mRNA expression with the expression of GAD₆₇ and GAT₁. The mRNA expressions of GAD₆₇, GAD₆₅ and GAT₁ also correlated with the expression of cholecystokinin. While the expression of GABA_Aα1 and GABA_Aα5 correlated with the expression of somatostatin in the OFC, no other correlations between GABA_A receptor mRNA and interneuronal markers were identified in the OFC. Therefore, altered GABA neurotransmission mediated by ionotropic and metabotropic receptors in the OFC may be localized to glutamatergic pyramidal cells.

Overall, the results of this thesis indicate that GABAergic neurotransmission plays an adaptive role in the PFC following sensitization to methamphetamine, with multiple inhibitory proteins, genes and cellular markers altered following sensitization to methamphetamine. However, a number of unexpected findings were revealed with respect to the GABAergic changes typically observed in schizophrenia. As such, these findings provide molecular evidence that schizophrenia and methamphetamine psychosis, at least with respect to the GABAergic system using the METH sensitization paradigm, may be associated with distinct inhibitory neuropathologies in the PFC. The functional, methodological and clinical implications of these results are also discussed throughout the thesis.

Author's Note

Each chapter has either been submitted to or has been prepared for a specific journal and these journals have different formatting requirements and spelling rules. Therefore, for this thesis, and in the interests of consistency, the same formatting and referencing style has been used throughout - for all chapters a USA dictionary has been used and APA 6th referencing style has been adopted. However, in light of the specific requirement for submission to proteomic journals, Chapter Two presents the results and discussion as one continuous section.

Chapter One

General Introduction

1.1 Methamphetamine

1.1.1 Historical Background

Amphetamines refer to a class of chemically related compounds that have been used extensively over the last century in both recreational and medicinal settings, with various amphetamine analogues used in the treatment of asthma, narcolepsy, attention deficit hyperactivity disorder (ADHD) and obesity (Anglin, Burke, Perrochet, Stamper, & Dawud-Noursi, 2000; Hart, Marvin, Silver, & Smith, 2012). Methamphetamine (METH; N-methyl-alpha-methylphenethylamine) is a highly potent and addictive amphetamine derivative that is frequently abused worldwide and has significant effects on physical, behavioral, cognitive and psychiatric output (Meredith, Jaffe, Ang-Lee, & Saxon, 2005). It was first derived from ephedrine in 1893 by Japanese chemist Nagai Nagayoshi and later synthesized to METH hydrochloride through ephedrine reduction by pharmacologist Akira Ogata in 1919 (Sulzer, Sonders, Poulsen, & Galli, 2005). METH, however, did not become widely used until it was distributed to German, American and Japanese soldiers during World War II (WWII) as a way of maintaining alertness and wakefulness (Roehr, 2005; Ujike & Sato, 2004). While the distribution of METH to American soldiers continued throughout the Vietnam, Korean and Iraq wars in various forms (Roehr, 2005), the extensive use of METH by Japanese soldiers during WWII generalized to the Japanese population, with the prevalence of METH use in Japan reaching epidemic status by the end of WWII (Ujike & Sato, 2004). Similarly, medications containing amphetamine were readily available without prescription until the late 1950s in the United States of America (USA), with 31 million prescriptions for amphetamine-containing medications recorded in 1967 (Anglin et al. 2000), suggesting that psychostimulants were being regularly used in the western world.

Illegal production of METH began in the 1960s by underground laboratories in the USA, with recreational use progressively and rapidly increasing throughout the 1980s and

1990s (Anglin et al., 2000). While METH was listed as a scheduled and controlled substance in the USA from the 1970s, increased use of METH throughout the latter part of 20th Century witnessed many media outlets describing its use as an “epidemic”, particularly in USA and Japan. Consequently, given the clear onset of abuse on a global scale, together with the significant negative side effects of addiction, overdose and psychiatric disturbances, governmental bodies enacted significant legal measures to regulate the manufacture, possession and distribution of METH from the middle of the 1990s. However, despite these efforts, METH continues to be produced by illegal laboratories and transported throughout the world where its use continues to increase.

1.1.2 The Epidemiology of Methamphetamine Use

Recent epidemiological studies place amphetamine-type stimulants as the most widely used and illicit drug in the world after cannabis (UNODC, 2009, 2011), with an estimated 16 to 51 million users globally between 15 and 64 years old (McKetin, Lubman, Baker, Dawe, & Ali, 2013; UNODC, 2013a, 2014b). Worldwide statistics on METH use describe it as a global phenomenon, with METH consumption reportedly independent of wealth, geographical location and culture (UNODC, 2003). Recent reports suggest an increased production of METH around the world and an increasing popularity of METH over the last 5–15 years, which has been linked to increased synthetic production in clandestine laboratories and augmented importation of METH from Mexico and Asia (UNODC, 2013a, 2014a). Indeed, worldwide seizures relating to METH have been greater than any other drug category (UNODC, 2014b), while admissions to publically funded substance abuse treatment programs for METH use have increased 255% from 1997 to 2007 in the USA (SAMHSA, 2006, 2008).

Australia has one of the highest rates of METH use in the world. According to the National Drug Strategy Household Survey report (AIHW, 2010, 2013), 7.0% of Australians

aged 14 years and over have used METH once or more in their lifetime. Additionally, the incidence of METH use in Australia in 2014 was 2.1%, or 400,000 (AIHW, 2013), which is particularly high given the incidence of METH use in the USA and United Kingdom is 0.5% and 1.0%, respectively (UNODC, 2009) with the worldwide average between 0.3% and 1.3% (UNODC, 2014). In Australia, males typically use METH more than their female counterparts, with 2.5% of males reporting METH use over the previous 12 months compared to 1.7% of females (AIHW, 2013). Furthermore, 8.2% of men surveyed had used METH in their lifetime compared 5.9% of females (AIHW, 2013), suggesting gender differences in the epidemiology of METH use in Australia.

While most users generally take METH infrequently (McKetin, McLaren, Riddell, & Robins, 2006), with 49.0% of recent users taking METH once or twice a year (NDSHSR, 2010), METH use presents a significant problem with respect to its high potential for addiction and subsequent burdens on the health care system and law-enforcement agencies. Specifically, between 73,000 and 97,000 people in the general Australian population are estimated to be dependent on METH (McKetin et al., 2014; McKetin, McLaren, Kelly, Hall, & Hickman, 2005), almost double the amount estimated to be regular users of heroin (45,000) (Degenhardt et al., 2008). This, together with the high incidence of METH use in Australia relative to other countries, has resulted in a significant increase in the regulation and law enforcement control of amphetamine-related substances over recent years, including a national government initiative to enforce a reduction in the importation, manufacture, sale and use of METH ("National Ice Taskforce," 2015). Indeed, there has been a steady increase in border seizures of amphetamine-type stimulants since 2001, with police reporting a 310.0% increase in the weight of METH-related border seizures throughout 2013. While the media consequently referred to the METH climate in Australia as a "pandemic", this may reflect improved capacity to identify illicit drug channels and easier detection of METH substances

at the border, as the prevalence and incidence of METH has remained relatively consistent over the past 5 to 10 years, particularly between 2010 and 2013 (AIHW, 2010, 2013). Nevertheless, while it is clear that legislation and law-enforcing bodies have attempted to cease the prevalence of METH use, it continues to be a serious drug of abuse on both domestic and international platforms.

1.1.3 Chemical Definition & Production of Methamphetamine

As a member of the amphetamine family (d-amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine and 3,4-methylenedioxymethamphetamine), METH is a cationic molecule and chiral compound based around a phenylethylamine core (Zorick, Rad, Rim, & Tsuang, 2008), although it is distinguishable from its amphetamine analogues by an additional methyl group (Figure 1). This methyl addition reportedly makes METH highly lipophilic, allowing it to increasingly penetrate the blood-brain barrier (Homer et al., 2008). Some researchers have suggested that the increased capacity to enter the central nervous system (CNS) mediates an elevated potency and addictive potential of METH over amphetamine (Rose & Grant, 2008). While some researchers have reported differences in behavior between METH and amphetamine (Hall, Stanis, Marquez Avila, & Gulley, 2008), other researchers have reported no difference in the physiological and behavioral effects between amphetamine and METH administration (Hart et al., 2012; Sevak, Stoops, Hays, & Rush, 2009). This suggests that the view that METH is pharmacologically superior to amphetamine may be due to limited research that explicitly compares the two compounds to each other, or biased due to METH's higher prevalence and use compared to amphetamine (Hart et al. 2012). Indeed, the lifetime incidence of METH use is 5.3% whereas it is 1.4% for amphetamine (Colliver et al., 2006; Hall et al., 2008).

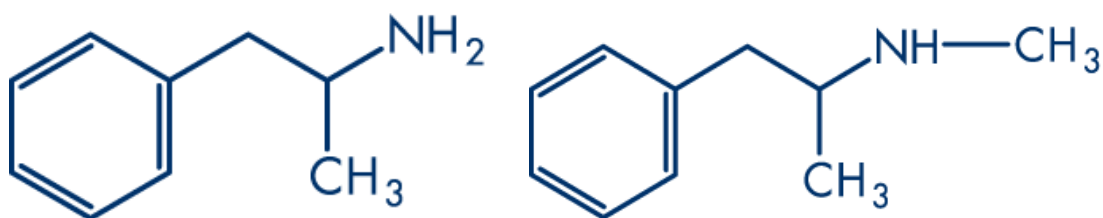


Figure 1. Chemical structure of amphetamine compared to methamphetamine (right).

The high prevalence of illicit METH use may be due to its ease and cost-effective synthesis in clandestine laboratories secondary to the high availability of primary ingredients. While METH is traditionally manufactured through the use of phenyl-2-propane (P2P)(Armstrong & Noguchi, 2004), a restricted and heavily regulated chemical since 1988 in the USA, METH can also be derived from ephedrine or pseudoephedrine - nasal decongestants commonly found in cold and flu medications. As such, the increased access to the requisite precursors for its production through over-the-counter and inexpensive ingredients has led to an increased prevalence of not only METH on the illegal drug market, but also high purity forms, as pseudoephedrine reduction results in both higher quality product and allows for greater amount of production from one cycle (Meredith et al., 2005). Furthermore, while the supply of other drugs of abuse, such as cocaine and heroin, are geographically restricted due to plant cultivation in specific terrains and climates, METH can be produced locally from common compounds, highlighting the ease of its global distribution. Greater restrictions, however, on precursor chemicals in westernized countries has ultimately resulted in a shift in the production of METH around the world, as there now appears to be an increase in the production and METH seizures in Mexico and areas of political unrest, such as Asia and West Africa, where profits may help fund criminal groups (McKetin, Sutherland, Bright, & Norberg, 2011).

1.1.4 Forms of Methamphetamine and Routes of Administration

METH is available in various forms and at different levels of chemical purity, with the addictive potential varying considerably depending on the form and route in which it is ingested. Speed ('goey', 'wizz'), the lowest purity of METH (approximately 10.0%), is a bitter tasting and odor-less powder that is usually administered nasally, orally, or as a suppository. Base, with a purity of approximately 20.0%, is a liquid or paste that is typically brown in appearance with an oily or waxy texture. It can be administered either orally, nasally, intravenously or as a suppository. METH in the form of a crystal ('ice', 'glass', 'crystal', 'crystal meth', 'tina', 'crank', 'shabu', 'shard', 'p') can reach levels of 80.0% purity or higher and typically appears as a white/translucent crystal of varying sizes. Crystallized METH is usually snorted, smoked or injected intravenously, thus providing the greatest potential for abuse (McKetin, Kelly, & McLaren, 2006).

The route of administration significantly impacts the effects of METH by differentially affecting bioavailability and degradation of the drug, which ultimately influences how long the drug lasts in the system. When injected, snorted or inhaled, the bioavailability is high and results in bioavailability as high as 79.0% to 100.0% (Cook et al., 1993; Harris et al., 2003), as METH has direct access to the circulatory system and therefore avoids first pass metabolism by the liver. Such administration results in the rapid onset of symptoms, typically within 10-20 minutes of METH administration (Cook et al., 1993; Cruickshank & Dyer, 2009). Oral administration, on the other hand, has lower bioavailability due to the first pass metabolism of the drug product, thereby increasing the latency of initial subjective experiences to 60-90 minutes (Hart, Ward, Haney, Foltin, & Fischman, 2001; Rose & Grant, 2008). The effects of METH typically last between 8-12 hours following administration (Harris et al., 2003; Meredith et al., 2005), consistent with METH's 12-hour half-life (Cruickshank & Dyer, 2009), before being degraded by the liver to amphetamine, 4-

hydroxymethamphetamine and norephedrine (Oyler, Cone, Joseph, Moolchan, & Huestis, 2002; Paul et al., 1994).

Given that the negative consequences of METH are associated with more potent forms of the drug and with hazardous routes of administration (i.e. injection), the increased availability of crystallized METH on the illegal drug market had resulted in a significant increase in its popularity amongst the dependent and intravenous drug-taking populations (Topp, Degenhardt, Kaye, & Darke, 2002). Indeed, 50.0% and 22.0% of METH users reported that powdered and crystallized METH was their drug of choice in 2010 (AIHW, 2010), respectively. However, there was a significant shift in the preference of amphetamine-type stimulants in 2013, with almost 50.0% of METH users then reporting that crystallized METH was their drug of choice while the preference for powdered METH decreased to 29.0% (AIHW, 2013). These trends are particularly salient given the potential for addiction, overdose, health and psychiatric disturbances with more potent forms of METH.

1.1.5 Pharmacology and Neurochemistry of Methamphetamine

1.1.5.1 Methamphetamine Pharmacology

Once absorbed into the blood stream, METH enters the CNS via the blood brain barrier to cause a cascade of changes to dopaminergic, serotonergic and noradrenergic systems through the stimulated release of monoamines and the inhibition of reuptake (Chuang, Karoum, & Jed Wyatt, 1982; Freye, 2010; Izawa, Yamanashi, Asakura, Misu, & Goshima, 2006; Weisheit, 2013). The acute inhibition of reuptake is achieved by METH binding to and blocking monoamine transporters to prevent the endocytosis of monoamines from the synapse to the presynaptic terminal (Meredith et al., 2005; Pierce & Kalivas, 1997). METH also reverses monoamine reuptake transporters through ‘reverse transportation’, which displaces monoamines from the cytosol into the synapse (Elliott & Beveridge, 2005; Goodwin

et al., 2009). These alterations induce a conformational change to reuptake transporters so that METH can be translocated to the presynaptic terminal, which further facilitates the release of noradrenaline (NA), serotonin (5-HT) and DA into the synapse via diffusion across the membrane (Rothman et al., 2001). Once in the cell, METH instigates secondary effects and disrupts the activity of vesicular monoamine transporter 2 (VMAT2) - which is responsible for the packaging of monoamines in vesicular stores - to redistribute stored intravesicular monoamines into the cytoplasm (Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007; Wimalasena, 2011). METH further inhibits metabolic enzymes (e.g. monoamine oxidase-A (MAO-A)) to limit the degradation of monoamines in the presynaptic terminal and thereby facilitate a greater pool of monoamines available for release (Mantle, Tipton, & Garrett, 1976; Osamu, Hideki, Minoru, Masakazu, & Yoshinao, 1980). The combined effect of stimulated release, reuptake inhibition, inactivation of VMAT and reduced efficacy of monoamine metabolic enzymes results in the transient accumulation of DA, 5-HT and NA in the synaptic cleft, which promotes post-synaptic receptor activation and efferent neuron excitability within monoamine terminal areas. Although all three monoamine systems are involved, the addictive and reinforcing properties of METH have typically been associated with dopaminergic neurotransmission, particularly in the mesocorticolimbic pathway (Elliott & Beveridge, 2005; Pierce & Kalivas, 1997; Pierce & Kumaresan, 2006; Torres, Gainetdinov, & Caron, 2003).

1.1.5.2 Pharmacology of Chronic Methamphetamine Administration

Findings from preclinical research studies suggest that chronic administration of METH results in long-term neurochemical changes to monoaminergic systems. These changes are consistent with the acute pharmacological effects of METH and include depletion of DA and 5-HT stores mediated primarily by the reduced activity and expression of the rate-limiting enzymes requisite for DA, NA and 5-HT synthesis, tyrosine and tryptophan

hydroxylase, respectively (Bakhit & Gibb, 1981; Bakhit, Morgan, Peat, & Gibb, 1981; Hotchkiss & Gibb, 1980; Peat, Warren, & Gibb, 1983; Schmidt, Ritter, Sonsalla, Hanson, & Gibb, 1985). Chronic METH use is also associated with reduced metabolites for DA and 5-HT (Bakhit et al., 1981; Clemens et al., 2004), a chronic reduction in the activity of DA and 5-HT reuptake transporters (Haughey, Fleckenstein, Metzger, & Hanson, 2000; Kokoshka, Vaughan, Hanson, & Fleckenstein, 1998), and reduced expression of vesicular monoamine transporters (VMAT2) (Frey, Kilbourn, & Robinson, 1997). Interestingly, NA appears to only be degraded following repeated METH administration at very high doses (Friedman, Castaneda, & Hodge, 1998; Kita, Wagner, & Nakashima, 2003), while NA depletion in the prefrontal cortex (PFC) has been shown to be specifically evident following repeated intermittent administration and not following binge administration (Clemens, Cornish, Hunt, & McGregor, 2007; Clemens, Cornish, Li, Hunt, & McGregor, 2005). Overall, these findings suggest that chronic METH administration is associated with long-term monoamine loss.

Accumulating evidence also shows that repeated administration and/or high doses of METH promote the formation of reactive oxidative species, suggesting chronic METH may induce a neurodegenerative process (Imam & Ali, 2001). It has been suggested these processes are mediated by excessive dopaminergic activity in the cytoplasm, and to a lesser extent 5-HT, which enter via diffusion across the plasma membrane (Cadet, Jayanthi, & Deng, 2005; Davidson, Gow, Lee, & Ellinwood, 2001) and through VMAT attached to synaptic vesicles. These processes raise the level of DA in the extravesicular environment and increase DA oxidation to create quinones, hydrogen peroxide and additional reactive oxygen species that cause cell injury (Cadet & Krasnova, 2009; Davidson et al., 2001). As such, the potential for high neurotoxicity following METH has raised potential concerns about the deleterious effect of chronic METH exposure on the CNS. Indeed, markers for lipid peroxidation have been reported in the PFC and striatum following chronic METH exposure

(Açikgöz et al., 2000) while swelling to dopaminergic nerve terminals, general terminal degeneration (Elliott & Beveridge, 2005; Tata, Raudensky, & Yamamoto, 2007) and neuronal injury have been reported in the striatum (Ricaurte, Guillery, Seiden, Schuster, & Moore, 1982) and parietal cortex (Eisch & Marshall, 1998) following chronic METH administration. Evidence has also shown reduced grey-matter, hippocampal volumes and white-matter atrophy in METH abusers (Thompson et al., 2004), with different brain structures more susceptible to the neurotoxic effects of METH, particularly those with high DA content (Friedman et al., 1998; Peat et al., 1983). Given that METH predominantly functions by increasing the activity of the nervous system, it serves that METH induces multiple physical, behavioral and cognitive changes following acute and chronic administration. However, the intensity of such symptoms – and the degree to which each monoaminergic system is affected - typically varies as a function of the amount and/or frequency of METH administered and the route of administration (Hart et al., 2012).

1.1.6 The Effects of Acute and Chronic Methamphetamine Use

1.1.6.1 Physical Effects of Methamphetamine Administration

METH produces significant changes to the sympathetic nervous system, causing increased heart rate, blood pressure, perspiration and body temperature together with dilation of pupils, hyperthermia and body tremor (Harris et al., 2003; Hart et al., 2008; Mendelson, Jones, Upton, & Jacob, 1995; Meredith et al., 2005; Sevak et al., 2009). Indeed, our own data demonstrate that acute systemic METH administration in the rat decreases cardiovascular functions (sympathetic nerve activity and cutaneous blood flow) and increases heart rate, metabolic function and central respiratory function, consistent with these previous findings (Hassan, Wearne, Cornish, & Goodchild, Submitted).

Health consequences following chronic METH abuse include insomnia (Gibb, Bush,

& Hanson, 1997), malnutrition (Werb, Kerr, Zhang, Montaner, & Wood, 2010), liver damage (Halpin, Gunning, & Yamamoto, 2013) and kidney damage (Tokunaga, Kubo, Ishigami, Gotohda, & Kitamura, 2006). In addition, chronic METH abuse is also associated with elevated infection rates for HIV and Hepatitis C secondary to needle sharing and unsafe sexual behavior (Bluthenthal et al., 2001; Gonzales, Marinelli-Casey, Shoptaw, Ang, & Rawson, 2006; Halkitis, Parsons, & Stirratt, 2001; Shoptaw et al., 2005). A common complication from chronic METH administration is teeth grinding stereotypy, resulting in tooth decay, or what is colloquially referred to as “meth mouth” (Hamamoto & Rhodus, 2009). Furthermore, severe cardiovascular complications and alterations to autonomic output are evident following chronic METH use (Cruickshank & Dyer, 2009) and include hypertension (Chin, Channick, & Rubin, 2006), hyperpyrexia (Kojima et al., 1984), tachycardia, hemorrhage, cardiomyopathy (Sharlene Kaye, Darke, Duflou, & McKetin, 2008; Kaye, McKetin, Duflou, & Darke, 2007) and ischemic stroke (Perez, Arsura, & Strategos, 1999; Westover, McBride, & Haley, 2007). Indeed, METH users have a 3.7-fold risk of cardiomyopathy relative to the general population (Hong, Matsuyama, & Nur, 1991; Karch, Stephens, & Ho, 1999; Yeo et al., 2007). Lastly, chronic METH use is associated with increased mortality. Not only can METH-related mortality be due to excessive use or overdosing, but also due to pulmonary edema, pulmonary congestion, ventricular fibrillation and acute aortic dissection (Cruickshank & Dyer, 2009; Davis & Swalwell, 1994; Kiely, Lee, & Marinetti, 2009; Wako, LeDoux, Mitsumori, & Aldea, 2007). As a result of the severe physical effects of chronic METH use, the number of Emergency Department admissions associated with METH was 94,000 in the USA in 2005 (SAMHSA, 2006). In Australia, METH-related hospital admissions have steadily increased from 1990 to 2012, and are the second highest drug-related cause for presentations after opioids (Sindlicich & Burns, 2012).

1.1.6.2 Behavioral Effects of Methamphetamine Administration

Subjective effects of METH use include increased attentiveness, alertness, confidence, energy, euphoria, excitability, productivity and sexuality and physical activity (Barr et al., 2006; Hart et al., 2012; Nordahl, Salo, & Leamon, 2003). METH administration is also associated with decreased appetite and sleep, together with increased anxiety (Hart et al., 2012). However, the acute effects of METH use also include aggressiveness, headaches, sweating, insomnia and restlessness (Hart et al., 2001; Nordahl et al., 2003). METH use has also been associated with increased psychosocial issues, such as increased violence (Plüddemann, Flisher, McKetin, Parry, & Lombard, 2010), recidivism, criminal behavior and incarceration (Cartier, Farabee, & Prendergast, 2006), together with homicidal behavior secondary to increased impulsivity (Scott et al., 2007). METH users are more likely to engage in high-risk sexual behaviors (Forrest et al., 2010) and are therefore more likely to transmit sexually transmitted diseases (Colfax & Guzman, 2006; Halkitis et al., 2001).

1.1.6.3 Cognitive Effects of Methamphetamine Administration

Numerous research studies have reported enhanced cognitive performance following acute METH administration for drug naïve volunteers in the domains of attention, concentration, psychomotor functioning, speed of processing information, visuospatial perception and on tasks of learning and memory (Hart, Haney, Foltin, & Fischman, 2002; Johnson, Ait-Daoud, & Wells, 2000; Marrone, Pardo, Krauss, & Hart, 2010; Mohs, Tinklenberg, Roth, & Kopell, 1978; Mohs, Tinklenberg, Roth, & Kopell, 1980; Silber, Croft, Papafotiou, & Stough, 2006). Consistent with these findings, qualitative reports and literature on the use of other amphetamines also show increased attention and speed of information processing following acute administration (Soetens, Hueting, Casaer, & D'Hooze, 1995). Additional studies have not found any changes across any cognitive domain assessed; at least

in the acute stages of METH use (Comer et al., 2001; Hart et al., 2001; Sevak et al., 2009). Such findings, however, should be interpreted with caution in light of significant limitations in experimental design and methodological considerations in this area, specifically with regard to different dosing regimens and variations in the cognitive tests administered (Hart et al., 2012; Scott et al., 2007). Furthermore, it is difficult to determine the validity of these findings to first time METH users, as it is common for these individuals to use METH intravenously or through inhalation (smoking) during their first exposure, as opposed to the oral administration regimes typically used in acute human studies.

A proliferation of research over the past two decades has shown that long-term METH use is associated with cognitive dysfunction, with deficits reflecting specific neurobiological and neurochemical changes within specific regions of the brain. Review and meta-analyses have concluded that chronic METH use is associated with moderate neuropsychological impairment in the domains of episodic memory, executive functioning and speed of information processing, with smaller deficits in language, visuoconstruction and motor skills (Barr et al., 2006; Fernandez-Serrano, Perez-Garcia, & Verdejo-Garcia, 2011; Scott et al., 2007). Furthermore, it has been suggested that as many as 40.0% of METH dependent individuals demonstrate global cognitive impairment (Rippeth et al., 2004), suggesting that cognitive difficulties may be quite prevalent following repeated METH use. However, there are several limitations to these findings. Firstly, many studies that assess cognition in chronic METH users rely on individual tests across one or two cognitive domains, rather than implementing complete neuropsychological test batteries (Hart et al., 2012; Scott et al., 2007). Additionally, given the ethical constraints in assessing cognitive performance in human subjects following repeated METH use in an experimental setting, the majority of studies that examine cognitive performance following METH use assess abstinent samples. While this is not necessarily a limitation, there appears to be some evidence of cognitive recovery with

abstinence from METH use, as performance on neuropsychological tests of verbal memory, gross motor activity and psychomotor speed appear to be grossly intact for abstinent users (Chang et al., 2002). Therefore, these results may not necessarily reflect the true effects of chronic METH exposure, but rather, the combined effect of chronic METH and abstinence. As such, the interpretation of these comparisons, and the cognitive field relating to METH more generally, should be cautiously made, as abstinence may be a significant confound in the assessment of long-term cognitive deficits following METH use. Furthermore, while several studies may find that METH users are statistically reduced compared to controls, chronic METH users still perform within the average range across these cognitive domains (Hart et al., 2012; Kalechstein, Newton, & Green, 2003), which questions the clinical significance of the findings.

1.1.6.4 Psychiatric Symptoms following Methamphetamine Administration

METH use is associated with significant psychological symptoms in the ‘coming down’ or withdrawal phases from acute and repeated use, respectively, and include dysphoria, depression, anxiety, and irritability (Meredith et al., 2005). It has been proposed that the immediate negative symptoms experienced following METH use is the result of depleted monoamine levels and loss of neurotransmitter stores at neuron terminals (Kokoshka et al., 1998). However, the symptoms secondary to withdrawal from repeated METH abuse are experienced at an increased severity and duration than those of acute METH use, with some symptoms persisting up to 12 months following abstinence (Rose & Grant, 2008). This is likely due to the loss of monoaminergic terminals and neurons following chronic use, particularly in the dopaminergic and serotonergic systems. Furthermore, long-term psychological and psychiatric changes are associated with METH use and include symptoms of depression, apathy, anxiety, obsessive compulsive disorder (OCD) and aggression (Darke,

Kaye, McKetin, & Duflou, 2008; Homer et al., 2008; Lawton-Craddock, Nixon, & Tivis, 2003; Looby & Earleywine, 2007; Meredith et al., 2005; Zweben et al., 2004), with METH users more likely to receive a psychiatric diagnosis than the general population.

Psychological and psychiatric disorders are prevalent amongst METH-dependent individuals, with considerable literature proposing that METH increases the risk of an Axis 1 diagnosis (American Psychiatric Association, 2013). Of those who had used METH in the previous month, 28.6% and 32.6% endorsed moderate and severe levels of psychological distress (AIHW, 2013), respectively, while 30.0% of those who had not used METH for 12 months continued to report at least moderate levels of psychological distress (AIHW, 2013). Additionally, 34.3% of current METH users and 13.5% of users who had not used in the previous 12 months reported being diagnosed or treated for a mental illness (AIHW, 2013), suggesting that METH is associated with a high degree of psychological distress during both periods of use and abstinence. Furthermore, 46.0% of an Australian METH-dependent treatment-seeking sample reported a previous Axis 1 disorder diagnosis (Dyer & Cruickshank, 2005), while in another study 34.0% and 48.1% of a METH-dependent sample met criteria for a current or lifetime psychiatric disorder other than a substance use disorder, respectively, with the most prevalent diagnoses being mood disorders, anxiety disorders and antisocial personality disorder (Glasner-Edwards et al., 2010). Depression and anxiety are the most common Axis 1 disorders amongst METH users (Glasner-Edwards et al., 2008a; Zweben et al., 2004), with 35.0% of an Australian sample reporting a previous diagnosis of depression (Dyer & Cruickshank, 2005) while 11.0% of regular METH users have been diagnosed with an anxiety disorder (Darke et al., 2008).

It should be noted that nature of the relationship between METH use and psychiatric disturbances is unclear, as it is not certain whether depression and anxiety were a cause or a consequence of METH use. While many METH users report a lifetime history of depression,

with some using METH to ease depressive symptoms (Lecomte et al., 2010), others only report depression and anxiety upon withdrawal and abstinence from METH (Darke et al., 2008; Rose & Grant, 2008). Furthermore, little research has examined the prevalence of subthreshold symptoms of depression and anxiety amongst METH using samples, as the majority of literature has studied the prevalence rates of present or lifetime formal diagnoses of psychiatric disorders. Nevertheless, there is evidence to suggest a relationship between METH use and the symptoms of depression and anxiety.

In light of the relationship between METH and Axis 1 disorders, it follows that METH use is associated with increased rates of self-harm and suicide compared to the general population. In one study, 20.0% of the total sample of METH-dependent participants had been hospitalized at some point in their life for self-harm, while 28.0% of METH-dependent women and 13.0% of METH-dependent men reported a lifetime history of suicide attempts (Zweben et al., 2004). While these findings could be secondary to multiple antecedent events and their interaction with significant psychosocial factors, previous research on METH-dependent samples has shown that gender, intravenous METH use and a history of psychiatric history are significant risk factors for suicidality (Glasner-Edwards et al., 2008b), suggesting an important interaction between psychiatry and METH use in self-harm and suicide.

Overall, the above findings are particularly salient given that comorbid psychiatric conditions and METH dependence is associated with greater psychosocial problems and poorer prognosis. That is, METH users with psychiatric disturbances are more likely to experience greater functional decline compared to those without a psychiatric disorder (Glasner-Edwards et al., 2010), and psychiatric disorders may also affect the response to treatment for METH use. In light of the significant psychiatric disturbances following METH use and METH dependence, patients may require access to treatment of both substance abuse and mental health issues if they hope to attain positive functional outcomes (Moos, 2007;

Ouimette, Moos, & Finney, 1998). Nevertheless, while these findings indicate that METH use is associated with depression, anxiety and suicidality, an increasingly prevalent psychiatric concern following METH use is psychosis, which has been likened to schizophrenia.

1.2 Methamphetamine Psychosis as a Psychotic Disorder

1.2.1 Schizophrenia

Schizophrenia is a severe, complex and debilitating neuropsychiatric disorder that is traditionally associated with poor treatment outcomes relative to other psychiatric disorders. Epidemiological evidence suggests that schizophrenia affects 21 to 60 million people worldwide (Jablensky, 2000; Shetty & Bose, 2014), with recent statistics suggesting that 3.0% of Australians will be affected by a psychotic disorder at some point in their life (SANE, 2005). Current lifetime prevalence rates for schizophrenia range from 2.7 to 8.3 per 1000 in the general population (McGrath, Saha, Chant, & Welham, 2008) while the incidence rate is approximately 0.2 per 1000 each year (Messias, Chen, & Eaton, 2007). The gender ratio indicates that males are 1.4-fold more likely to develop schizophrenia relative to women (Aleman, Kahn, & Selten, 2003). The typical age of onset is the late teenage to early adulthood years, with the peak onset age between 20-28 years for males and 26-32 years for females (Castle, Wessely, Der, & Murray, 1991; Shetty & Bose, 2014). However, psychotic episodes concomitant with schizophrenia tend to run a recurrent, chronic and relapsing course, with episodes persisting well into a patient's adulthood. Although studies have found significant geographical variation in the global epidemiology of schizophrenia (Bresnahan, Menezes, Varma, & Susser, 2003; Jablensky et al., 1992; Saha, Chant, Welham, & McGrath, 2005), there is no discrimination of schizophrenia as a function of economic status (Saha, Welham, Chant, & McGrath, 2006).

In light of its early age of onset and chronic course, the burden of schizophrenia to the

individual and society is substantial, with schizophrenia ranked as one of the top 20 leading causes of disease burden for all age groups (Begg et al., 2007; Murray & Lopez, 1997) and one of the top 5 burdens for disease for people between the ages of 14-44 (Begg et al., 2007). Schizophrenia is particularly debilitating with regard to occupational and day-to-day functioning, with sufferers more likely to be unemployed (Sevy & Davidson, 1995), homeless or live in poverty (Herman, Susser, Jandorf, Lavelle, & Bromet, 1998). Schizophrenia has also been associated with poor physical health (Buhagiar, Parsonage, & Osborn, 2011; McCreadie, 2003; Osborn, 2001), substance abuse (Green, Salomon, Brenner, & Rawlins, 2002; Westermeyer, 2006), comorbid mental health issues (Buckley, Miller, Lehrer, & Castle, 2009) and reduced quality of life (Pinikahana, Happell, Hope, & Keks, 2002). Consequently, schizophrenia has a high rate of suicide, with a lifetime prevalence of approximately 10.0% (Meltzer, 2002); nine times higher than the general population (Harris & Barraclough, 1997). In light of its debilitating and severe course, a significant number of patients with schizophrenia require consistent hospitalization and lifelong care, which places a huge financial burden on the health care system. Indeed, the annual costs associated with the management of schizophrenia are estimated to be billions of dollars locally and globally (Desai, Lawson, Barner, & Rascati, 2013; Knapp, Mangalore, & Simon, 2004). In fact, 2.0% of Australia's health expenditure was accounted for by schizophrenia-related costs (Carr et al., 2004), with an average of \$79,537 spent on public mental health services per person with schizophrenia in 2010 (Neil et al., 2014).

1.2.2 The Symptoms Profile of Schizophrenia

Schizophrenia is a significantly heterogeneous disorder, with symptoms so diverse and idiosyncratic from patient to patient that the clinical profile has to be 'clustered' into different domains. While there is no symptom that is sufficient for a person to be diagnosed with

schizophrenia, there are particular symptoms that aid in differential diagnosis. ‘Positive symptoms’ refer to symptoms experienced by those with schizophrenia and not the general population, and include distortions in perceptions (hallucinations), false beliefs or distorted thought content (delusions), unclear or confused thinking (thought disorder) and disorganized speech. These symptoms are generally interpreted as a loss of touch with reality and usually only present at discrete times during ‘psychotic episodes’, which are considered a core feature of the disorder (Keffe, 2007). ‘Negative symptoms’, on the other hand, refer to symptoms or experiences that are usually absent or diminished in individuals with schizophrenia but are present in the general population. These include social withdrawal, anhedonia, flattened affect, motor retardation and poverty of speech (Blanchard and Cohen, 2006; Liemburg et al., 2013). Negative symptoms have a significant bearing on functional engagement and independence, with negative symptoms shown to predict the status of future functioning, employment, independence and social contact (Breier, Schreiber, Dyer, & Pickar, 1991).

Both positive and negative symptoms are established as core symptom dimensions and criteria for schizophrenia diagnosis in the DSM-V (American Psychiatric Association, 2013), and a third core symptom domain reported in schizophrenia is cognitive dysfunction. A wide range of cognitive domains appear to be compromised in schizophrenia, with many reviews and meta-analyses concluding moderate to severe deficits in general intelligence, attention, working memory, verbal learning and memory, speed of information processing, visuospatial deficits and executive dysfunction (Aleman, Hijman, de Haan, & Kahn, 1999; Elvevag & Goldberg, 2000; Goldberg & Gold, 1995; Green, Kern, & Heaton, 2004; Heinrichs & Zakzanis, 1998; Weickert et al., 2000). The cognitive deficits in schizophrenia are stable across the course of the disorder (Bozikas & Andreou, 2011; Dawes, Jeste, & Palmer, 2011; Lewandowski, Cohen, & Öngur, 2011; Szöke et al., 2008) and are consistent between those with first episode psychosis and chronic schizophrenia (Barder et al., 2013; Mesholam-

Gately, Giuliano, Goff, Faraone, & Seidman, 2009; Saykin et al., 1994). Executive functions appear to be the most compromised and are conserved deficits of cognition across patients with schizophrenia (Hutton et al., 1998; Minzenberg, Laird, Thelen, Carter, & Glahn, 2009), which has prompted extensive research into therapeutic markers that could specifically target executive dysfunction in schizophrenia (Kluwe-Schiavon, Sanvicente-Vieira, Kristensen, & Grassi-Oliveira, 2013). Additionally, the fact that the cognitive issues in schizophrenia are deleterious to social functioning, functional outcomes (Milev, Ho, Arndt, & Andreasen, 2005; Sharma & Antonova, 2003), independence (Dodge, Du, Saxton, & Ganguli, 2006; MacNeill & Lichtenberg, 1997), recovery (Jaeger, Berns, Loftus, Gonzalez, & Czobor, 2007) and well-being (Fillit et al., 2002), has prompted the argument that cognitive dysfunction should be regarded as one of the core dimensions in the disease, particularly with respect to DSM-V diagnostic criteria (Keefe, 2008).

1.2.3 Etiological Explanations of Schizophrenia

Over the past century, researchers within the field of mental health have sought to determine the etiology of psychiatric diseases and behavior. Traditional work in this area placed the origin of mental health problems in the context of either a genetic predetermination or the result of aberrant environmental factors. The evidence for a genetic basis of schizophrenia derived from familial studies that showed consistent concordance for schizophrenia amongst monozygotic twins and dizygotic twins (Gottesman, II & Shields, 1973; Gottesman & Shields, 1966). Indeed, multiple genetic studies on large schizophrenia populations have attempted to deliver a range of candidate genes that could be the basis for the development of psychotic disorders, e.g. catechol-o-methyl transferase (COMT), d-amino acid oxidase activator (DAOA), disrupted in schizophrenia 1 (DISC1) and brain-derived neurotrophic factor (BDNF)(Ivleva, Thaker, & Tamminga, 2008). However, as more studies

attempted to pinpoint the etiology of such disorders to genetic polymorphisms, it became clear that the human genome alone was insufficient for the development of schizophrenia. For example, studies of monozygotic twins reared together or reared apart often illustrate contentious findings for a sole genetic basis of schizophrenia, and polymorphism research is often characterized by small effect sizes that often fail to be replicated (Fallin & Pulver, 2001; Tiwari, Zai, Muller, & Kennedy, 2010).

It has long been known that environmental factors and significant life events increase the likelihood of developing psychiatric symptoms. Over the last half-century, numerous studies have been performed in order to identify specific environmental factors that could increase the risk of psychotic disorders. Epidemiological and case-control research has revealed a long list of potential environmental factors that could mediate the onset of schizophrenia, and included: stress (van Os & Selten, 1998), mental and physical abuse (Sideli, Mule, La Barbera, & Murray, 2012), maternal infection (Brown & Derkits, 2010), maternal diet during pregnancy (Kirkbride et al., 2012), vitamin D deficiency (McGrath, Burne, Féron, Mackay-Sim, & Eyles, 2010), advanced paternal age (Miller et al., 2011), obstetric complications (Cannon, Jones, & Murray, 2002), famine (Susser et al., 1996), migration (Cantor-Graae & Selten, 2005), exposure to infections (Müller, 2004) and use of cannabis (Callaghan et al., 2012; Louisa Degenhardt, Hall, & Lynskey, 2003; Henquet, Di Forti, Morrison, Kuepper, & Murray, 2008). However, a more recent finding in the literature on the environmental triggers of psychosis is METH use.

1.2.4 Acute Methamphetamine Psychosis

Repeat administration, or high doses of METH, can induce an acute psychosis that is behaviorally indistinguishable from schizophrenia (Ujike & Sato, 2004), whereby users experience auditory and visual hallucinations, delusions, ideas of reference and disorganized

speech (Bramness et al., 2012; McKetin, McLaren, Lubman, & Hides, 2006; Zweben et al., 2004). The idea that METH could induce a psychotic state has long been recognized by clinicians in Japan, who increasingly observed psychosis in their METH-dependent patients (Sato, 1992). While this was later acknowledged by clinicians around the world – at least with respect to acute METH psychosis - the early identification of this relationship was due, in part, to the high prevalence of METH use together with the absence of polydrug use in Japan. This enabled clinicians to isolate the link between METH and psychosis without the confound of additional substance use (Ujike & Sato, 2004).

More recent studies around the world have shown that METH psychosis is a prevalent health concern among recreational and chronic users. Indeed, an Australian study of non-treatment seeking METH users found that 13.0% of the sample were positive for psychosis at the time of assessment (McKetin, McLaren, Lubman, & Hides, 2006) while 23.0% reported ‘clinically significant’ symptoms of psychosis over the previous year, such as unusual thoughts, hallucinations, hostility and suspiciousness. Importantly, 52.0% of the sample reported mild psychotic symptoms, suggesting that METH users could potentially experience subthreshold psychotic symptoms following METH use that may go unreported and undetected by formal psychiatric facilities. An additional study found that 60.0% of METH-dependent individuals sampled in the USA reported at least one type of psychotic symptom (Mahoney, Hawkins, De La Garza, Kalechstein, & Newton, 2010). Overall, recreational users of METH are two to three times more likely to experience psychotic symptoms than the general population (McKetin, Hickey, Devlin, & Lawrence, 2010), with their risk significantly increasing if they begin using METH at a younger age or if large amounts of METH are administered (Chen et al., 2003). Regular METH users, however, are 11 times more likely to experience psychosis than the general population (McKetin et al., 2006), with the average time between first use and psychosis being 1.7 years (Matsumoto et al., 2002).

In support of the idea that METH users are more susceptible to the psychotic effects of METH whilst they are using the drug, McKetin et al. (2013) found that chronic METH users were 5 times more likely to experience psychotic symptoms during periods of METH use than during periods of abstinence. They also found dose-response effects between the frequency of METH use and psychotic symptoms, with psychosis reaching a peak likelihood of 48.0% following 16 days or more of chronic METH use. Importantly, these findings were still significant once they controlled for polydrug use, suggesting that the psychotic symptoms were attributable to the effects of METH and not due to the interaction of additional drug consumption. Overall, these findings suggest that METH use is associated with a high prevalence of psychotic symptoms, which may present a significant burden on the healthcare system due to increased demand for care and management of METH-related psychoses. Indeed, METH psychosis accounts for 10.0% of admissions to psychiatric facilities in Thailand (Farrell et al., 2002) and in Australia, METH psychosis is responsible for 10.3 hospital admissions per 1000 (McKetin, 2005).

1.2.5 Chronic Methamphetamine Psychosis

Methamphetamine psychosis typically follows a transient course, with psychotic symptoms subsiding once the user has stopped taking the drug (Meredith et al., 2005). Some consumers, however, can experience a prolonged psychosis that persists even after the drug has been removed from the body. Indeed, up to 60.0% of METH psychoses can take up to 10 days to resolve following cessation of METH (Connell, 1957), with the majority of psychotic symptoms resolving within 1 month (Deng et al., 2012; Sato, 1992). However, research findings, particularly from Japan, have indicated that METH psychosis can develop into an enduring form of psychosis. Reports have suggested that up to 30.0% of those with METH psychosis may have symptoms that continue up to 6 months following abstinence (Deng et

al., 2012), with specific studies reporting 15-28% of patients admitted to hospital with METH psychosis needed to be hospitalized for more than 2-3 months following admission (Iwanami et al., 1994; Nakatani et al., 1989). Other studies have found that 10.0% to 28.0% of patients with METH psychosis continued to display psychosis for more than 6 months (Iwanami et al., 1994; Ujike & Sato, 2004) while in another study, 28.0% of METH-users continued to display 'schizophrenia-like symptoms' 8 to 12 years following abstinence (Teraoka, 1967). Additional findings outside of Japan have reached similar conclusions. McKetin et al. (2013) reported that even abstinent METH users had a 7.0% risk of experiencing psychotic symptoms, while another study reported that at 3 years follow up, 5.0% of abstinent METH-dependent users met criteria for a current psychotic disorder (Glasner-Edwards et al., 2010). Furthermore, METH can induce a chronic psychosis in those with no premorbid psychiatric risk factors (Grelotti, Kanayama, & Pope, 2010), suggesting that METH could potentially induce persistent physiological changes consistent with psychosis that are independent to genetic and personality predispositions.

Overall, these findings suggest that METH psychosis can result in a persistent psychotic syndrome that is resistant to spontaneous recovery, and in light of the high use of METH use globally, chronic METH psychosis will undoubtedly continue to be an issue for health-care professionals. As such, understanding the factors that subserve the neurobiology and maintenance of chronic psychosis induced by METH abuse will be important for delineating diagnostic markers and avenues for treatment.

1.3 The Relationship between METH-induced Psychosis and Schizophrenia

1.3.1 Diagnostic Ambiguity

Previous research suggests that a subset of users can experience an enduring form of psychosis following METH use, which has been noted to closely resemble paranoid

schizophrenia (Salo et al., 1992). However, there is uncertainty of the diagnostic status of chronic METH psychosis as a primary psychotic disorder. That is, clinicians from Japan were early to accept that METH psychosis could develop into a persistent and enduring form of psychosis. However, other clinicians, particularly in the west, were less amenable to the idea that METH use alone could result in a psychotic disorder, with METH-induced, and other substance-induced psychoses, clearly distinguished between schizophrenia and other primary psychoses in the DSM-V. In fact, any psychosis in the presence of withdrawal from a substance requires the diagnosis of “substance-induced psychotic disorder” (American Psychiatric Association, 2013). Diagnostic guidelines, however, become ambiguous should the psychosis persist for an extended period of time. For example, early reports suggested that regardless of the type and severity of drug use, psychotic symptoms that extended beyond six weeks of abstinence should not be linked to the abused drug (Boutros & Bowers, 1996). Furthermore, the DSM-V outlines that any psychosis that persist longer than six months should warrant the diagnosis of a primary psychotic illness (American Psychiatric Association, 2013). Indeed, a Thai study of METH abusers, who were initially hospitalized for METH psychosis (n= 400, excluding those with current METH use), found that 38.8% (n = 174) of the sample had been diagnosed with schizophrenia due to persistent psychosis at 5 years follow up (Kittirattanapaiboon et al., 2010), while 5.0% of Chinese patients with METH-induced psychosis had their diagnosis changed to schizophrenia (Deng et al., 2012). Furthermore, a large study conducted over a 10 year period in California, USA, determined that individuals who were hospitalized for METH-related causes (n = 42,412) had a higher risk of receiving a subsequent schizophrenia diagnosis, with this risk comparable to those in the cannabis-related group (Callaghan et al., 2012), a substance that has significant links to schizophrenia (D'Souza, Sewell, & Ranganathan, 2009). While these findings support that METH use is associated with an enduring psychosis, there are several interpretations to the

link between METH use and schizophrenia.

A possible interpretation of these findings is that METH use could induce schizophrenia, potentially by eliciting an underlying vulnerability/predisposition to a primary psychotic disorder. Early research on amphetamine psychosis attributed the continuation of psychotic symptoms to “latent paranoia” (Connell, 1958). Additionally, a growing body of literature has examined the role of gene and environmental interactions in the development of METH psychosis, with some studies showing convergence of genetic risk factors for METH psychosis with the genetic risk factors for schizophrenia (Bousman, Glatt, Everall, & Tsuang, 2009; Grant et al., 2012). Additionally, one study found a significant enrichment of ‘risk allele’ single nucleotide polymorphisms (SNPs) for METH psychosis in patients with schizophrenia (Ikeda et al., 2013). A total of 67 genes were found to overlap between the METH-induced psychosis and schizophrenia conditions, such as NOTCH4, suggesting a shared genetic risk between both conditions that was independent to METH dependence. Furthermore, a family history of schizophrenia is a risk factor for the development of METH psychosis (Chen et al., 2005; Chen et al., 2003; Matsumoto et al., 2002). These findings suggest that the development of a persistent psychotic syndrome, such as schizophrenia, may be the interaction between a predetermined vulnerability (i.e. a diathesis) and/or the direct effects of METH as an environmental trigger (i.e. the two hit hypothesis), and may provide an explanation as to why only a small percentage of METH users, and those with METH psychosis, go on to develop a persistent psychotic syndrome. More recently, however, there has been discussion surrounding the possibility that METH use could actually *cause* the onset of schizophrenia (Callaghan et al., 2012; Flaum & Schultz, 1996; Grelotti et al., 2010), potentially by inducing schizophrenia pathology (e.g. dysfunctional dopaminergic and/or glutamatergic expression). Even though this does not explain why only a percentage of users develop a persistent psychotic syndrome, both explanations suggest that METH psychosis and

schizophrenia may be the same disorder on a continuum of pathology, converging with the idea that schizophrenia is a neurobiological disorder with multiple etiologies.

More recent reports, however, have documented the development of a prolonged METH psychosis in the absence of a psychotic diagnosis or personal and family predisposition to schizophrenia (Grelotti et al., 2010; Matsumoto et al., 2002; Ozaki, 2004). These reports indicate that alternative explanations for the development of METH-induced psychosis may also be possible. As such, it could be that METH psychosis and schizophrenia represent distinct and separate disorders, and indeed, several researchers have proposed that METH use in isolation can produce a persistent psychotic syndrome that should be diagnosed and treated as a functionally different syndrome to schizophrenia (Yeh, Lee, Sun, & Wan, 2001; Yui, Goto, Ikemoto, & Ishiguro, 2000). There are several implications to this idea. Given that any persistent psychosis beyond a 6-month period should be considered as a primary psychotic disorder, based on the current diagnostic criteria in the DSM-V, METH psychosis may be routinely misdiagnosed and treated as a schizophrenia disorder. Therefore the diagnosis of schizophrenia secondary to METH use described in the aforementioned studies may merely reflect adherence to diagnostic protocol and may not be a true reflection of the status and prevalence of chronic METH psychosis in the general population. That is, individuals who present with METH psychosis may be diagnosed with schizophrenia, which may therefore underestimate the degree to which METH use results in a persistent psychotic disorder in epidemiological research studies.

Overall, there appears to be uncertainty about whether METH causes schizophrenia or whether chronic METH psychosis represents a biologically and symptomatically distinct disorder that should be distinguished from other primary psychoses. While there appears to be similarity between the two conditions, there is limited research that has explicitly compared the behavioral and cognitive markers that appear to be conserved and/or different between the

disorders. To understand the similarities and distinguishing features of METH psychosis and schizophrenia is of benefit, as not only will this assist in determining the diagnostic entity of METH psychosis, but will also help develop differential diagnostic markers for clinicians, better treatment options for long-term METH psychosis suffers and will help to delineate common biological markers across syndromes that may initiate and maintain a persistent vulnerability to psychosis. This knowledge may enable a deeper theoretical understanding of the specific biological factors that subserve the symptoms that are commonly observed across psychotic disorders.

The following section describes and critiques the literature that has compared the clinical profile of METH psychosis and schizophrenia, with particular focus on the convergence of positive, negative and cognitive symptoms. If the use of METH does cause a primary psychotic disorder, then the presentation and symptoms of chronic METH psychosis should match those typically reported in schizophrenia. Consequently, persistent METH psychosis could be regarded as the same diagnostic entity and could allude to similar neurobiology and etiological mechanisms. However, if METH psychosis represents a biologically and clinically distinct disorder, then there should be divergence in the behavioral, cognitive and biological markers between METH psychosis and schizophrenia. To this end, METH psychosis should be carefully and critically distinguished from schizophrenia in the diagnostic literature and in the clinical setting.

1.3.2 Positive Symptoms

Early findings on METH induced psychosis reported hallucinations and delusions as a predominant presenting factor (Ellinwood, 1967; Sato, 1992), with later findings acknowledging that the similarities between METH psychosis and schizophrenia were largely directed towards positive symptoms. In a small study of 11 patients with METH psychosis,

Tomiyama (1990) reported that 5 subjects experienced visual hallucinations, 7 experienced delusions of reference and persecutory delusions while all experienced auditory hallucinations. Similarly, McKetin et al. (2006) found that unusual thoughts, hallucinations and suspiciousness were present in one-quarter of chronic methamphetamine users diagnosed with METH psychosis. Additional studies have also reported that METH psychosis is associated with a high prevalence of persecutory delusions, auditory hallucinations, visual hallucinations, odd speech and delusions of reference (Chen et al., 2003; Fasihpour, Molavi, & Shariat, 2013; Srisurapanont et al., 2003). Given that all of these findings were derived from different samples and geographical locations, the type of positive symptoms exhibited by those with METH psychosis appeared to be consistent, suggesting that METH psychosis could be similar to the clinical presentation of schizophrenia.

Of studies that have directly compared METH psychosis with schizophrenia, Srisurapanont et al. (2011) compared the psychotic symptoms of 168 patients with METH psychosis and 169 patients with schizophrenia. They found no difference in the type, frequency and severity of positive symptoms between METH psychosis and schizophrenia. Furthermore, Medhus, Mordal, Holm, Morland, and Bramness (2013) compared 42 individuals with METH psychosis and schizophrenia in 2 acute psychiatric wards. They found no difference in the ratings of delusions, grandiosity, suspiciousness and hallucinatory behavior on the Positive and Negative Syndrome Scale (PANSS) between drug-negative individuals with schizophrenia and those with psychosis induced by amphetamines. These findings suggest that the positive symptoms of METH-induced psychosis appear to be qualitatively and quantitatively comparable to the positive symptoms of schizophrenia.

In consideration of the above findings, there are potential limitations to the research that should be addressed. Firstly, Mehduş et al. (2013) did not control for polydrug use, meaning that the symptoms of psychosis may not be exclusively attributed to METH

administration. Additionally, the length of abstinence from METH is an issue with the interpretation of these findings. Mehdu et al. (2013) required subjects to test positive for METH to be included in the study while Srisurapanont et al. (2011) assessed their METH psychosis subjects within a week of their admittance to hospital. Therefore, these findings may not be generalizable to samples of chronic METH psychosis, as it is uncertain whether these behavioral responses may be referable to the direct effects of METH, acute stimulant psychosis or a chronic form of enduring psychosis. Furthermore, both of these studies were reliant on data from hospitalized samples that could bias the results, as those admitted to psychiatric facilities would have more severe psychosis than non-treatment-seeking individuals with METH psychosis in the community. Lastly, these studies compared METH psychosis to schizophrenia using the PANSS or the Manchester scale (Medhus et al., 2013; Srisuraponont et al., 2011). A significant limitation of these scales is that they do not differentiate the qualitative nature of the hallucinations or delusions experienced, as they quantify the status of positive symptoms with a total score. Given that specific types of hallucinations may be more prevalent in one type of psychosis, these scales may be unable to detect differences that may differentiate the two conditions.

Studies that have use more comprehensive scales in conjunction with these screener items have been able to differentiate the types of hallucinations and delusions commonly experienced in METH psychosis and schizophrenia. Studies that have used the Mini-International Neuropsychiatric Interview-Plus (MINI-Plus), have found that auditory hallucinations are the most common form of hallucination in both METH psychosis and schizophrenia (Ali et al., 2010; Chen et al., 2003; Srisurapanont et al., 2003). Indeed, auditory hallucinations are the most prevalent positive symptom in schizophrenia, occurring in 67-90.0% of cases (Bauer et al., 2011). This finding converges with research that has reported a lifetime prevalence of auditory hallucinations of 72.6% (Srisurapanont et al., 2003) and

84.5% (Chen et al., 2003) for individuals with METH psychosis. Additionally, approximately 70.0% of individuals with both METH psychosis and schizophrenia experience delusions, with the most common content being persecutory delusions followed by delusions of reference (Appelbaum, Robbins, & Roth, 1999; Chen et al., 2003; Curran, Byrappa, & McBride, 2004). Less common similarities between the two conditions refer to motor activity, with individuals with METH psychosis and schizophrenia often engaging in repetitive stereotypies (Meredith et al., 2005) and sluggish behavior (Bell, 1965).

Despite the considerable overlap in positive symptoms between METH psychosis and schizophrenia, there are also several differences across both conditions. While hallucinations are common across both METH psychosis and schizophrenia, visual and tactile hallucinations appear to be more prominent in METH psychosis compared with schizophrenia (Bell, 1965; Zorick et al., 2008). Chen et al. (2003) reported that 46.5% and 21.3% of their METH psychosis sample reported visual and tactile hallucinations, respectively. Additional findings have also confirmed visual hallucinations in 68.8% of METH abstinent individuals (Akiyama, 2006) while others have reported that visual hallucinations are the fourth most reported positive symptom in METH psychosis (Fasihpour et al., 2013). However, visual hallucinations are typically only reported in severe cases in schizophrenia (Mueser, Bellack, & Brady, 1990), with the prevalence rate ranging from 16.0 to 27.0% (Mueser et al., 1990; Waters et al., 2014). Additionally, formication, a tactile hallucination where individuals believe that one's skin has been infested by bugs, is typically only reported in METH psychosis (Rusyniak, 2013). Therefore, while auditory hallucinations appear to be the most common hallucination of both METH psychosis and schizophrenia, visual and tactile hallucinations appear to be more prominent in METH psychosis. Thus there appears to be qualitative differences in the prevalence of specific hallucinations across both conditions, which could ultimately be used to distinguish between METH psychosis and schizophrenia.

An additional distinction between METH psychosis and schizophrenia is thought disorder. Thought disorder refers to disorganized thinking and is characterized by the loosening of associations and fragmented speech (Yui, Ikemoto, Ishiguro, & Goto, 2000). Research findings suggest that thought disorder is a defining and salient feature in schizophrenia but does not appear to be prominent in METH psychosis (Angrist, Sathananthan, Wilk, & Gershon, 1974; Bell, 1965; Dore & Sweeting, 2006; Yui, Ikemoto, & Goto, 2002). Initial work by Bell (1965) distinguished between schizophrenia and amphetamine-induced psychosis with the appearance of thought disorder, as this symptom was only seen in schizophrenic cases. Additionally, Yui et al. (2002) found that while individuals with METH psychosis experienced paranoid hallucinations and delusions, the same participants did not exhibit thought disorder or disorganized speech. Furthermore, in their study comparing METH psychosis and schizophrenia, Srisurapanont et al. (2011) found that incoherent speech, a distinguishing marker of thought disorder, was the only symptom with a greater differential item functioning score greater than .1, indicating it was the only symptom that differed between schizophrenia and METH psychosis (Srisurapanont et al., 2011). Therefore, the absence of thought disorder may be a discriminating feature associated with METH psychosis that can be used to differentiate this condition from schizophrenia.

1.3.3 Negative Symptoms

While stimulant-induced psychotic disorders have been predominantly characterized by positive symptoms, negative symptoms such as flat affect, social withdrawal, apathy, loss of drive, anhedonia and poverty of speech have also been reported in METH psychosis samples (Ali et al., 2010; Chen et al., 2003; Srisurapanont et al., 2011; Yui, Ikemoto, et al., 2000; Zorick et al., 2008). Research has shown no difference in the type of negative symptoms experienced between METH psychosis and schizophrenia. For example,

Tomiyama (1990) found that the rating of avolition and anhedonia were similarly high in both groups while Srisurapanont et al. (2011) found that the negative symptoms of alogia, psychomotor retardation and flat affect severity were similarly rated for both METH-induced psychosis and schizophrenia groups. Furthermore, symptoms of anxiety/depression syndrome were similar in severity between both conditions (Srisurapanont et al., 2011).

Even though negative symptoms have been reported in both schizophrenia and METH psychosis, there appears to be differences in the prevalence and severity of these symptoms. That is, negative symptoms are common in schizophrenia, with negative symptoms considered a central feature of its phenomenology and diagnostic criteria (Foussias, Agid, Fervaha, & Remington, 2014; Möller, 2007). Indeed, 58.0% of individuals with schizophrenia experience negative symptoms (Bobes, Arango, Garcia-Garcia, & Rejas, 2010), with 50-90% of those with schizophrenia displaying negative symptoms in first-episode psychosis (Makinen, Miettunen, Isohanni & Koponen, 2008). On the other hand, Ali et al. (2010) found that only 25.0% of individuals hospitalized with METH psychosis exhibited negative symptoms while Srisurapanont et al. (2003) similarly found that only 21.4% of their sample met criteria for negative symptoms in a clinical interview using the MINI-plus. While these lower prevalence rates may be attributable to limited research in the area, specifically with respect to inclusion and appropriate assessment of negative symptoms in research studies, these findings suggest that negative symptoms may be experienced at a considerably lower rate in METH psychosis compared with schizophrenia.

Negative symptoms in schizophrenia tend to be most pronounced between psychotic episodes (Möller, 2007) – although positive and negative symptoms can co-occur – yet patients with schizophrenia continue to have negative symptoms throughout the course of their disorder. Indeed, 20-40% of individuals with schizophrenia experience persistent negative symptoms throughout the course of the disorder (Makinen et al., 2008). However,

negative symptoms concomitant with METH psychosis tend to resolve within six months of diagnosis (Yeh et al., 2001), indicating that the progression and stability of negative symptoms appears to differ across the two conditions.

Differences in the type of negative symptoms experienced between METH psychosis and schizophrenia may exist. Specifically, Tomiyama (1990) found that flattened affect and poverty of speech were less prevalent in METH psychosis compared to schizophrenia. Additionally, Panenka et al. (2013) found that individuals with METH psychosis had lower scores on measures of flattened affect and social withdrawal compared to schizophrenia, indicating that these symptoms were less severe than those typically experienced in schizophrenia (Panenka et al., 2013).

1.3.4 Cognition¹

Recent work has further examined the prevalence and severity of cognitive dysfunction following METH psychosis in comparison with schizophrenia. Jacobs, Fujii, Schiffman, and Bello (2008), in an exploratory cross-sectional study, compared the cognitive profile of individuals hospitalized with METH psychosis (n = 20) with patients with paranoid schizophrenia (n = 19) across eight cognitive domains, including premorbid intellectual ability, learning and memory, executive functioning, general intellectual functioning, attention and concentration, motor abilities together with non-verbal and verbal skills. They found no significant differences between the two groups in any cognitive domain examined, suggesting that both METH psychosis and schizophrenia may have similar cognitive profiles and may therefore share underlying brain pathology, particularly with respect to dysfunction of the frontal and temporal lobes. While there was no control group, the results were

¹ For the purposes of this review, consideration was made in distinguishing the cognitive profile of chronic METH use with and without psychosis. This section describes research that specifically examined the cognitive profile concomitant with METH psychosis. The cognitive profile associated with chronic METH use can be found in section 1.1.6.3.

qualitatively and quantitatively representative of a typical schizophrenia cognitive profile. However, there are several limitations to these findings. Firstly, the study was hampered by a small sample size, meaning that there may not have been enough statistical power to detect any meaningful difference between the two groups. Secondly, there were between-group differences in age, ethnicity and place of birth between those with METH psychosis and schizophrenia, suggesting that these factors may have been confounds in the study. Lastly, it was not known how long the sample had been abstinent from METH nor was it reported how long the METH psychosis sample had been taking METH prior to their participation in the study. Regardless, this initial study provided evidence that METH psychosis showed cognitive deficits that were similar to those typically reported in schizophrenia.

Ezzatpanah, Shariat, and Tehrani-Doost (2014) compared cognitive function in individuals with METH-induced psychosis and schizophrenia to healthy controls, with all subjects matched for age, sex and education. All three groups were compared across a wide range of cognitive abilities including executive functioning, working memory, episodic memory and sustained attention. They found that both METH psychosis and schizophrenia were characterized by reduced performance on all cognitive tasks examined when compared to healthy controls, while there were no significant differences in the performance of those with METH psychosis and schizophrenia across tasks of memory, sustained attention, selective attention and executive functioning. Specifically, METH psychosis and schizophrenia groups demonstrated difficulty in inhibiting, manipulating and suppressing information, together with difficulties learning and retaining verbal information over time. These findings indicate that both disorders may be characterized by comparable deficits of cognition mediated by the temporal and frontal lobes, specifically the PFC, and further extends Jacobs et al. (2008)'s findings that both METH psychosis and schizophrenia may be the product of similar pattern of brain pathology. Furthermore, these similar findings are

strengthened by the fact that these two studies were derived from different cultural samples - America (Jacobs et al., 2008) and Iran (Ezzatpanah et al., 2014) – and through the use distinct cognitive tools. The fact that these profiles were reliably replicated adds further validation to the fact that both disorders may be characterized by robust cognitive phenotypes. However, these studies were based on recent abstinent METH users, and may not be generalizable to those with chronic METH psychosis.

In light of these limitations, more recent research has shown that cognitive dysfunction is specific to METH users with persistent psychosis and not to the direct effects of METH use or acute METH psychosis. Chen et al. (2015) conducted a cross-sectional study on METH users without psychosis ($n = 25$), METH users with acute psychosis (operationalized as METH users who had psychotic symptoms that dissipated within one month following cessation of METH, ($n = 50$)), METH users with persistent psychosis ($n = 56$), individuals with schizophrenia ($n=54$) and controls ($n=67$). Using the Brief Assessment of Cognition in Schizophrenia (BACS), all groups were assessed across a range of cognitive functions including verbal memory, working memory, motor speed, verbal fluency, attention and processing speed and executive functioning. Interestingly, METH users with persistent psychosis performed comparably to those with schizophrenia across all cognitive domains, with both these groups performing cognitively worse than the other METH and control groups. These findings extend the findings of Jacobs et al., (2008) and Ezzatpanah et al. (2014) by clearly distinguishing between METH users with acute and chronic psychosis, suggesting that schizophrenia and only persistent psychosis secondary to METH use are associated with similar cognitive profiles. These findings indicate that chronic METH psychosis is associated with brain changes that should be carefully distinguished from the changes concomitant with METH use and acute METH psychosis.

Despite the overwhelming similarities in cognitive dysfunction between METH

psychosis and schizophrenia, some studies have reported several minor differences in cognitive functioning between those with schizophrenia and METH psychosis. For example, Salo, Ravizza, and Fassbender (2011) suggested that METH users, during the early stages of abstinence, demonstrated worse cognitive functioning than in patients with schizophrenia. Furthermore, Ezzatpanah et al. (2014) report that individuals with schizophrenia and METH psychosis demonstrated difficulties with sustained visual attention compared to controls, yet those with schizophrenia performed significantly poorer on the VSAT, the Visual Search and Attention Test, than subjects with METH psychosis. As selective visual attention is primarily correlated with the parietal cortex, these findings indicate that dysfunction of the parietal cortex may be more pronounced in schizophrenia than METH psychosis. Consistent with these findings, Yui et al., (2000) previously reported qualitative differences in attention deficits between individuals with METH psychosis and schizophrenia. That is, while both conditions were associated with attention disturbances, individuals with METH psychosis had heightened attention to their environment while those with schizophrenia were likely to be indifferent to their surroundings (Yui et al., 2000).

1.3.5 Summary

Based on the aforementioned findings, research has shown both similarities and differences in the positive, negative and cognitive symptoms between METH-induced psychosis and schizophrenia. There appears to be a high degree of concordance in the type, prevalence and severity of positive symptoms between METH psychosis and schizophrenia, confirming that it would be difficult to distinguish between the two conditions in the clinical setting based on the positive symptoms alone. However, while auditory hallucinations appear to be the most common hallucination reported in METH psychosis and schizophrenia, visual and tactile hallucinations appear to be more prominent in METH psychosis, with thought

disorder the most pronounced symptom in schizophrenia. Furthermore, even though negative symptoms occur in both METH psychosis and schizophrenia, some research has indicated that there are differences in the type, severity and progression of negative symptoms throughout both conditions, with METH psychosis associated with reduced frequency and severity of several negative markers, such as flattened affect. Lastly, from a cognitive perspective, most cognitive domains appear to be similarly perturbed across METH psychosis and schizophrenia. However, more recent findings have highlighted that some functions subserved by the parietal cortex, such as selective visual attention, may be more pronounced in schizophrenia than METH psychosis.

Therefore, while there is considerable overlap in the behavioral and cognitive symptoms between METH psychosis and schizophrenia, research has shown that there are unique and divergent aspects to each condition. Although both disorders may be characterized by common underlying biological pathologies and phenotypes, METH psychosis could represent a distinct psychotic disorder to schizophrenia and may be clinically distinguished from a primary psychotic disorder based on the distinct behavioral and cognitive sequelae identified above. However, one of the biggest limitations with METH psychosis research is that little effort is made to distinguish between those with chronic METH psychosis and acute METH psychosis, with the majority of the findings portraying a blended representation. Given that only those with a persistent psychotic syndrome appear to display the cognitive dysfunction typically associated with schizophrenia (Chen et al., 2015), it is possible that the differences in positive, negative and cognitive symptoms reported in additional studies may be referable to acute METH psychosis or the result of methodological limitations in the literature, such as differences in sample size, the effects of psychotropic medication and polydrug use or selection bias in non-blind sample selection. It will be important for future research to specifically examine the effect of persistent METH psychosis and how this relates to the

behavioral, cognitive and biological changes typically reported in schizophrenia in order to elucidate whether chronic METH psychosis represents a distinct psychotic disorder.

1.4 The Role of Sensitization in Chronic Psychosis

1.4.1 Behavioral Sensitization

Given only a subset of METH users develop a persistent psychotic syndrome, with positive, negative and cognitive deficits that are comparable to schizophrenia, certain biological factors must be involved in mediating a persistent vulnerability to psychosis and shared symptomatology between these conditions. Researchers have placed this vulnerability in the context of behavioral sensitization (Boileau et al., 2006; Featherstone, Kapur, & Fletcher, 2007; Robinson & Becker, 1986; Ujike, 2002; Ujike & Sato, 2004; Yui, Goto, et al., 1999). Behavioral sensitization refers to the unique phenomenon whereby repeat exposure to stimulus, such as a drug, results in a progressively increased behavioral and neurochemical response to that stimulus following a period of abstinence. While repeated exposure to a drug will cause a progressive reduction in the responsiveness to the effects of the drug, or ‘tolerance’, repeated administration of psychostimulants can lead to an increased sensitivity to the behavioral (motor stimulant) and neurochemical (dopamine) effects (Robinson & Becker, 1986), and consequently, sensitization has been referred to as ‘reverse tolerance’. For example, even though psychostimulants induce locomotor activity when administered acutely, a chronic intermittent administration regime will induce significantly more locomotor activity and striatal dopamine release when the subject is re-exposed to the same drug following a withdrawal period (Pierce & Kalivas, 1997; Ujike & Sato, 2004).

There are several important characteristics to behavioral sensitization. Firstly, sensitized behavior is most apparent after a period of abstinence and re-exposure to a drug. Therefore, when studying the mechanisms that subserve the behavioral and neural changes

associated with sensitization, the phenomenon can be separated into two distinct domains that differ in terms of their temporal and anatomical characteristics (Cador, Bjijou, & Stinus, 1995; Pierce & Kalivas, 1997). *Initiation* refers to the transient cellular and molecular changes that coincide with repeated drug exposure that cause an increase in behavior, such as increased locomotor activity. A large body of literature has shown that the initiation of sensitization is critically dependent on dopamine regulation in the mesolimbic pathway, specifically within the ventral tegmental area (Chen, Chen, & Chiang, 2009; Pierce & Kalivas, 1997; Pierce & Kumaresan, 2006; Vanderschuren & Kalivas, 2000; Vezina, 2004). *Expression*, on the other hand, refers to the enduring neural changes that follow from the initiation process that maintain a persistent behavioral sensitivity to the stimuli. While the molecular mechanisms that characterize the initiation process have been well established, the expression of sensitization is more ambiguous, particularly given that the neuronal events that coincide with expression of sensitization appear to be distributed throughout the motivational circuit, including the nucleus accumbens, ventral tegmental area, the ventral pallidum and the prefrontal cortex (Pierce & Kalivas, 1997; Steketee, 2003).

Secondly, sensitization is an enduring behavioral transformation, meaning that once sensitization has developed, subsequent exposure to a drug can result in a sensitized behavioral response even after long-term abstinence. Indeed, sensitized rats have been observed to demonstrate elevated sensitized responding for up to a year following abstinence (Paulson, Camp, & Robinson, 1991). This suggests that sensitization is more than a reflection of the drug working acutely on the brain and that enduring neuroplastic changes must be mediating a persistent vulnerability to sensitized behavior following withdrawal.

Additionally, sensitized responding can be elicited by exposure to alternative drugs of abuse or environmental cues, a phenomenon known as cross-sensitization. That is, animals sensitized to one drug may demonstrate an increased behavioral response if exposed to other

drugs, stress or environmental cues, such as being placed in the same environment to which drugs had been administered previously (Antelman, Eichler, Black, & Kocan, 1980; Cauli, Pinna, Valentini, & Morelli, 2003; Cunningham, Finn, & Kelley, 1997; Horger, Giles, & Schenk, 1992; Lamarque, Taghzouti, & Simon, 2001; Shaham, Erb, & Stewart, 2000). These findings suggest the sensitization must have a common underlying pathology that is independent to the acute effects of the individual stimuli.

1.4.2 Evidence of Sensitization in Humans

Behavioral sensitization has been traditionally studied in animals, and a large body of literature extending over several decades has shown robust and reliable evidence of behavioral sensitization across many stimulant drugs, including METH, and across a large range of animal subjects (Pierce & Kalivas, 1997; Steketee, 2003; Vanderschuren & Kalivas, 2000). However, even early research noted evidence of sensitization in humans (Down & Eddy, 1932), and specifically, studies have shown humans sensitize to the effects of amphetamines (Sax & Strakowski, 2001; Strakowski, Sax, Rosenberg, DelBello, & Adler, 2001; Strakowski, Sax, Setters, & Keck, 1996). For example, subjects that are given three separate administrations of amphetamine show increased motor activity and eyeblink responses following their third administration compared to the first or second exposures (Strakowski & Sax, 1998), suggesting that behavioral sensitization can be produced in humans under procedures similar to those used in animal studies. In light of the evidence of sensitization in humans following psychostimulant administration, sensitization is traditionally interpreted in the context of addiction, with the mechanisms likened to increased craving associated with drug use that may lead to relapse following withdrawal (Cornish & Kalivas, 2001; Kalivas, Pierce, Cornish, & Sorg, 1998; Robinson & Berridge, 1993). However, the sensitization paradigm can also explain certain aspects of chronic psychoses.

1.4.3 The Role of Sensitization across Methamphetamine Psychosis and Schizophrenia

There are several lines of evidence that suggests sensitization may be implicated in the maintenance of chronic METH psychosis. Firstly, as noted above in section 1.2.4, METH use in humans has been shown to induce an acute psychosis that resembles schizophrenia (Dore & Sweeting, 2006; McKetin et al., 2006). This psychotic state typically subsides following cessation of the drug, however, observational and empirical data has shown that psychosis can be elicited following a single low-dose re-exposure to METH (Sato, 1992; Yui, Goto, et al., 1999; Yui, Ikemoto, Goto, Nishijima, & Kato, 2003; Yui, Ishiguro, Goto, & Ikemoto, 1997), suggesting that certain neuronal changes must be involved in mediating a persistent vulnerability to psychotic relapse. Secondly, psychotic relapse can occur even after years of abstinence, with studies reporting ranges from a few months to more than 4 years (Sato, Numachi, & Hamamura, 1992). It has also been documented that symptoms associated with relapsed psychostimulant psychosis tend to be of quicker onset and more severe, converging with the observation that sensitization is an enduring behavioral transformation and that abstinence is critical for the development of amplified behavior. Thirdly, psychotic relapses can be induced by not only the resumption of a particular drug of abuse, but also stressors and additional substances, suggesting METH psychosis demonstrates cross-sensitization to additional triggers (Sato, 1992; Yui, Goto, et al., 2000; Yui et al., 2002). Additionally, METH psychosis can be prevented or attenuated through the use of neuroleptic medication (Ohmori, Ito, Abekawa, & Koyama, 1999; Olivares, Sermon, Hemels, & Schreiner, 2013), suggesting that similar neurochemical mechanisms are involved in mediating the behavioral symptom profile between METH psychosis and schizophrenia. Indeed, the induction of sensitization appears to critically depend on mesolimbic dopaminergic transmission (Paulson & Robinson, 1996; Pierce & Kalivas, 1997), with amphetamine-induced behavioral changes in humans accompanied by a decrease in dopamine D2 receptor binding in the striatum following re-

exposure to amphetamine, which is indicative of increased dopaminergic activity in the mesolimbic pathway (Boileau et al., 2006). These data indicate that repeated psychostimulant exposure might induce both long-term behavioral and neurochemical sensitization in humans.

In light of the significant similarities between sensitization and chronic METH psychosis, psychostimulant-induced locomotor sensitization is often regarded as an animal model of stimulant-induced psychosis in humans (Robinson & Becker, 1986; Snyder, 1973, Ujike, 2002). As such, the induction of psychostimulant sensitization may provide insight into the etiology and biological factors that subserve first episode psychosis, while the expression of sensitization may determine the neural pathways and factors that maintain a persistent sensitivity to psychotic relapse. While some have argued that the sensitized model lacks construct validity (Jones, Watson, & Fone, 2011), partly due to the lack of objective evidence that animal models display psychotic symptoms, psychotic behavior has been observed in non-human primates as a result of amphetamine administration (Castner, Vosler, & Goldman-Rakic, 2005). For example, monkeys exhibit behaviors that are believed to represent positive symptoms of psychosis, including staring at empty spaces or picking at imaginary parasites following repeated (Castner & Goldman-Rakic, 1999, 2003) or acute administration of amphetamine (Sams-Dodd & Newman, 1997).

Schizophrenia is also associated with behavioral changes that could be interpreted through mechanisms of behavioral sensitization. For example, once remission has been effectively reached through neuroleptic medication, individuals with schizophrenia can relapse to a more severe psychosis once their medication is discontinued (Ohmori et al., 1999). Furthermore, psychotic episodes can also be experienced following a significant life stressor (Olivares et al., 2013), with some research suggesting that patients can relapse to a more severe form of psychosis (Bramness et al., 2012), consistent with the ideas of cross-sensitization and augmented behavioral response of sensitization. Furthermore, individuals

with schizophrenia can also experience a psychotic relapse following exposure to amphetamine at a dose that does not cause psychosis in healthy controls (Curran et al., 2004; Lieberman, Kane, & Alvir, 1987), suggesting that psychostimulants specifically target sensitized circuitry. Consistent with this idea, neuroimaging evidence has shown increased subcortical dopamine release following amphetamine challenge in schizophrenia (Howes et al., 2012; Laruelle et al., 1996), demonstrating the behavioral and neurochemical augmentation consistent with sensitization to repeated stimulant administration.

These findings suggest that both schizophrenia and METH-induced psychosis share common neuronal mechanisms that initiate and maintain a persistent vulnerability to psychosis, with sensitization as a key mediating factor that links these conditions to each other. As such, it will be important to examine the neural changes that mediate sensitization to chronic METH, as this will enable understanding of the mechanisms that are responsible for mediating different aspects of psychotic disease states, including the behavioral and cognitive phenotypes that appear to be similarly perturbed across the two conditions.

1.4.4 The Relevance of Sensitization to Negative and Cognitive Symptoms of Psychoses

Early research typically focused exclusively on the positive symptoms of schizophrenia and the neurobiological correlates in sensitization with the mesocorticolimbic pathways, however, more recent research has examined the potential for sensitization to extend to the negative and cognitive symptoms of psychoses. Specifically, immediate withdrawal from psychostimulants in animals has been associated with reduced locomotor activity and spontaneous nocturnal behavior (Paulson et al., 1991; Robinson & Camp, 1987), consistent with the depressed mood (anhedonia) and elevated sleep patterns following amphetamine withdrawal in humans (McGregor et al., 2005; Newton, Kalechstein, Duran, Vansluis, & Ling, 2004). Rats have also shown anhedonia as decreased motivation to respond

for sucrose and reduced sexual behavior following withdrawal from stimulant administration (Barr, Fiorino, & Phillips, 1999; Barr & Phillips, 1999, 2002). These findings, however, are typically transient and dissipate quickly following the initiation of withdrawal (Barr et al., 1999; Barr & Phillips, 1999; Featherstone et al., 2007). While these effects are consistent with the resolution of negative symptoms in METH psychosis, which tend to resolve within six months of diagnosis (Yeh, Lee, Sun, & Wan, 2001), negative symptoms in schizophrenia tend to have a chronic course, suggesting that negative symptoms following sensitization may be more reflective of METH psychosis as opposed to schizophrenia. Furthermore, other studies have shown mixed findings for reduced locomotor activity (Segal & Kuczenski, 1997) and anhedonia (Russig, Pezze, et al., 2003) following withdrawal, while social behavior appears to be unchanged following repeated psychostimulant exposure (Sams-Dodd, 1998). These findings suggest that sensitization to psychostimulants may not reflect all the negative symptoms reported across psychoses, and given that these behaviors are not maintained following abstinence, these symptoms may be more appropriately explained by acute withdrawal rather than the effect of sensitization on the brain.

A large body of literature has shown cognitive deficits following sensitization to psychostimulants, particularly amphetamine (for review, see Featherstone et al., 2007). Specifically, prepulse inhibition - the attenuation of a startle-response to a high-intensity stimulus when a weaker prepulse precedes it – has been linked to dysfunctional sensorimotor gating and the filtering of external stimuli (Braff, Grillon, & Geyer, 1992; Geyer, Swerdlow, Mansbach, & Braff, 1990), with deficits in prepulse inhibition routinely reported in schizophrenia (Braff, Geyer, & Swerdlow, 2001; Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001; Parwani et al., 2000). Previous studies have found reduced prepulse inhibition following sensitization to psychostimulants (Peleg-Raibstein, Sydekum, & Feldon, 2006; Peleg-Raibstein, Sydekum, Russig, & Feldon, 2006; Tenn, Fletcher, & Kapur, 2003; Tenn,

Kapur, & Fletcher, 2005) while other studies have found no change to PPI following sensitization (Murphy, Fend, Russig, & Feldon, 2001), although it has been proposed that these discrepancies may be secondary to differences in the adopted escalation sensitization regimes (Featherstone et al., 2007). Individuals with schizophrenia also tend to show deficits with latent inhibition, the phenomenon whereby reinforcement and conditioning take longer to develop for familiar stimuli than unfamiliar stimuli. Several studies have shown sensitization disrupts latent inhibition on tasks of conditioned avoidance (Murphy et al., 2001; Russig, Kovacevic, Murphy, & Feldon, 2003; Russig, Murphy, & Feldon, 2002; Tenn et al., 2003; Tenn et al., 2005). Sensitization to repeated psychostimulant administration is also associated with cognitive deficits in the areas of sustained attention (Fletcher, Tenn, Sinyard, Rizos, & Kapur, 2007; Kondrad & Burk, 2004), spatial working memory (Castner et al., 2005), reversal learning (Featherstone, Kapur, & Fletcher, 2005) and attentional set-shifting (Fletcher, Tenn, Rizos, Lovic, & Kapur, 2005). While long-term memory and working memory appear to be conserved (Featherstone et al., 2005; Featherstone, Rizos, Kapur, & Fletcher, 2008; Russig, Durrer, Yee, Murphy, & Feldon, 2003) other results are mixed (Bisagno, Ferguson, & Luine, 2003; Stefani & Moghaddam, 2002). Nevertheless, these findings indicate that sensitization is associated with specific cognitive deficits, suggesting that psychostimulant sensitization could potentially be used to study the neurobiology of cognitive deficits typically observed in chronic psychoses.

1.5 The Prefrontal Cortex and Behavioral Sensitization

1.5.1 Anatomy and Circuitry of the Prefrontal Cortex

The PFC is situated at the anterior portion of the frontal lobe in front of the motor and limbic cortices, and includes Brodmann areas 8, 9, 10, 11, 44, 45, 46 and 47 (Figure 2). Its boundaries can be marked by gross morphological features including the presylvian fissure,

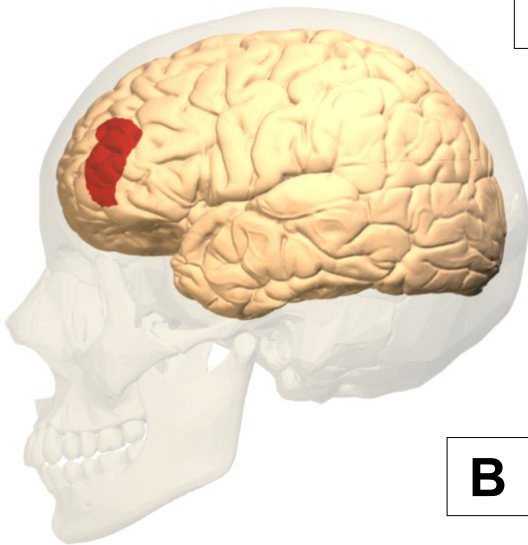
arcuate sulcus, inferior precentral fissure and the anterior curvature of the cingulate sulcus (Figure 2). Distinct regions of the PFC, particularly the orbitomedial and lateral areas, have distinct reciprocal connections with other brain structures. For example, the orbital and medial PFC (Figure 2B) is connected with the hypothalamus, amygdala, medial temporal cortex, limbic system and medial thalamus while the lateral prefrontal cortex (Figure 2C) is connected with the neocortex, dorsal caudate nucleus and lateral thalamus (Fuster, 2008a).

The neuronal activity of the PFC is dependent on the activity of two main neuronal subtypes: pyramidal cells and interneurons. Pyramidal cells use glutamate as their primary neurotransmitter, which are predominantly excitatory (Krnjevic, 2010), and are the most abundant cells in the PFC. Pyramidal cells are responsible for the communication between brain regions, thus making the axons of pyramidal cells important for PFC output. Interneurons, on the other hand, are predominantly characterized by the presence of gamma-aminobutyric acid (GABA), thereby making them predominantly inhibitory (Kresimir, 2010). Interneurons have a salient role in regulating the output of pyramidal cells, and will be discussed in more detail in section 1.6.2.

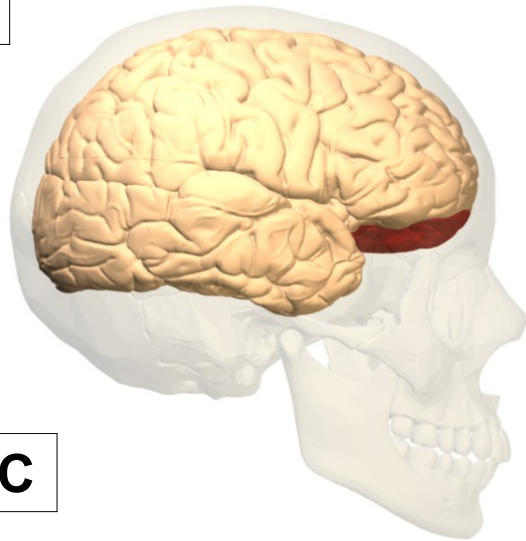
The PFC consists of six distinct cell layers that are differentiated based on their cytoarchitecture. Each cell layer, or lamina, has specific extrinsic and intrinsic connectivity, with the PFC sending and receiving projections from many cortical and subcortical structures. Importantly, all projection axons show little colocalization to their extrinsic targets (Gabbott, Warner, Jays, Salway, & Busby, 2005; Pinto & Sesack, 2000), meaning that efferents and afferent projections of the PFC have a high degree of specificity with their target sites. Layers II to VI of the PFC contain glutamatergic spiny pyramidal neurons that are important for efferent output and are organized topographically. Layers II and III specifically project to cortical areas while Layers V and VI project predominantly to subcortical regions, with a large proportion of Layer V projecting to the nucleus accumbens, lateral hypothalamus and



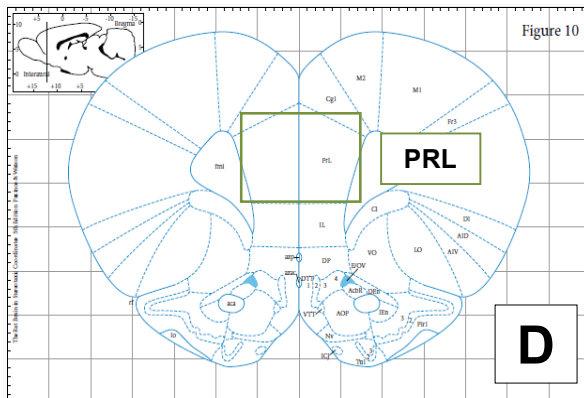
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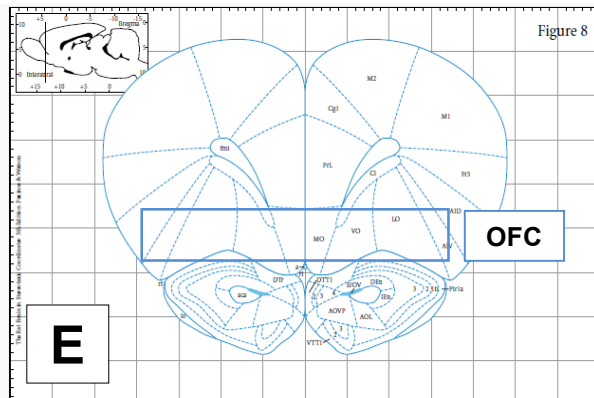
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Figure 2. Global and localized regions of the prefrontal cortex (PFC) in the adult and rat brain. Figure A depicts the PFC from a coronal view of the adult brain, with the PFC covering the frontal lobe pole. Figure B and C show the localization of the dorsolateral prefrontal cortex (DLPFC) and orbitofrontal cortex (OFC) in the adult brain, respectively. Figure D shows the localization of the prelimbic cortex (PRL) in the rat brain (green), a brain region believed to be analogous to the DLPFC in the human brain (Faron & Pucet, 2000). Figure E shows the localization of the OFC in the PFC of the rat brain (blue). Figures A, B and C are from BodyParts3D, generated by Science Database Centre for Life Science (SBCLS), and copied with permission under the license “Creative Commons Attribution-Share Alike 2.1 Japan”. Figures D and E are taken from the rat atlas (Paxinos & Watson, 1986).

the basolateral amygdala. Layer VI primarily projects to the thalamus, with 37.0% of projecting neurons from this layer specifically targeting the mediodorsal nucleus of the thalamus (Gabbott et al., 2005). In fact, the PFC was originally described in terms of its strong reciprocal connections with the mediodorsal nucleus of the thalamus (Groenewegen & Uylings, 2000; Kolb, 1984), as afferents from this brain region project to the anterior and ventral aspects of the frontal lobe (Preuss & Goldman-Rakic, 1991). While this criterion can still be used (Fuster, 2008a), more recent studies have shown that projections from the mediodorsal nucleus do not project solely to the PFC, meaning that this definition of the PFC may not be completely valid. With regard to afferent projections, the middle layers of the PFC, specifically layers II and III, receive both cortical and subcortical inputs (Cenquizca & Swanson, 2007; Giguere & Goldman-Rakic, 1988; Hoover & Vertes, 2007; Jay & Witter, 1991; Krettek & Price, 1977; Swanson, 1981).

1.5.2 The Role of the Prefrontal Cortex in Cognition and Behavior

Simple and familiar behaviors are routinely performed below the level of consciousness and without the demands on attention through ‘bottom-up’ processing, where behavioral output is determined by the nature of the stimuli and the associated neural pathways that mediate automatic processing. While a large proportion of these behaviors are innate, other autonomic behaviors are contingent on experienced-based and associative learning, and develop as a gradual process over time as a function of exposure. Although these behaviors can be performed quickly and automatically, they can take a long time to develop, are inflexible and do not generalize to novel situations (Miller & Cohen, 2001).

Purposeful and flexible behavior, on the other hand, is achieved through top-down processing, where internal goals and intentions are executed with the coordination of sensory input and motor output to produce an adaptive behavioral response that is context-appropriate

(Cohen & Servan-Schreiber, 1992; Grafman, 1994; Miller, 1999, 2000b; Miller & Cohen, 2001; Passingham, 1993; Wise, Murray, & Gerfen, 1996). Given that the PFC has strong connections with both cortical and subcortical regions, the PFC is in a position to regulate the top-down control of multiple brain systems that regulate sensory and motor behavior (Miller & Cohen, 2001) leading to the modulation and coordination of purposeful and goal-directed output. An argument for the role of the PFC in driving top-down control of behavior is strengthened by findings that greater surface area is dedicated to the PFC in animals that require greater cognitive control in mediating a diverse behavioral repertoire, such as humans and primates (Miller & Cohen, 2001). The PFC has consequently received significant scientific examination with respect to its role in coordinating complex behavior. A large body of neuroimaging, neurophysiological, neuropsychological and lesion studies have shown that PFC function is linked to many cognitive processes including attention, working memory, planning, decision-making, problem solving, flexibility and abstraction (Bechara, Damasio, Damasio, & Lee, 1999; Bechara, Tranel, & Damasio, 2000; Curtis & D'Esposito, 2003; Euston, Tatsuno, & McNaughton, 2007; Fuster, 2008b, 2008c, 2008d; Hashimoto & Sakai, 2002; Jones, 2002; Kane & Engle, 2002; Koechlin, Corrado, Pietrini, & Grafman, 2000; Miller, 2000a; Waltz et al., 1999). The PFC is also hypothesized to play a central role in moderating social behavior (Yaling Yang & Raine, 2009) and personality (DeYoung et al., 2010). Importantly, given that executive functioning appear to be similarly perturbed across METH sensitization, METH psychosis and schizophrenia, it follows that the PFC has received considerable attention across these conditions.

1.5.3 The Role of the Prefrontal Cortex in Behavioral Sensitization and Psychoses

Dysfunctional processing of the PFC has been linked to many of the cognitive symptoms associated with neurological and neuropsychiatric disorders, including chronic

psychoses. The PFC has been extensively examined in schizophrenia, particularly given that associated executive deficits are considered diagnostically salient and functionally debilitating (Keefe & Harvey, 2012; Minzenberg et al., 2009). Neuroimaging studies have shown volumetric changes (Davidson & Heinrichs, 2003), cortical thinning (Fornito, Yucel, Patti, Wood, & Pantelis, 2009), altered neuronal integrity (Bertolino et al., 1999) and altered task-dependent neural activity of the PFC (Eich, Nee, Insel, Malapani, & Smith, 2014; Glahn et al., 2005). Post-mortem studies of schizophrenic brains show changes to oligodendrocytes (Kim & Webster, 2010; Uranova, Vostrikov, Orlovskaya, & Rachmanova, 2004), white matter neuronal density (Yang, Fung, Rothwell, Tianmei, & Weickert, 2011) and astrocytes (Feresten, Barakauskas, Ypsilanti, Barr, & Beasley, 2013), together with alterations to mitochondria and oxidative phosphorylation (Karry, Klein, & Ben Shachar, 2004; Prabakaran et al., 2004), synaptic proteins (Wesseling et al., 2013) and inflammatory markers (Fillman, Cloonan, Catts, et al., 2013; Fillman, Cloonan, Miller, & Weickert, 2013).

Some research has also shown PFC changes in individuals with METH psychosis. For example, Aoki et al. (2013) found grey matter volume reductions in the posterior inferior frontal gyrus and anterior superior temporal gyrus in individuals with METH psychosis compared to healthy controls, with these findings correlating with the severity of positive symptoms. Similarly, reduced density of white matter fibers have also been shown in individuals with METH psychosis, which also correlated with the severity of psychiatric symptoms (Tobias et al., 2010). Interestingly, functional neuroimaging has identified differential activation of the PFC depending on the point of drug use, with the PFC activated during periods of chronic use yet inactive during withdrawal (Goldstein & Volkow, 2011; Goldstein, Volkow, Wang, Fowler, & Rajaram, 2001). This finding suggests that changes to the PFC following drug consumption are time-specific, and are consistent with the idea that sensitization represents distinct temporal phases characterized by different brain pathology.

Nevertheless, psychoses appear to be associated with widespread PFC changes, and place the PFC as a key target in the maintenance of psychotic symptoms.

Extensive research has shown that the PFC is implicated in the biology of sensitization to psychostimulants. As noted above, the PFC mediates many of the cognitive deficits that coincide with sensitization to psychostimulants, including prepulse inhibition, latent inhibition and attentional set-shifting (Castner & Williams, 2007; Lacroix, Broersen, Feldon, & Weiner, 2000; Lacroix, Broersen, Weiner, & Feldon, 1998). Additionally, Castner and Goldman-Rakic (2003) found that bilateral lesions of the PFC suppressed psychotic behaviors following sensitization to amphetamine in primates, suggesting that the PFC could mediate both positive and cognitive symptoms of sensitization. While the VTA and the NAc are critically involved in the initiation and expression of locomotor sensitization, respectively (Cador et al., 1995; Paulson et al., 1991; Pierce & Kalivas, 1997; Steketee, 2003), excitatory projections to the mesolimbic pathway are pivotal for locomotor sensitized behavior. Indeed, glutamatergic efferents from the PFC project to the mesoaccumbens pathway (Geisler, Derst, Veh, & Zahm, 2007; Overton & Clark, 1997) and several lines of evidence suggest that the PFC could modulate activity in this system. For example, stimulation of the PFC potentiates firing of dopamine neurons in the VTA and increases dopamine levels in the NAc (Karreman & Moghaddam, 1996; Taber, Das, & Fibiger, 1995; Taber & Fibiger, 1995; Tong, Overton, & Clark, 1996), while reversible blockade of the PFC with lidocaine attenuates firing activity and reduced dopamine release in the NAc (Murase, Grenhoff, Chouvet, Gonon, & Svensson, 1993). As such, these findings indicate that the PFC is in a position to modulate activity, and by extension, sensitized output controlled by the mesolimbic system. Indeed, lesions of the PFC block the expression of sensitization to various psychostimulants (Li et al., 1999; Li & Wolf, 1997; Pierce, Reeder, Hicks, Morgan, & Kalivas, 1998; Sorg, Li, & Wu, 2001; Wolf, 1998), suggesting a relationship between the PFC, projections to the mesolimbic pathways

and sensitization. In support for this relationship, Cador, Bjijou, Cailhol, and Stinus (1999) found that combined VTA infusions of amphetamine and a NMDA receptor antagonist dose-dependently blocked the induction of sensitization, suggesting that glutamatergic transmission in the VTA is requisite for the initiation of sensitization. Furthermore, sensitization to repeated VTA and systemic administration of amphetamine was blocked following ibotenic acid lesion of the PFC but not the amygdala, confirming that glutamatergic afferents from the PFC are critically involved in sensitization (Cador et al., 1999).

Additional work has extended these findings to examine the role of the PFC in the expression of sensitization. While lidocaine-induced blockade of the PFC potentiates locomotor response to acute amphetamine administration, it blocks the locomotor and rearing behavior associated with chronic sensitization (Degoulet, Rostain, David, & Abraini, 2009), indicating that the PFC has an inhibitory role of locomotor behavior to acute psychostimulant administration while it has an excitatory role in the expression of sensitization to amphetamine (Degoulet, Rostain, David & Abraini, 2009). Consistent with this, Aguilar-Rivera, Casanova, Gatica, Quirk, and Fuentealba (2015) found that while acute METH caused an inhibitory effect on burst firing rate in the PFC, specifically within the prelimbic cortex (PRL), sensitization to amphetamine resulted in increased neuronal burst firing rate, suggesting that the PFC is hyperexcitable following sensitization. These findings suggest that increased excitability of the PFC may strengthen the connection between the PFC and the nucleus accumbens. Indeed, sensitization is accompanied by neuroplastic and morphological changes in the PFC, including increased dendritic spine length and density (Robinson & Kolb, 1997) and an increased number of synapses onto spines (Morshedi, Rademacher, & Meredith, 2009). However, these findings are not specific to sensitization, as activity of the PFC is correlated with increased striatal dopamine release (Meyer-Lindenberg et al., 2002) and striatal D₂ receptor binding in schizophrenia (Bertolino, 1999), suggesting that the role of the

PFC may be biologically conserved across psychoses.

Collectively, these findings suggest that common neuronal mechanisms in the PFC are involved in neurobiology of behavioral sensitization, chronic METH psychosis and schizophrenia. Accordingly, investigation of the neurobiology underlying behavioral sensitization in the PFC is warranted, as this could lead to more precise understanding of the mechanisms that subserve the vulnerability to relapse in schizophrenics and METH psychosis. In light of the clear role of pyramidal projections from the PFC to the mesolimbic pathway, together with the fact that the PFC is innervated by dopaminergic efferents from the VTA, the role of glutamate and dopamine in the PFC following sensitization has been well described (for reviews see Steketee, 2003; Pierce & Kalivas, 1997). However, the role of inhibitory regulation of the PFC via GABAergic neurotransmission and interneurons has received considerably less attention.

1.6 Inhibitory Regulation of the Prefrontal Cortex

1.6.1 The GABAergic System

γ -aminobutyric acid (GABA) is the most ubiquitous inhibitory neurotransmitter in the central nervous system. Discovered in the early 1950s, GABA was proposed to play a prominent role at inhibitory synapses (Awapara, Landua, Fuerst, & Seale, 1950; Roberts & Frankel, 1950; Roberts, Frankel, & Harman, 1950), and indeed, additional studies have found that 25-50% of synapses contain GABA, 20-30% of neurons synthesize GABA and every neuron expresses GABA receptors (Koella, 1981; Sivilotti & Nistri, 1991). While GABA has both excitatory and inhibitory roles across various stages of brain development (Ben-Ari, Tseeb, Raggozzino, Khazipov, & Gaiarsa, 1994; Gao, Stricker, & Ziskind-Conhaim, 2001; Obrietan & van den Pol, 1995), GABA in the adult mammal CNS is inhibitory, where it regulates neuronal excitability through the inhibition of pyramidal cells and interneurons.

1.6.1.1 GABA Synthesis, Release, Reuptake and Metabolism

Reliable inhibitory regulation of the PFC relies on a large network of genes/proteins that control GABA synthesis, release, reuptake and metabolism. GABA is synthesized from the α -decarboxylation of glutamate by the rate-limiting enzyme glutamate-decarboxylase (GAD) (Roberts et al., 1950). GAD is present as two allelic isoforms, GAD₆₇ and GAD₆₅, which differ with respect to genetic homology and molecular size (67 and 65 kiloDaltons). While both GAD₆₇ and GAD₆₅ are present in all GABAergic cells, they diverge in terms of their relative concentration and subcellular localization (Erlander, Tillakaratne, Feldblum, Patel, & Tobin, 1991; Erlander & Tobin, 1991; Esclapez, Tillakaratne, Tobin, & Houser, 1993; Esclapez, Tillakaratne, Kaufman, Tobin, & Houser, 1994; Feldblum, Erlander, & Tobin, 1993), with both playing different roles in GABA production. The activity of GAD isoforms is regulated by a pyridoxal 5'-phosphate cofactor, which either activates or silences when it is attached or released to the enzyme, respectively (Phillips, 2015). GAD₆₇ is typically saturated with the cofactor, meaning that it predominantly exists in its active state. As such, GAD₆₇ is localized throughout the neuronal body and regulates the cytoplasmic pool of GABA (Figure 3)(Soghomonian & Martin, 1998), with over 90.0% of GABA production derived from GAD₆₇ activity (Asada et al., 1997). GAD₆₅, on the other hand, is primarily expressed at axon terminals, where it regulates synaptic inhibitory neurotransmission through GABA vesicular filling only under sustained periods of elevated synaptic phasic activity (Choi, Morales, Lee, & Kirkwood, 2002; Hensch et al., 1998; Kash et al., 1997; Patel, De Graaf, Martin, Battaglioli, & Behar, 2006; Stork et al., 2000; Tian et al., 1999).

GABAergic neurotransmission is also regulated by specific mechanisms that control the release and reuptake of GABA at the synapse. GABA is packaged into vesicles and stored in the neuronal terminal until it is released by neuronal depolarization with the assistance of vesicular GABA transporter (VGAT). VGAT is found at nerve terminals of GABAergic

neurons in the brain where it facilitates the Ca^{2+} dependent vesicular transport of cytoplasmic GABA to the neuronal membrane for exocytosis into the synapse (Bellocchio et al., 1998; Chaudhry et al., 1998; Dumoulin et al., 1999; Fattorini, Antonucci, Menna, Matteoli, & Conti, 2015; Freneau et al., 2001), placing VGAT as pivotal for GABA-mediated inhibitory neurotransmission. The activity of synaptic GABA is terminated by Na^+ dependent GABA transporters, which are responsible for the reuptake of GABA at inhibitory synapses (Conti, Minelli, & Melone, 2004). Four distinct transporters for GABA are known: GAT_1 , GAT_2 , GAT_3 and BGT_1 (Borden, Smith, Gustafson, Branchek, & Weinshank, 1995; Borden, Smith, Vaysse, et al., 1995). GAT_2 and BGT_1 are found exclusively outside the brain in the liver, kidney and leptomeninges, although there is some evidence for GAT_2 in some blood vessels of the brain (Zhou et al., 2012). The reuptake of GABA at inhibitory synapses in the PFC is regulated by the expression of GAT_1 and GAT_3 (Dalby, 2003). GAT_1 is predominantly localized to terminals and glia (Conti et al., 2004; Gadea & Lopez-Colome, 2001) where it regulates basal GABA levels in the extracellular space, while GAT_3 is exclusively found in glia (Conti et al., 2004). As such, GAT_3 is critically involved in the reuptake of GABA to the glutamate/glutamine cycle where it is metabolized by GABA-aminotransferase (GABA_T) to glutamate and succinic-semialdehyde (Figure 3)(van der Laan, de Boer, & Bruinvels, 1979).

1.6.1.2 GABA_A Receptors

GABA exerts its inhibitory effects by binding to GABA receptors on cortical neurons. There are two main types of GABA receptors: ionotropic GABA_A receptors and metabotropic GABA_B receptors. GABA_A receptors are hetero-oligomeric membrane proteins composed of five receptor subunits that form a central chloride channel pore. GABA_A receptors are structurally and pharmacologically complex, with each receptor composed from a pool of 19 distinct subunits, with each subunit encoded by separate genes and categorized into different

family classes (Table 1; α 1 - 6, β 1 - 3, γ 1 - 3, δ , ϵ , π , θ , ρ 1 - 3). 70-80% amino acid sequence homology exists within each subunit class while 30% homology is evidence between classes (Costa, 1998), indicating some degree of similarity between specific subunits.

Receptors comprised of different subunits combinations are considered distinct receptor subtypes. Given the hetero-pentameric arrangement of GABA_A receptors, together with the diversity of subunits available, it follows that a large number of permutations of GABA_A receptors are possible; however, interestingly, only 20 have been clearly identified in the mammalian CNS (McKernan & Whiting, 1996). Indeed, GABA_A receptor stoichiometry typically consists of 2 α , 2 β and 1 γ , with the α 1 β 2 γ 2 receptor combination the most abundant in most brain regions (McKernan & Whiting, 1996) and combinations of α 1, α 2, α 3, α 5, β 2, β 3 and γ 2 subunits representing over 80% of benzodiazepine sensitive GABA_A receptors in the adult brain (Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000). Variability across the individual subunits that compose the GABA_A receptor is also salient, as more than one type of subunit is required (Schofield et al., 1987; Sieghart et al., 1999), subunits cannot be expressed in isolation and not all subunits can colocalize together to produce a functional receptor (Verdoorn, Draguhn, Ymer, Seeburg, & Sakmann, 1990). These findings suggest a high degree of specificity in the collection of subunits that compose GABA_A receptors. Indeed, staining studies across the brain have shown considerable overlap in the expression of α 1 and β 2, α 2 and β 3, α 4/6 and δ , suggesting that these combinations are generally expressed together (Jechlinger, Pelz, Tretter, Klausberger, & Sieghart, 1998; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000b; Sur et al., 1999). Furthermore, GABA_A receptors appear to be differentially expressed across subregions of the brain (Table 1).

Depending on their arrangement, GABA_A receptors represent distinct entities with specific functional and spatial-temporal profiles (Boileau, Pearce, & Czajkowski, 2005; Olsen & Sieghart, 2009; Patel, Mortensen, & Smart, 2013). While GABA_A receptors were

traditionally described as mediators of phasic inhibitory transmission at the synapse, a growing body of literature has shown that GABA_A receptors can be located either synaptically or extrasynaptically (Figure 3), with extrasynaptic GABA_ARs mediating tonic inhibition secondary to ambient levels of GABA or GABAergic spillover (Brickley & Mody, 2012; Farrant & Nusser, 2005; Wei, Zhang, Peng, Houser, & Mody, 2003). Synaptic GABA_A receptors that mediate phasic inhibition and the hyperpolarization of postsynaptic neurons typically comprise of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 2/3$ and $\gamma 2$ subunits, whereas extrasynaptic GABA_A receptors comprise of $\alpha 4$, $\alpha 5$, $\alpha 6$, together with $\beta 2/3$ and δ subunits, although combinations with $\alpha 1$ and γ have been reported (Barnard et al., 1998; Crestani, Assandri, Tauber, Martin, & Rudolph, 2002; Mortensen & Smart, 2006; Nusser, Sieghart, & Somogyi, 1998).

1.6.1.3 GABA_B Receptors

GABA_B receptors are metabotropic G-protein inhibitory receptors with 7 transmembrane domains. GABA_B receptors are found as two subtypes, GABA_{B1} and GABA_{B2}. While GABA_A receptors are located postsynaptically, GABA_B receptors are found both pre and postsynaptically (Figure 3) (Farb et al., 2005), thereby having the capacity to alter both afferent and efferent inhibitory neurotransmission at the synapse, respectively. The activation of GABA_B receptors can alter multiple cellular processes via slow synaptic transmission, either through the inhibition of neurotransmitter release (presynaptically) or through the inhibition of adenylate cyclase and downstream molecular pathways (postsynaptically). Activation of postsynaptic GABA_B receptors can also increase the intracellular potassium concentration by opening potassium ion channels, which ultimately hyperpolarizes the postsynaptic neuron and produces a slow inhibitory post-synaptic current (IPSC).

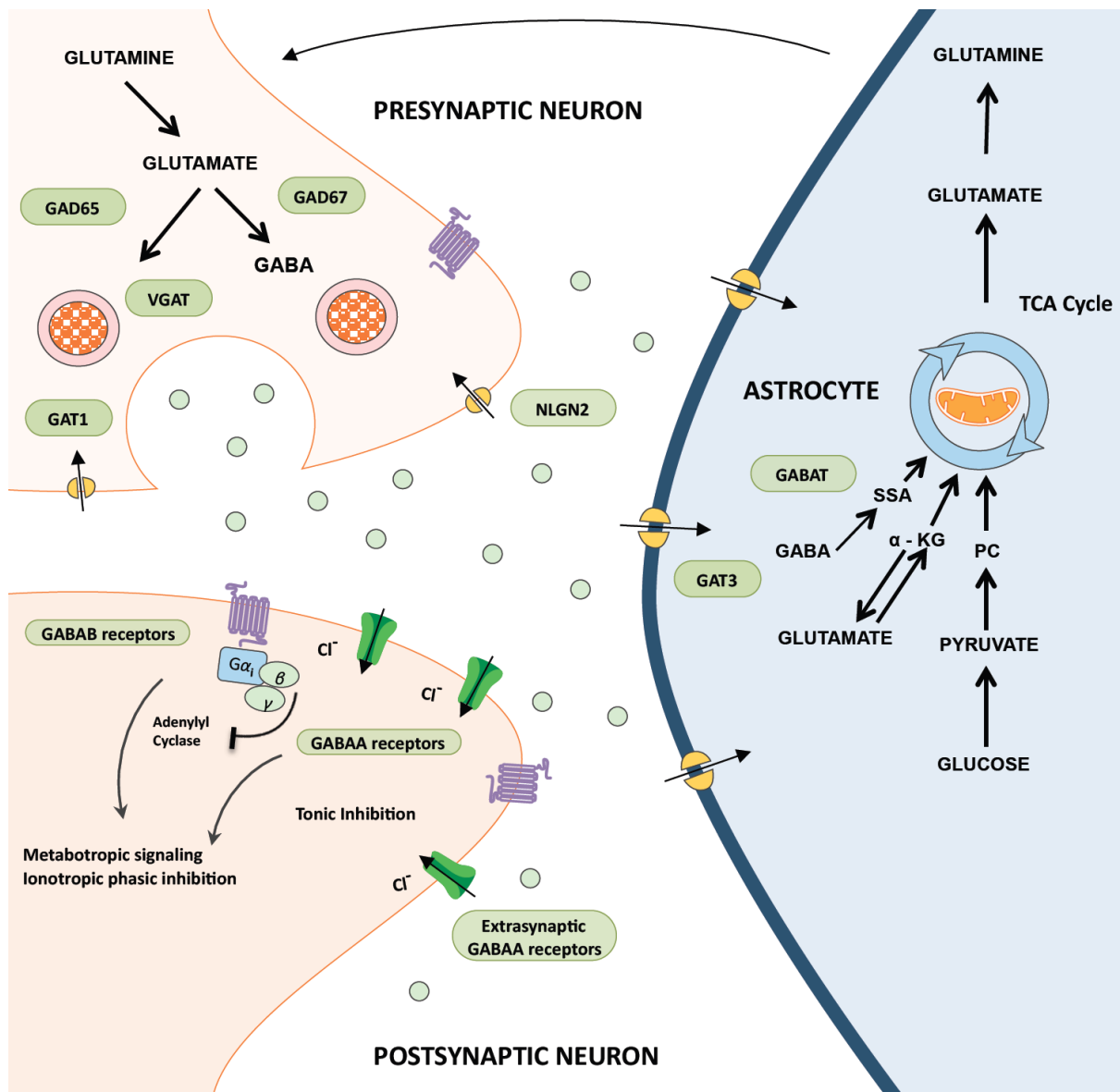


Figure 3. A diagrammatic representation of the production, transport, reuptake, action and degradation of GABA (γ -aminobutyric acid) at an inhibitory synapse in the cortex. GABA is produced by the α -decarboxylation of glutamate by the rate-limiting enzyme, glutamate-decarboxylase (GAD). GAD is present as two isoforms, GAD₆₇ and GAD₆₅, which mediate the production of the cytoplasmic and synaptic stores of GABA in the presynaptic neuron, respectively. GABA is transported to the neuronal membrane and synapse through the affinity of VGAT to bind to inhibitory vesicles. Exocytosis of GABA into the synapse activates a constellation of postsynaptic ionotropic (GABA_A) and metabotropic (GABA_B) receptors that mediate phasic inhibition and slow synaptic inhibitory neurotransmission, respectively. GABA spillover can also activate extrasynaptic GABA_A receptors that are responsible for the tonic inhibition of postsynaptic neurons. GABAergic signaling is terminated through the reuptake of GABA to either the presynaptic membrane, via the GABA transporter 1 (GAT₁), or to astrocytes through the action of GABA transporter 3 (GAT₃). GABA is metabolized to glutamate and succinic-semialdehyde via the action of GABA transaminase (GABA_T). Drawing designed and created by adapting various slides purchased from Motifolio (<http://motifolio.com/>).

Table. 1 GABA_A Receptor Subunits in the Brain

Subunit	Gene	NCBI Reference	Other information	Localization	References
α 1	GABRA1	NM_183326.2	Highly co-assembled with β2 and γ2 subunits in cortical GABAergic neurons (43% of GABA _A receptors). Synaptically located on neuronal cell bodies.	Throughout the brain, highest expression in the cortex and thalamus. Less prominent in the striatum, reticular thalamic nucleus or internal granular layer of the olfactory bulb.	Fritschy & Mohler, 1995; B. Gao & Fritschy, 1994; Pirker et al., 2000b; Vinkers, Mirza, Olivier, & Kahn, 2010; Zimprich, Zezula, Sieghart, & Lassmann, 1991
α 2	GABRA2	NM_001135779.1	Co-distributed with a β3 and γ2 subunits. Often located on the axon initial segment of pyramidal cells. Associated with alcohol dependence	Highest expression in olfactory bulb, outer layers of the cortex, dentate molecular layer, hippocampus area CA3, central and lateral amygdala, striatum, nucleus accumbens and hypothalamus.	Edenberg et al. 2004; Matthews, Hoffman, Zezza, Stiffler, & Hill, 2007; Pirker et al., 2000; Zimprich et al, 1991; Fritschy & Mohler, 1995
α 3	GABRA3	NM_017069.2	The mRNA expression of the α3 subunit is developmentally regulated. It is the dominant subunit in the forebrain tissue at birth gradually decreasing in prominence as alpha subunit 1 takes over. Also experiments with mice have demonstrated that editing of pre-mRNA alpha 3 subunit increases from 50% at birth to nearly 100% in adult. Associated with unipolar depression.	Most prominent in the olfactory bulb, Inner layers of the cortex, the endoperiform nucleus, amygdala, the lateral septum, claustrum and in the superior colliculus. Less abundant in the basal ganglia, hippocampus, thalamus, hypothalamus and cerebellum. Expressed in monoaminergic neurons such as the raphe nuclei and the locus coeruleus in the brainstem and cholinergic neurons in the forebrain.	Pirker et al., 2000; Zimprich et al., 1991; Vinkers et al., 2005; Henkel et al., 2004; Wisden, Laurie, Monyer, & Seeburg, 1992
α 4	GABRA4	NM_080587.3	Generally diazepam-insensitive and co-assemble with a γ or δ subunit. They are located at extrasynaptic sites and responsible for tonic inhibition.	Highest concentrations in the thalamus (except ventral lateral geniculate, reticular and central medial thalamic nuclei), the striatum and nucleus accumbens, the tuberculum olfactorium, the molecular layer of the dentate gyrus. Less concentrated in the cerebral cortex, the CA1 sector of the hippocampus, the septum, the outer layers of the solliculus superior and the entire brainstem. Uniquely present in the thalamus, cortex and dentate gyrus	Pirker et al., 2000; Vinkers et al., 2005; Wisden et al., 1992; Fritschy & Mohler, 1995

Subunit	Gene	NCBI Reference	Other information	Localization	References
$\alpha 5$	GABRA5	NM_017295.1	Comprise 15-20% of the diazepam-sensitive GABA _A receptors in the hippocampus. Located extrasynaptically, co-assembling with $\beta 3$ and $\gamma 2$ subunits at the base of dendritic spines where they modulate excitatory glutamatergic input. Associated with bipolar disorder	High concentrations are found in the olfactory bulb (external plexiform layer and internal granular layer), inner layers of the cerebral cortex, endoperiform nucleus, subiculum, Ammon's horn and ventromedial hypothalamic nucleus.	Pirker et al., 2000; Vinkers et al., 2005; Wisden et al., 1992; Fritschy & Mohler, 1995; McKernan & Whiting, 1996; Otani et al., 2005
$\alpha 6$	GABRA6	NM_021841.1	Associated with neuroticism	Only in the granule cell layer of the cerebellum	Pirker et al., 2000; Vinkers et al., 2005; Wisden et al., 1992; Fritschy & Mohler, 1995; Sen et al., 2004; Wang et al., 2004
$\beta 1$	GABRB1	NM_012956.1		Distributed widely in the brain, notably in the cerebral cortex. Less expressed compared to $\beta 2$ and $\beta 3$.	Pirker et al., 2000
$\beta 2$	GABRB2	NM_012957.2	Highly co-assembled with $\alpha 1$ and $\gamma 2$ subunits in cortical GABAergic interneurons (43% of GABA _A receptors). Genetic deletion is not lethal and does not cause seizures.	Distributed widely in the brain, notably in the cerebral cortex and thalamus. Most highly expressed in the Cortex. Highly expressed in the pallidum of the basal ganglia to the striatum. Highly expressed in thalamic nuclei (except reticular nucleus)	Fritschy et al., 1992; Gao & Fritschy, 1994; McKernan and Whiting, 1996; Pirker et al., 2000
$\beta 3$	GABRB3	NM_017065.1	Mutations in this gene may be associated Angelman syndrome, Prader-Willi syndrome, and autism, particularly with regard to savant skills. Genetic deletion of the $\beta 3$ subunit produces non-viable offspring that die after birth. Gene deficient mice have impaired social and exploratory behaviors linked to autism spectrum disorders	Distributed widely in the brain, notably in the cerebral cortex and thalamus. Most abundant in the striatum, with higher concentrations in the striatum than the pallidum.	Nurmi et al., 2003; DeLorey, Sahbaie, Hashemi, Homanics, & Clark, 2008; Fritschy et al., 1992; Gao & Fritschy, 1994; McKernan and Whiting, 1996; Pirker et al., 2000.

Subunit	Gene	NCBI Reference	Other information	Localization	References
$\gamma 1$	GABRG1	NM_080586.1	Display a reduced affinity for classical benzodiazepines. Variants of this gene may be associated with alcohol dependence.	Preferentially located in the pallidum, substantia nigra, septum, medial and central amygdaloid nucleus and in the bed nucleus of the stria terminalis.	Fritschy et al., 1992; Gao & Fritschy, 1994; McKernan and Whiting, 1996; Pirker et al., 2000; Baulac et al., 2001
$\gamma 2$	GABRG2	NM_183327.1	75-80% of GABA _A receptors contain a $\gamma 2$ subunit. Highly co-assembled with $\beta 2$ and $\alpha 1$ (43% of GABA _A receptors). Mutations in this gene have been associated with epilepsy and febrile seizures.	Throughout the brain, highest expression in olfactory bulb, cortex, hippocampus, amygdale, septum and basal forebrain, pallidum, hypothalamus. Weak observations are observed in the thalamus	Fritschy et al., 1992; Gao & Fritschy, 1994; McKernan and Whiting, 1996; Pirker et al., 2000; Baulac et al., 2001
$\gamma 3$	GABRG3	NM_024370.3	Display a reduced affinity for classical benzodiazepines. Linked to alcoholism	Distributed in low concentrations throughout the brain. Present in distinct dendrites and somata and motor trigeminal nucleus and was unevenly disturbed in the olfactory bulb.	Pinker et al. 2000; Vinkers et al. 2005; Dick et al., 2004
δ	GABRD	NM_017289.1	Exclusively extrasynaptically located and co-expressed with $\alpha 4$ or the $\alpha 6$ subunit. GABA is a partial agonist at δ -containing GABA _A receptors. Mice lacking this subunit display spontaneous seizures.	Present in the thalamus, striatum, cortex, dentate gyrus (α) and in the cerebellum ($\alpha 6$)	Lenzen, Heils, Lorenz, Hempelmann, & Sander, 2005; Mihalek et al., 1999
ϵ	GABRE	/		Present in monoaminergic nuclei of the brainstem	Vinkers et al. 2005; Sinkkonen, Hanna, Kirkness, & Korpi, 2000
π	GABRP	/		Mainly peripherally located outside the brain	Vinkers et al. 2005
θ	GABRQ	/		Present in monoaminergic nuclei of the brainstem	Vinkers et al. 2005
ρ	GABRR1, GABRR2, GABRR3	/		Preferentially found in the retina	Vinkers et al. 2005; Cutting et al., 1991

1.6.2 Interneurons

The majority of neurons (70-80%) in the cortex are excitatory pyramidal neurons that provide major excitatory projections between brain regions and typically demonstrate similar anatomical, molecular and physiological characteristics (DeFelipe & Fariñas, 1992; Peters & Sethares, 1991). Interneurons, on the other hand, constitute the remaining 20-30% of neuronal cortical cell types and have diverse molecular, morphological, physiological and synaptic properties (Cauli et al., 1997; DeFelipe, 1993, 2002; Kawaguchi & Kubota, 1997; Markram et al., 2004). Unlike pyramidal cells, interneurons have aspiny dendrites, receive excitatory and inhibitory synapses onto their soma, and their axons typically project laterally across columns or within the same localized area, meaning that they do not project to distant brain regions (Markram et al., 2004). In light of their axon restrictions, interneurons are also called ‘local circuit neurons’. While some interneurons in the brain are excitatory, such as spiny stellate cells in layer IV of sensory areas (LeVay, 1973; Lund, 1973), the majority of interneurons are inhibitory and use GABA as their neurotransmitter.

A major characteristic of inhibitory interneurons is their capacity to target different subdomains of neurons, which ultimately gives rise to their considerable variability in somatic, dendritic and axonal morphology within the cortex (DeFelipe, 1997; Peter Somogyi, Tamás, Lujan, & Buhl, 1998). The heterogeneous nature of inhibitory interneurons ultimately allows for specific temporal-regulation of pyramidal compartments and for the inhibitory regulation of excitatory input and output across various brain regions, including the PFC. Classification of interneuron subtypes, however, is consequently difficult, particularly due to the lack of objective features that can be used as classifying criteria (Ascoli et al., 2008). For example, dendritic morphology is the most variable feature of GABAergic interneurons and interneuron type cannot be determined by dendritic information alone. However, axonal morphology is more specific across interneuronal subtypes, and therefore axonal targeting, in

conjunction with additional morphological and neurochemical phenotypes, can be used to differentiate amongst classes of inhibitory interneurons. While a range of interneuronal subtypes can be distinguished, the prevalence of each type varies as a function of species, brain region and neuronal layer.

Inhibitory interneurons can be distinguished based on their electrophysiological output and their molecular markers. Interneurons are typically characterized as representing three-broad classes of cells associated with irregular spiking (Porter et al., 1998), bursting (Kawaguchi & Kubota, 1993; Toledo-Rodriguez et al., 2004) and fast-spiking patterns (Chow et al., 1999; Toledo-Rodriguez et al., 2004). Fast-spiking interneurons have a constant high frequency firing rate with minimal interval between spikes, which is readily distinguishable from pyramidal cells and other interneuronal subtypes (Zaitsev et al., 2009). As such, it follows that fast-spiking neurons have homogenous membrane properties, such as large hyperpolarizations and fast action potentials, which are believed to be secondary to the expression of potassium channels (particularly Kv3.1), sodium channels and rapid activation kinetics (Jonas, Bischofberger, Fricker, & Miles, 2004; Rudy & McBain, 2001). Fast-spiking cells form axons that target the perisomatic region, proximal dendrites, the initial axon segment and the cell body of pyramidal cells (Figure 4). They are differentiated from other neuronal subtypes by the presence of the calcium binding protein, parvalbumin (Freund, 2003) and parvalbumin-expressing cells have the highest density in layers 3 and 4 of the PFC. Fast-spiking cells can be further differentiated into two distinct subtypes, basket and chandelier cells (Figure 4). Basket cells can be large, small or nested basket cells, all of which innervate the soma, the proximal dendrites and spines of pyramidal cells (Marin-Padilla, 1969; Wang, Gupta, Toledo-Rodriguez, Wu, & Markram, 2002). Chandelier cells, on the other hand, synapse onto the initial axon segment of pyramidal cells, and can therefore be referred to as axo-axonic cells (Somogyi, 1977). Parvalbumin-expressing neurons innervate

either the axon or soma of pyramidal cells, placing them in a position to provide strong inhibitory control over dendritic integration, postsynaptic action potentials and cell firing of excitatory efferents (Miles, Tóth, Gulyás, Hájos, & Freund, 1996; Zhu, Stornetta, & Zhu, 2004). Indeed, parvalbumin-expressing cells regulate feed-forward inhibition and neuronal output, and given they have contact with a vast number of postsynaptic targets, these are requisite for the neuronal oscillation and synchronization of large groups of neurons (Cobb, Buhl, Halasy, Paulsen, & Somogyi, 1995; Massi et al., 2012; Pouille & Scanziani, 2001; Sohal, Zhang, Yizhar, & Deisseroth, 2009; Volman, Behrens, & Sejnowski, 2011).

Non fast-spiking cells, on the other hand, show an adaptive firing pattern whereby the interval between spikes increases with stimulus duration, and compared with fast-spiking cells, they have slower membrane constants and have slower synaptic inputs and outputs (Jonas et al., 2004). The majority of non fast-spiking cells innervate at dendritic sites away from the soma or initial axon segment (Megias et al., 2001), suggesting that they have a distinct functional role compared to parvalbumin-expressing fast-spiking cells. Non fast-spiking cells are more heterogeneous than fast-spiking cells and contain a distinct collection of neuropeptides. Cholecystokinin containing basket cells also target the perisomatic region of pyramidal cells, (Figure 4), although their input is different compared to parvalbumin-expressing basket cells (Somogyi et al., 2004). Martinotti cells, which are characterized by the presence of somatostatin, innervate mostly the distal dendritic arbor of pyramidal cells (Figure 4) (Kawaguchi, 1995; Yasuo Kawaguchi & Kondo, 2002). Calbindin-containing double bouquet/neurogliaform cells have a dense axonal arbor around its own soma, suggesting that it projects predominantly to nearby dendrites (Kalinichenko, Dudina, & Motavkin, 2005). Overall, these findings suggest that non fast-spiking cells likely alter excitatory input to pyramidal cells, particularly given that they synapse onto pyramidal dendrites, and therefore have the capacity to modulate excitatory integration and the plasticity

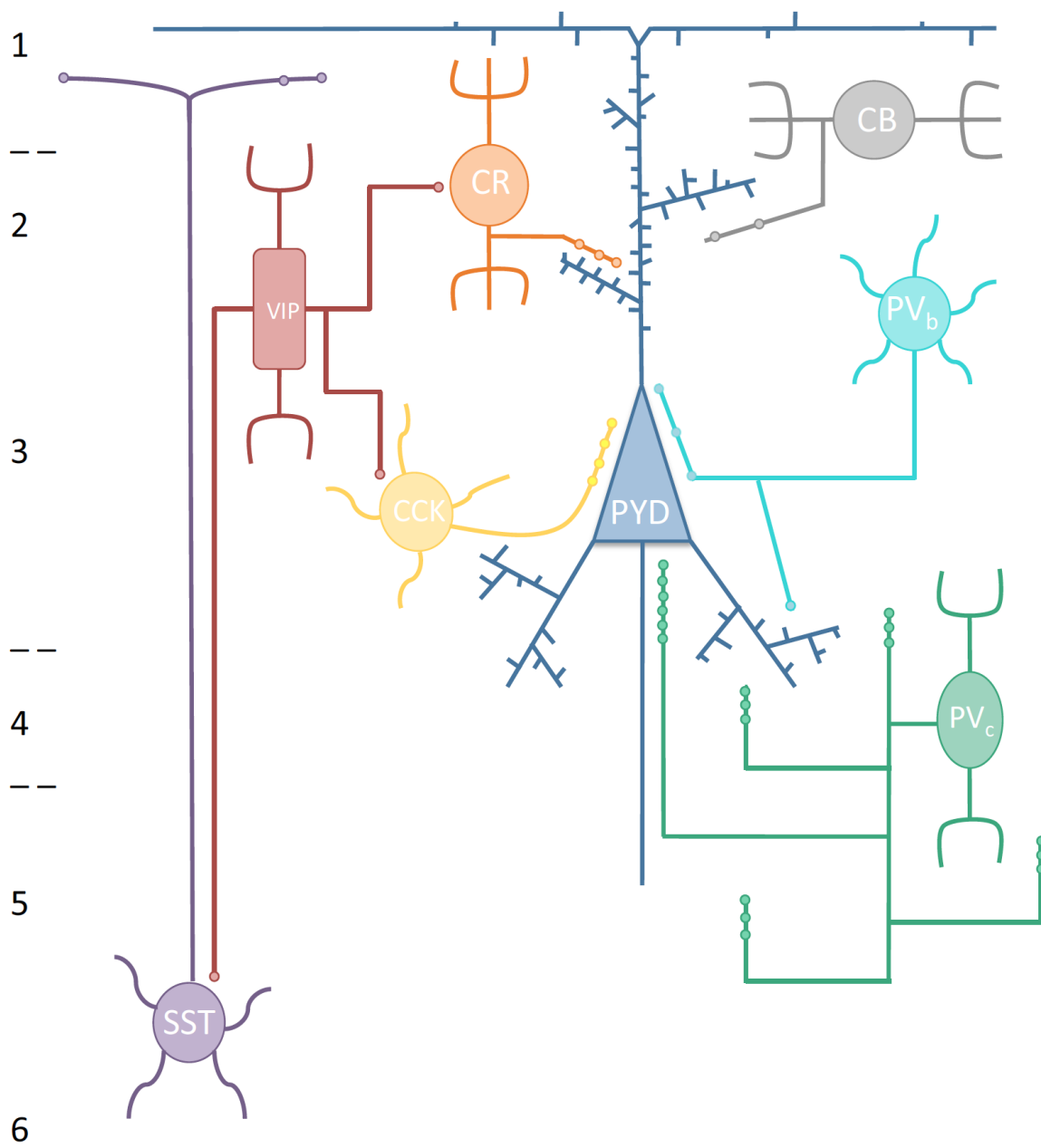


Figure 4. Graphical representation of the various interneuronal subtypes in the prefrontal cortex (PFC) as expressed by their molecular markers. Parvalbumin (PV) is present in fast-spiking chandelier (PVc; green) and basket cells (PVc; light blue), which target the initial axon segment and perisomatic region and pyramidal cells (PYD; blue), respectively. Similarly, cholecystokinin-containing basket cells (CCK; yellow) target the initial axon segment of the pyramidal neuron. Somatostatin (SST; purple) cells are intrinsic to martinotti cells that target the pyramidal distal dendritic arbor while vasoactive intestinal peptide (VIP; red) is localized to multipolar cells that innervate other interneurons, suggesting they play a role in neuronal disinhibition. The calcium binding proteins, calbindin (CB; grey) and calretinin (CR; orange) represent bouquet interneurons that are characterized by a bursting and irregular spiking patterns, respectively.

of glutamatergic synapses. However, vasoactive intestinal peptide-containing multipolar cells directly inhibit other interneurons (Pi et al., 2013), suggesting a prominent role of vasoactive intestinal peptide interneurons in the disinhibition of interneurons in the cortex.

1.6.3 GABAergic Dysfunction in Schizophrenia

Given that GABAergic system is heavily involved in the production of synchronized network oscillations during cognitive tasks (Başar, Başar-Eroglu, Karakaş, & Schürmann, 2001; Gonzalez-Burgos, Hashimoto, & Lewis, 2010), dysfunctional GABAergic signaling of the PFC is believed to mediate the altered gamma oscillatory patterns and executive deficits inherent to schizophrenia (Keefe & Harvey, 2012; Lewis, 2012; Minzenberg et al., 2010). In fact, various animal models have provided evidence that altered GABAergic signaling of the PFC, particularly with respect to GABA_A receptors, leads to cognitive, emotional and biological changes that are routinely observed in schizophrenia (Tse, Piantadosi & Floresco, 2007), providing support that GABAergic disturbances of the PFC may subserve the expression of cognitive impairment in the disorder.

Alterations to the GABAergic network are some of the most consistent molecular changes in the PFC of schizophrenic brains. For example, down-regulated mRNA and protein expression of GAD₆₇ has been consistently reported at the tissue level of the PFC using various genetic and protein analyses (Akbarian, Kim, et al., 1995; Blum & Mann, 2002; Curley et al., 2011; Gonzalez-Burgos et al., 2010; Guidotti, Auta, Davis, & et al., 2000; Hashimoto et al., 2008; Joshi, Fung, Rothwell, & Weickert, 2012; Straub et al., 2007; Volk, Austin, Pierri, Sampson, & Lewis, 2000), with the average expression reduced between 12.0% and 68.0% (Lewis, 2012). More specifically, GAD₆₇ mRNA-positive cells appear to be consistently reduced by 25.0 to 35.0% across layers 1 – 5 of the PFC in subjects with schizophrenia (Curley et al., 2011; Lewis, Hashimoto, & Volk, 2005). The fact that the total

number of GABA neurons are unchanged in the PFC of schizophrenia, with the majority of these cells exhibiting unaltered level of GAD₆₇ indicates that only a subset of inhibitory neurons are specifically affected in the disorder. Interestingly, mRNA and protein expression of GAD₆₅ is unchanged in the PFC of schizophrenia across tissue and cellular analyses (Hashimoto et al., 2008; Huang et al., 2007; Volk et al., 2012). As the expression of GAD₆₇ is activity dependent (Lau & Murthy, 2012), the preferential deficit of GAD₆₇ has been interpreted as a loss of cortical GABA in the PFC, which would have significant consequences on inhibitory neurotransmission and oscillation patterns (Gonzalez-Burgos et al., 2010; Volk & Lewis, 2010).

Additional GABAergic changes are evident in the PFC in schizophrenia. VGAT mRNA expression is decreased in the dorsolateral prefrontal cortex of schizophrenia (Hoftman et al., 2015) while the antipsychotic, clozapine, increases VGAT expression at GABAergic synapses in the rat (Bragina, Melone, Fattorini, & Conti, 2007). These findings indicate that GABA packaging and release is decreased in the PFC in schizophrenia, and that this may be normalized with clozapine administration. In light of the reduced synthesis and release of GABA at the synapse, GAT₁ expression, binding and the density of GAT₁ mRNA positive neurons is decreased in the PFC (Gonzalez-Burgos, Rotaru, Zaitsev, Povysheva, & Lewis, 2009; Hoftman et al., 2015; Ohnuma, Augood, Arai, McKenna, & Emson, 1999; Schleimer, Hinton, Dixon, & Johnston, 2004; D. Volk, Austin, Pierri, Sampson, & Lewis, 2001; Schleimer et al., 2004). Indeed, there is some evidence that GAD₆₇ and GAT₁ may be reduced in the same subset of inhibitory neurons, as within-subjects differences of density and laminar expressions have been found within the same matched controls and schizophrenia subjects, with GAD₆₇ and GAT₁ significantly correlated in the same subjects (Lewis, 2011; Lewis et al., 2005). These findings suggest that the changes to GAD₆₇ and GAT₁ are altered as a function of the other. There is also some evidence that GAT₃ binding is increased by

23.0% in the dorsolateral prefrontal cortex (DLPFC) of schizophrenia (Schleimer et al., 2004). Overall, these findings suggest that schizophrenia is associated with significant changes to the synthesis, release and reuptake of GABA at inhibitory synapses in the PFC.

GABA receptors are also implicated in the neurobiology of the PFC in schizophrenia. Several studies have indicated that schizophrenia is associated with increased number of GABA_A receptor in the PFC, as total [3H]muscimol binding at is increased in the PFC of schizophrenia (Benes, Vincent, Alsterberg, Bird, & SanGiovanni, 1992; Benes, Vincent, Marie, & Khan, 1996; Dean et al., 1999; Deng & Huang, 2006; Hanada, Mita, Nishino, & Tanaka, 1987). Indeed, mRNA expression of $\alpha 1$, $\alpha 2$ and $\alpha 5$ and protein expression for $\alpha 1$, $\alpha 2$ and $\beta 2/3$ appear to be upregulated in the PFC of schizophrenic brains (Impagnatiello et al., 1998; Ishikawa, Mizukami, Iwakiri, Hidaka, & Asada, 2004b; Ohnuma et al., 1999; Volk et al., 2002), although there is evidence of reduced mRNA expression of $\alpha 1$, $\alpha 5$ and $\beta 2$ in layers 3 and 4 of the PFC of schizophrenia (Akbarian, Huntsman, et al., 1995; Glausier & Lewis, 2011; Beneyto et al., 2010; Lewis, Curley, Glausier, & Volk, 2012). Nevertheless, as $\alpha 1$ and $\alpha 2$ receptors are localized on the synapses of pyramidal neurons in the PFC (Ishikawa, Mizukami, Iwakiri, Hidaka, & Asada, 2004a; Wisden et al., 1992), the upregulated expression of GABA_A receptors has been interpreted in the context of compensatory input to pyramidal neurons due to decreased GABA at the synapse (Benes et al., 1992; Benes et al., 1996; Ishikawa et al., 2004a; Weickert & Kleinman, 1998). Furthermore, not all GABA_A receptors appear to be similarly perturbed in the disorder. For example, $\gamma 1$ and $\gamma 3$ protein expression is unchanged in the PFC of schizophrenia (Ishikawa et al., 2004a) while the mRNA and protein expression of the $\gamma 2$ subunit is reduced (Akbarian et al., 1995; Huntsman, Tran, Potkin, Bunney, & Jones, 1998). As the $\gamma 2$ is requisite for benzodiazepine binding (Pritchett, Luddens, & Seeburg, 1989), several studies have found that GABA_A receptor binding at benzodiazepine-sites is unchanged (Owen, Cross, Crow, Lofthouse, & Poulter, 1981) or

reduced (Squires, Lajtha, Saederup, & Palkovits, 1993). These findings indicate that alterations to GABA_A receptors in the PFC of schizophrenia are subtype specific, with receptors devoid of the $\gamma 2$ subunit, and therefore benzodiazepine binding, being preferentially upregulated in the disorder. With regard to GABA_B receptor expression, protein studies have found reductions in the expression of GABA_B labelling in pyramidal cells within the PFC of schizophrenia (Ishikawa, Mizukami, Iwakiri, & Asada, 2005).

Given the range of inhibitory interneurons present in the cortex, it is possible that the changes to inhibitory neurotransmission in schizophrenia are dependent on the type of interneuron affected. Indeed, parvalbumin mRNA expression is downregulated in the PFC and GAD₆₇ mRNA expression is reduced in parvalbumin-expressing neurons in the PFC of schizophrenia (Gonzalez-Burgos et al., 2010; Hashimoto et al., 2003), with in situ hybridization studies demonstrating the 50.0% of parvalbumin mRNA positive neurons lack GAD₆₇ mRNA (Hashimoto et al., 2003). Laminar specific analyses have shown that the reduced expression of parvalbumin and GAD₆₇ is most prominent in layers 3 and 4 while approximately 50.0% of GAD₆₇ protein expression is reduced in parvalbumin basket cells (Glausier, Fish, & Lewis, 2013). Consistent with the relationship between GAT₁ and GAD₆₇, parvalbumin and GAD₆₇ mRNA expression are positive correlated in the PFC (Lewis et al., 2005). As an extension of these findings, the reduction of GAT₁ expression is most prominent in parvalbumin-expressing chandelier interneurons (Curley et al., 2011; Hashimoto et al., 2003; Nakazawa et al., 2012); cells believed to mediate the tonic inhibition of pyramidal neurons and the neuronal synchronization and oscillations during cognitive tasks (Başar et al., 2001; Gonzalez-Burgos et al., 2010), particularly within the PFC (Benchenane, Tiesinga, & Battaglia, 2011). Indeed, parvalbumin chandelier cells are associated with a 40.0% reduction of GAT₁ protein expression in schizophrenia (Pierri, Chaudry, Woo, & Lewis, 1999; Woo, Whitehead, Melchitzky, & Lewis, 1998). As such, there appears to be a close relationship in

the expression of GAT₁, GAD₆₇ and parvalbumin in the PFC of schizophrenic brains. In addition to these findings, mRNA expressions of cholecystokinin (Hashimoto et al., 2008), somatostatin (Fung et al., 2010; Hashimoto et al., 2008) and neuropeptide Y (Hashimoto et al., 2008) are reduced in the PFC of patients with schizophrenia. Interestingly, calretinin appears to be unaltered (Lewis et al., 2005), and given that 40.0 to 50.0% of interneurons express calretinin, this finding strengthens the argument that schizophrenia is associated with a selective inhibitory deficit in the PFC.

Overall, it is clear that GABAergic dysfunction of the PFC in schizophrenia has been well described, with schizophrenia characterized by reduced expression of GAD₆₇, GAT₁ and GABA_B receptors, upregulated expression of GAT₃ and GABA_A receptors together with unaltered GAD₆₅ expression. These changes appear to be most prevalent in parvalbumin expressing interneurons, although there is some evidence that cholecystokinin and somatostatin, but not calretinin interneurons, are implicated in the inhibitory pathophysiology of the PFC of schizophrenia. These findings are potentially salient in describing the changes to oscillations and cognitive impairments of the disorder. However, research surrounding PFC GABA-mediated neurotransmission following METH sensitization has been less studied.

1.6.4 GABAergic Dysfunction in Sensitization to Psychostimulants

A growing body of literature has shown that psychostimulant use is associated with the GABAergic system. At the genetic level in humans, METH use has been associated with genes for the GABA_Aα1 (Lin, Chen, Ball, Liu, & Loh, 2003) and the GABA_Aγ2 subunits, with the latter being replicated across several studies (Lin et al., 2003; Nishiyama et al., 2005). In light of the fact that sensitization is typically associated with increased activity of the mesolimbic pathway, METH sensitization is associated with decreased GAD₆₇ and GABA_Aα2 protein expression in the NAc core and shell in animal subjects, while the same

proteins are increased in the striatum (Zhang et al., 2006). While increased GAD₆₇ protein expression in the striatum has also been reported in amphetamine sensitization (Pereira et al., 2008), others animal studies have found reduced GAD₆₇ mRNA and protein expression following sensitization to METH (Jayanthi, Deng, Noailles, Ladenheim, & Cadet, 2004; Pereira et al., 2012). The differences in expression may be related to the dosage of METH used. For example, Jayanthi et al. (2004) administered 40 mg/kg of METH to rats across 7 days, and therefore the reduced expression of GAD₆₇ mRNA and protein may be related to increased apoptosis of GABAergic neurons following this neurotoxic dose.

A more specific role of GABAergic neurotransmission in sensitization to psychostimulants has derived from animal research that has specifically targeted the GABA system through specialized compounds. For example, clonazepam, a GABA-benzodiazepine agonist, attenuated the acquisition of sensitization when it was systemically administered prior to METH administration (Ito, 1999; Ito, Ohmori, Abekawa, & Koyama, 1997, 2000). Furthermore, sensitization was not affected by the administration of flumazenil, a GABA-benzodiazepine antagonist, prior to METH administration, nor was sensitization affected by the administration of clonazepam prior to METH challenge (Ito et al., 1999, 2000), suggesting a specific role of GABA_A receptors in the initiation of sensitization to amphetamine. Additionally, the GABA_B receptor agonist, baclofen, when administered systemically, has been shown to inhibit the development (Bartoletti, Gubellini, Ricci, & Gaiardi, 2005; Cedillo & Miranda, 2013) and the expression of amphetamine sensitization (Bartoletti, Gubellini, Ricci, & Gaiardi, 2004). Overall, these findings suggest a role of GABA receptors in the development and the expression of sensitization to psychostimulants. However, given the systemic approach used, these studies provide little insight into the brain regions that mediate these altered behavioral responses.

1.6.5 The Prefrontal Cortex GABAergic System and Behavioral Sensitization

Although PFC GABAergic neurons do not directly project to the mesolimbic pathway, interneurons are able to modulate the mesolimbic system through inhibition of excitatory projections from the PFC, meaning that the PFC GABAergic network is in a position to mediate sensitized behavior. Indeed, some research findings have suggested a role of PFC GABAergic neurons in sensitization to psychostimulants. Specifically, using metabonomics, Bu et al. (2013) found that GABA was reduced in the PFC following sensitization to METH, a finding they believed was secondary to increased GABAergic metabolism in the PFC. Peleg-Raibstein et al. (2008), by using immunohistochemistry, also found that GAD₆₇ protein expression was downregulated in the inner layers of the PFC following amphetamine sensitization, although this finding was only evident after long-term (70 days) and not short-term (6 days) withdrawal. Additional studies have also shown molecular GABAergic changes following sensitization. For example, parvalbumin protein expression is downregulated in layer 5 of the PRL following sensitization to amphetamine (Morshedi & Meredith, 2007). Some evidence also suggests that METH use is associated with loss of calbindin interneurons in the cortex. For example, METH users with HIV have selective loss of calbindin interneurons compared to HIV positive non-users (Langford et al., 2003), suggesting this loss may be specific to METH. Furthermore, Kuczenski et al. (2007) found decreased calbindin expression in the neocortex following extended exposure to METH. While these findings suggest that calbindin cells are implicated in GABAergic dysfunction of the PFC following METH, these findings should be interpreted with caution in light of the limited regional specific descriptions provided by Kuczenski (2007) and given the compounding neurodegenerative effects of HIV and METH (Liu et al., 2009; Reiner, Keblesh, & Xiong, 2009). Consequently, it is uncertain whether these changes reflect the effect of METH sensitization on calbindin-positive interneurons in the PFC or the effects of neurodegradation.

With regard to GABA receptor function, sensitization to amphetamine is associated with unchanged GABA_A receptor binding in the global PFC (Gruen, Stoker, Friedhoff, & Bradberry, 1999) while Armstrong and Noguchi (2004) found no change in GABA_A receptor binding in the anterior cingulate following 5 days of subcutaneous injections of METH (32 mg/kg/day), as measured by autoradiography with [3H]-Flunitrazepam. Furthermore, sensitization to amphetamine is not associated with altered GABA_B receptor binding in the mPFC (Zhang, Tarazi, Campbell, & Baldessarini, 2000), although the same study showed that GABA_B receptor coupling to G proteins was increased, suggesting that sensitization may be associated with increased inhibitory metabotropic receptor signaling (Zhang et al., 2000). Indeed, Arai et al. (2009) found that chronic METH administration produced cognitive deficits in recognition memory that were ameliorated by baclofen but not gaboxadol, a GABA_A receptor agonist, suggesting that GABA_B receptors may be specifically involved in mediating cognitive dysfunction associated with METH use and sensitization. In support for the role of GABA_B receptors in the PFC, baclofen has been shown to ameliorate METH induced PPI, a cognitive deficit typically associated with hyperdopaminergic PFC function (Mizoguchi & Yamada, 2011). Interestingly, intra-PFC administration of GABA_B receptor agonists, SK597541 and baclofen, significantly reduces basal and amphetamine-induced dopamine levels (Balla et al., 2009), suggesting that GABA_B compounds may inhibit dopaminergic neurons that are otherwise hyperexcitable following sensitization, with this inhibition normalizing cognitive dysfunction.

1.7 Summary and Thesis Aims

In summary, while evidence of dysfunctional GABAergic processing following sensitization to psychostimulants has begun to accrue, there are a number of limitations in the extant literature. Specifically, the majority of aforementioned research has focused on the

striatum, have been based on the effects of chronic METH independent to sensitization, and of those that have examined sensitization, the conclusions have predominantly derived from research on amphetamine and/or cocaine. Consequently, there is a paucity of research that has specifically assessed changes to the PFC GABAergic network following METH sensitization, particularly with respect to molecular changes. Indeed, while some molecular studies have assessed individual components of the GABA system in the PFC following sensitization (e.g. GABA, GAD₆₇, GABA_A & GABA_B receptor binding), these have been assessed across separate experiments that are concomitant with variations in sensitization protocols and/or dosing regimens. Therefore, drawing conclusions around the functional significance subserved by these changes is difficult to ascertain. The aim of this thesis, therefore, was to investigate changes to the PFC GABAergic system following sensitization to METH. The current thesis contributes to this existing body of empirical work by providing a multi-method evaluation of the proteomic, genomic and cellular changes associated with the GABAergic system across global and localized regions of the PFC following METH sensitization.

Furthermore, few studies of METH sensitization have placed these results in the context of psychosis. Consequently, there is a need to elucidate whether METH sensitization and schizophrenia have overlapping inhibitory neuropathologies of the PFC, as this will assist in determining whether chronic METH psychosis represents a biologically distinct disorder to schizophrenia and whether the GABAergic system could mediate psychosis symptoms and vulnerability to relapse. As such, the findings determined throughout the subsequent chapters are continuously compared with research that has examined the role of similar neurological factors in the PFC of schizophrenia.

Four studies were conducted for this thesis and are presented as separate journal articles:

- The aim of chapter 2 was to determine the differential changes to the proteome of the

prefrontal cortex following METH sensitization, with particular emphasis on pathways and proteins that had been previously implicated in the neurobiology of schizophrenia. The findings of the study served to identify particular biological substrates that could represent common biological markers across METH sensitization and schizophrenia and thereby informed the progression of the subsequent experiments in this thesis.

- In light of the significant changes to the GABAergic proteomic network identified in the PFC following METH sensitization identified in Chapter 2, the aim of Chapter 3 was to determine the relative expression of GABAergic mRNA expression in the PFC following METH sensitization.
- The aim of Chapter 4 was to extend the findings found in Chapter 3 by isolating and examining the changes to GABAergic mRNA expression of the prelimbic and orbitofrontal cortices of the PFC following METH sensitization, with further analysis of the role of inhibitory metabolism and transport in mediating altered inhibitory environments across subregions of the PFC.
- In light of the fact that specific GABAergic changes may represent alterations secondary to changes in interneurons, Chapter 5 addressed whether mRNA encoding for interneuronal inhibitory markers was differentially expressed across the prelimbic and orbitofrontal cortex of rats sensitized to METH. An additional aim of this chapter was to correlate the expression of altered GABAergic mRNA expression and interneuronal mRNA in METH-sensitized rats in order to elucidate whether GABAergic deficits corresponded to a particular inhibitory cellular phenotype.
- The final chapter discusses the major findings of this thesis, how these relate to the role of the PFC in sensitized behavior, the clinical implications of this research together with the strengths and limitations of this thesis.

Chapter Two

Methamphetamine-induced Sensitization is Associated with Alterations to the Proteome of the Prefrontal Cortex: Implications for the Maintenance of Psychotic Disorders

Chapter published as:

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Please see Appendix A for a copy

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1. Introduction

Psychotic disorders, such as schizophrenia, represent a class of heterogeneous, chronic and complex illnesses that are deleterious for quality of life and traditionally associated with poor treatment outcomes (Harvey et al., 2012; Jablensky, 2000; Sharma & Antonova, 2003). While seminal work in the psychiatric field placed the pathogenesis of psychotic disorders in the context of dysfunctional monoamine and neurotransmitter systems in the brain, with particular emphasis on dopamine (Howes & Kapur, 2009) and glutamate (Laruelle, 2014), treatment regimes that aim to rectify the catecholamine changes in the central nervous system are unable to ameliorate all psychotic symptomatology, such as affective states and cognitive dysfunction (Keefe & Harvey, 2012; Leucht, Arbter, Engel, Kissling, & Davis, 2009). As such, research has consequently attempted to unravel the molecular mechanisms underlying psychotic disorders with the expectation of finding downstream mediators that could represent therapeutic targets that normalize all aspects of psychotic pathology. Even though research describing these biological markers has accrued, knowledge concerning the etiology of a psychotic state, or the biological underpinning that maintain this vulnerability, is still relatively unknown.

Methamphetamine (METH) is a potent psychostimulant that has a high prevalence of psychotic symptoms amongst both recreational (McKetin, Hickey, Devlin, & Lawrence, 2010) and chronic users (Chen et al., 2003; Farrell et al., 2002; McKetin, McLaren, Lubman, et al., 2006), with METH contributing to the development of psychosis even after controlling for additional drug use (McKetin et al., 2013). While most psychotic symptoms are transient and resolve once the drug is ceased, anecdotal and observational research has suggested that METH-induced psychosis may develop into a more persistent psychotic syndrome that is indistinguishable from schizophrenia (Lecomte et al., 2013; Medhus et al., 2013; Sato, 1992; Yui, Ishiguro, Goto, & Ikemoto, 2000), with METH-users more likely to have a

schizophrenia diagnosis than controls (Callaghan et al., 2012; Kittirattanapaiboon et al., 2010). The fact that chronic METH use can result in the development of a chronic schizophrenia-like psychosis suggests that METH may induce certain brain changes that are consistent with schizophrenia pathology, or that chronic METH use and schizophrenia share a common biological vulnerability.

Researchers have placed the neurobiological vulnerability to psychosis in the context of behavioral sensitization (Ujike, 2002; Yui, Goto, et al., 1999). Behavioral sensitization refers to the unique phenomenon whereby repeat exposure to a stimulus results in a progressively increased behavioral response to the stimulus following a period of abstinence (Pierce & Kalivas, 1997). Indeed, METH users can experience a relapsed psychotic state from a single low-dose re-exposure to METH or alternative psychostimulants after decades of abstinence (Akiyama, Saito, & Shimoda, 2011; Sato, Chen, Akiyama, & Otsuki, 1983), while schizophrenia patients can relapse to a more severe psychosis once their medication is discontinued (Ohmori et al., 1999) or after they experience a significant stressor (Olivares et al., 2013). Interestingly, patients with schizophrenia can experience a psychotic relapse following exposure to METH at a dose that does not cause psychosis in healthy controls (Lieberman et al., 1987). These findings suggest that both schizophrenia and METH-induced psychosis share common neuronal mechanisms that initiate and maintain a persistent vulnerability to psychosis, with sensitization as a key mediating factor that links these conditions to each other. Importantly, sensitization to psychostimulants can be reliably and effectively induced in experimental animals, with locomotor sensitization (hyperlocomotion) regarded as an animal model of human stimulant-induced psychosis (Featherstone et al., 2007; Ujike, 2002). As such, to further understand the molecular markers that characterize sensitization will not only help understand the etiology of drug-induced psychosis, but also the pathogenesis of schizophrenia in humans.

The prefrontal cortex (PFC) is critically involved in mediating higher-order cognitive processes relating to behavioral and cognitive control (Anderson, Bechara, Damasio, Tranel, & Damasio, 1999; Ridderinkhof, van den Wildenberg, Segalowitz, & Carter, 2004). It is therefore not surprising that a large body of neuroimaging (Minzenberg et al., 2009), neuropsychological (Reichenberg & Harvey, 2007) and post-mortem studies (Mistry, Gillis, & Pavlidis, 2013) have implicated significant PFC dysfunction in the pathogenesis of schizophrenia, with executive impairment regarded as an inherent characteristic of the disorder (Keefe & Harvey, 2012). Similarly, chronic METH-use is associated with executive dysfunction and damage to the PFC (Kamei et al., 2006), with METH-induced psychosis associated with cognitive dysfunction that is indistinguishable from schizophrenia (Srisurapanont et al., 2011). Further, METH sensitization is associated with PFC-mediated cognitive dysfunction, such as deficits to attention, prepulse and latent inhibition (Featherstone et al., 2007), suggesting common executive dysfunction across psychotic syndromes. While numerous transmitter systems within the PFC are proposed to mediate the etiology and maintenance of behavioral sensitization (Pierce & Kalivas, 1997; Steketee, 2003), understanding of the underlying biological mechanisms that serve as common substrates across METH sensitization, METH-induced psychosis and schizophrenia within the PFC is ongoing.

Proteomics provides a high-throughput method of evaluating the differential expression of multiple proteins, and therefore functional output, of a biological system or diseased state. While previous research has examined the effect of METH at the mRNA and protein level for multiple researcher-selected targets, the proteome of the PFC has only been examined at 24 hours after acute METH administration exposure (Kobeissy et al., 2008) or after 8 days of METH exposure in adolescent rats (Faure, Hattingh, Stein, & Daniels, 2009). As such, no research has examined the effect of behavioral sensitization to chronic METH

exposure on the PFC proteome in adult rats, nor have any studies placed their results in the context of psychotic disorders.

The aim of the current study was to investigate differential changes in protein expression in the PFC following behavioral sensitization to chronic METH exposure using quantitative label-free shotgun proteomics. Using this information, we provide an analysis of the proteins identified as being differentially expressed with respect to previous schizophrenia and psychoses literature. By identifying common molecular pathways that mediate the PFC-driven symptomatology in these disorders, the molecular pathogenesis and maintenance of psychosis following METH and in schizophrenia can be better understood.

2. Materials and Methods

2.1 Animals

Twelve experimentally naïve male Sprague-Dawley rats (Animal Resource Centre, WA, Australia), weighing an average of 261 ± 6 g at the start of testing, were used. Animals were housed in groups of four in plastic high top cages [(64 cm (L) x 40 cm (W) x 20 cm (H))] that were kept in a humidity- and temperature-controlled room (21 ± 2 °C, 60% humidity) and maintained on a 12 h light: 12 h dark cycle (lights on at 0600 h). All experimentation was carried out during the light period. The rats were given *ad libitum* access to food and water in their home cages for the duration of the experiment. Rats were acclimated to their new surroundings for one week and then handled daily for an additional week prior to drug treatment and behavioral testing. All experimental procedures were approved by the Macquarie University Animal Ethics Committee (reference number ARA 2010/045; Appendix C) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2004).

2.2 Methamphetamine-induced Behavioral Sensitization

2.2.1 Drug Schedule

On Day 1, all rats received an injection of saline (0.9%, 1 ml/kg, i.p.) and were placed into the measurement apparatus to record locomotor activity. Rats were then allocated to treatment groups (METH n=6 or Saline n=6) based on baseline locomotor activity such that there was no significant difference between groups prior to the commencement of the drug schedule ($p = 0.67$). Rats in the METH group were treated with once daily 1.0 mg/kg of METH (i.p.) on days 2 and 8, while the same rats received once daily injections of 5.0 mg/kg METH (i.p.) on days 3 – 7. These doses were selected to represent low to moderate administration whereby exposure would unlikely cause neurotoxicity while simultaneously

inducing locomotor sensitization and are consistent with previous methods (Iwazaki, McGregor, & Matsumoto, 2008; Morshedi & Meredith, 2008). Rats in the control group received daily injections of 1.0 ml/kg saline (i.p.) from day 2 to 8. On days 9 to 22, all rats were given a 14-day withdrawal period in their home cages. On day 23, all rats were injected with 1.0 ml/kg saline (i.p.) to test for conditioned baseline responding. The next day, all rats were assessed for methamphetamine-induced sensitization via a challenge dose of 1mg/kg METH (i.p.).

2.2.2 Behavioral Measures

In order to quantify the behavioral effects of the METH administration schedule and to confirm the development of behavioral sensitization, locomotor activity was recorded on Day 1, 2 and 8 of the drug regime and on Day 15 and 16 of drug withdrawal, respectively. Sixteen standard chambers [25 cm (L) × 31cm (W) ×50 cm (H)] consisting of aluminum tops and side panels, together with plexi-glass front and back panels with a metal rod floor (16 rods, 6 mm diameter, 15 mm apart), were used. Each chamber was equipped with four infrared photobeam detectors (Quantum PIR motion sensor, part no. 890-087-2, NESS Security Products, Australia) positioned on the front and back panels approximately 50 mm apart and 30 mm above the floor. Locomotor activity was quantified as the number of photobeam interruptions and recorded via a computer equipped with Med-IV PC software (Med Associates, St Albans, VT, USA). Rats were placed in the test chamber 15 minutes prior to drug injection to reduce novelty-induced activity before locomotor activity was recorded (60 mins). Each chamber was cleaned with ethanol solution (70%) between trials.

2.2.3. Drugs

Methamphetamine hydrochloride (METH) was purchased from the Australian

Government Analytical Laboratories (Pymble, NSW) and was dissolved in 0.9% saline. Intraperitoneal injections (i.p.) were made at a volume of 1 ml/kg with control rats treated with saline (0.9%).

2.3 Statistical Analysis for Methamphetamine-induced Sensitization

All results are reported as means \pm SEM. To determine locomotor sensitization, mean locomotor activity in response to METH challenge was compared between Saline and METH pretreated rats using a two-tailed independent t-test. Additionally, a paired t-test was used to compare mean baseline locomotor activity on day1 for METH pretreated rats to the mean locomotor activity in response to METH challenge. Statistical analyses were performed using SPSS version 17 and the significance level was set at $p < 0.05$.

2.4 Brain Dissection and Proteomic analyses

2.4.1 Sacrifice

One hour following the METH challenge, rats were euthanized via rapid decapitation, their brains removed, rinsed and cooled in wet ice/water mixture. The PFC was dissected out on dry ice stored at -80°C until analysis. The dissection method has previously been described in detail (Harkin, Connor, Mulrooney, Kelly, & Leonard, 2001).

2.4.2 Protein Extraction and Fractionation by SDS-PAGE

PFC samples were homogenized in buffer (0.32mM sucrose, 2mM EDTA, 1% SDS) with a dounce homogenizer. The solution was centrifuged at 14 000 rpm for 15 minutes at 4°C , the supernatant removed and the pellet stored at -20°C until further analysis. 50 μl aliquots of protein were combined with 20 μl of a 5x SDS sample buffer containing 200mM DTT and then separated using a Bio-Rad 10% Tris-HCl SDS-PAGE gel. Coomassie Brilliant

Blue G-250 (Bio-Rad) was used to stain the gel overnight. A solution of 50% H₂O, 40% Methanol and 10% Acetic Acid destained the gel for 2 hours prior to in-gel digestion.

2.4.3 Trypsin in-gel Digestion

Using a scalpel, each of the 6 gel lanes was cut into 16 equal pieces, with each piece further divided into 4 equal pieces before being transferred to a 96-well plate. Gel pieces were briefly washed with 100mM NH₄HCO₃ and then 3 times with 200µl of ACN (50%)/100mM NH₄HCO₃ (50%), each for 10 mins. Fractions were then dehydrated with 100% ACN for 5 minutes, air dried, and then reduced using 50µl of 10mM DTT/NH₄HCO₃ (50mM) at 37°C for 1 hr. Samples were cooled at room temperature in the dark before being alkylated with 50µl of 50mM iodoacetamide/NH₄HCO₃ (50%) for 45 mins, then washed with 100mM NH₄HCO₃ for 5 minutes and washed twice with 200µl of ACN (50%)/100mM NH₄HCO₃ (50%) each for 10 minutes. Gel pieces were then dehydrated with 100% ACN and air-dried. Finally, samples were placed on ice and digested with 20µl of trypsin (12.5ng/ml 50mM NH₄HCO₃) for 30 minutes before being covered and left to digest overnight at 37°C.

2.4.4 Peptide Extraction

Remaining solutions from trypsin digestion were transferred to individual Eppendorf tubes and 50µl of ACN (50%)/formic acid (2%) was added before being incubated for 30 mins. This was repeated twice to give a final extraction volume of approximately 90µl for each of the 16 fractions. Extracts were then dried using a vacuum centrifuge and reconstituted to 10µl with 2% formic acid.

2.4.5 Nanoflow LC-MS/MS

Nanoflow liquid chromatography/tandem mass spectrometry with a LTQ-XL linear

ion top mass spectrometer (Thermo, San Jose, CA) was used as previously described (Gammulla, Pascovici, Atwell, & Haynes, 2011; Mirzaei, Pascovici, Atwell, & Haynes, 2012). Briefly, reversed-phase columns were packed in-house using 100A, 500m Zorbax C18 resin (Agilent Technologies, CA, USA) to approximately 7cm (100mm id) in a fused silica capillary with an integrated electrospray tip. The tip was homemade with an inner diameter of approximately 10 to 15 μ m. A 1.8kV electrospray voltage was applied upstream of the C18 column via a liquid junction. Using a surveyor autosampler, each sample was injected onto the column followed by a wash of Buffer A (5% v/v ACN, 0.1% v/v formic acid) for 10 mins at 1 mL/min. Samples were then eluted from the column using Buffer B (95% v/v ACN, 0.1% v/v formic acid) at 500 nL/min at 0-50% for 58 mins followed by 50-90% for 5 mins and were directed into the mass spectrometer's nanospray ionization source. Spectra over the range of m/z 400-1500 were scanned, and automated peak recognition, dynamic exclusion (repeat count 1, repeat duration 30 seconds, list size 500, exclusion duration 90 seconds, exclusion by mass with 1.5 Dalton tolerance) and MS of the top six most intense precursor ions at 35% normalization collision energy were performed using Xcalibur software (Version 2.06, Thermo). The samples were injected in rows of 8 from the 96 well plate, with the three replicates of control tissue analyzed before the three replicates from the METH condition. Standards were run before and after data acquisition to ensure optimum system performance.

2.4.6 Database Search for Protein/peptide Identification

Raw data files were converted to mzXML format and analyzed using the global proteome machine software (GPM version 2.1.1) and the X!Tandem algorithm by searching Tandem Mass Spectra against the NCBI *Rattus Norvegicus* Reference Sequence database (94699 proteins, April 2013). To evaluate the false discovery rate (FDR), additional searching against a reversed sequence database was used. For each replicate, the 16 fractions were

individually and sequentially searched and the output merged into a single file using GPM software. The merged data only contained protein identifications with $\log(e)$ values < -1 . A 0.4 Da fragment mass error was used for peptide identification. Variable modifications were set for oxidation of methionine while fixed modifications were set for Carbamidomethylation of cysteine. For X! Tandem searches, the mass tolerance for fragment ions was 0.4 Daltons and the tolerance for parent ions was +3 daltons and -0.5 daltons. The enzyme specificity was set to trypsin.

2.4.7 Data Processing and Quantitation

GPM output files were combined for each condition using the Scrappy program (Neilson, Keighley, Pascovici, Cooke, & Haynes, 2013). Proteins were retained for quantification if they were present across all three replicates with a total spectral count greater than or equal to 6. Normalized spectral abundance factors (NSAF) were calculated for each protein as previously described (Neilson et al., 2013). When summarizing protein abundance for experimental conditions, the mean NSAF value across triplicates was used.

2.5 Statistical Analysis of differentially Expressed Proteins

Only proteins (i.e. spectral count > 6) present in all three replicates for at least one experimental condition were included in the data set. The protein false discovery rate was calculated using the reverse database as decoy (i.e. $FDR = \# \text{ reverse proteins identified} / \text{Total proteins}$) in addition to the peptide false discovery rate (Peptide $FDR = \# \text{ reverse peptide identification} / \text{Total peptides}$). In order to determine whether proteins were differentially expressed between experimental conditions, independent t-tests were performed on the log transformed NSAF values for individual proteins. The significance level was set at $p < 0.05$ for all comparisons. Differentially expressed proteins were then further differentiated based

on their protein-protein relationships, signaling pathways and cellular and biological functions using Ingenuity Pathway Analysis (IPA) software. The data was searched against the Ingenuity Pathways Knowledge Base (IPKB), a continuously updated knowledge base of known proteins from peer reviewed scientific publications.

3. Results and Discussion

3.1 Sensitization to Methamphetamine

Methamphetamine challenge resulted in a significant increased locomotor response in METH pre-treated rats compared to saline controls, $p < .05$ (Figure 1A). METH pre-treated rats also showed a significant increased locomotor response on challenge when compared to their locomotor response on Day 1 of METH exposure, $p < .01$ (Figure 1A). Furthermore, as shown in Figure 1B, time-course analysis of the locomotor response to METH challenge revealed that METH pre-treated rats were significantly more active than saline pre-treated controls from 15 min to 35 min following challenge administration, $p < .001$. Collectively these results suggest that repeated METH administration induced locomotor sensitization in response to METH challenge and are consistent with a large body of literature that has shown increased hyperlocomotion following repeated psychostimulant administration (Ago et al., 2012; Pierce & Kalivas, 1997). Importantly, given that locomotor sensitization is regarded as an animal model of human stimulant-induced psychosis (Featherstone et al., 2007; Ujike, 2002), with sensitized behavior observed in both METH psychosis (Akiyama et al., 2011; Sato et al., 1983) and schizophrenia (Ohmori et al., 1999; Olivares et al., 2013), the clear sensitized response to METH would suggest that any protein changes detected could represent biological factors that may contribute to the vulnerability to psychosis.

3.2 Proteomic Analysis

Table 1 represents a summary of proteins and peptides identified across replicates in our experimental conditions. A total of 1317 nonredundant proteins were identified across both control and METH-sensitized conditions, with 1312 protein reproducibly identified in controls and 1314 proteins identified across the METH-sensitized group (Table 1). False discovery rates (FDRs) were calculated after combining replicate data and were consistently

low at the peptide level (FDR = 0.00768%; Table1) and protein level (FDR = 0.0759%; Table 1), indicating that the data was of adequate stringency and further filtering was not necessary. There was also minimal variability between the numbers of peptides counted in each nano LC-MS/MS run between biological replicates and across conditions, with a relative standard deviation of approximately 3% across treatment groups. This suggests that the number of identified peptides was highly consistent and given our previous experience has shown that protein expression can be reliably detected and confirmed by additional molecular approaches when the calculated %RSD is less than 10% (Lee et al., 2011; Pascovici, Keighley, Mirzaei, Haynes, & Cooke, 2012), the low variability between replicates confirmed that validation through the use of additional measures was not required.

3.3 Proteins related to METH Sensitization and Schizophrenia

Given that the replicates were highly reproducible, with little variability between samples (<3% RSD), a cut-off of 1.3 fold-change was applied to the differentially expressed proteins. METH-treated rats showed a significant down-regulation of 32 proteins (Table 2) in the PFC while 64 proteins were significantly up-regulated (Table 3) compared to saline controls. To discuss and interpret the biological significance of each of these changes individually is beyond the scope of this article, although each variation in protein expression could represent a significant area of potential investigation. To overcome this, differentially expressed proteins were further characterized based on their biological functions and cellular processes through the use of IPA (Table 4). The top biological functions in order of significance were cellular assembly and organization, cell-to-cell signaling and interaction, cellular function and maintenance, small molecule biochemistry and cell morphology. There were 235 canonical pathways linked to METH sensitization in the PFC, with the top

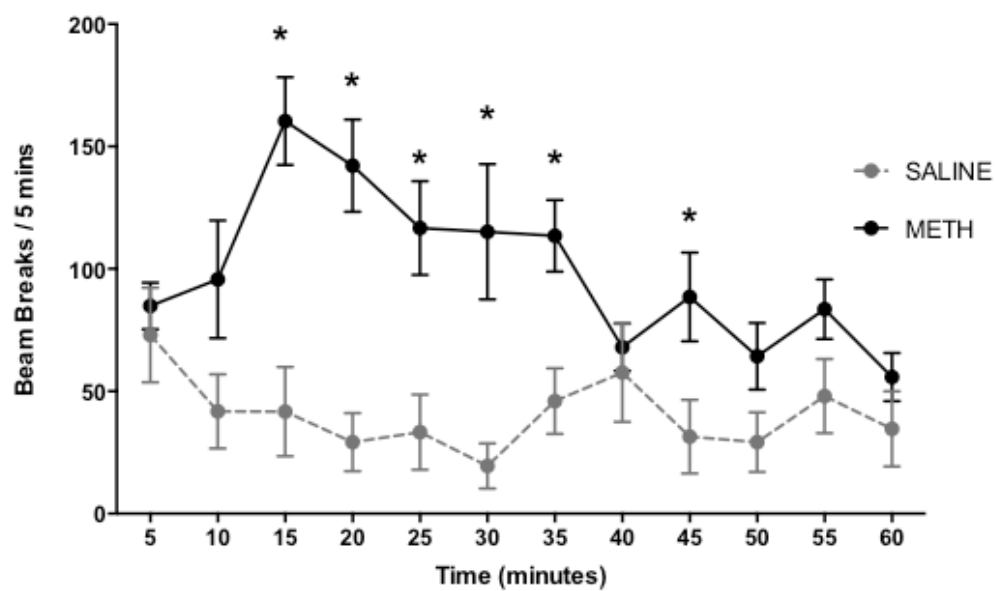
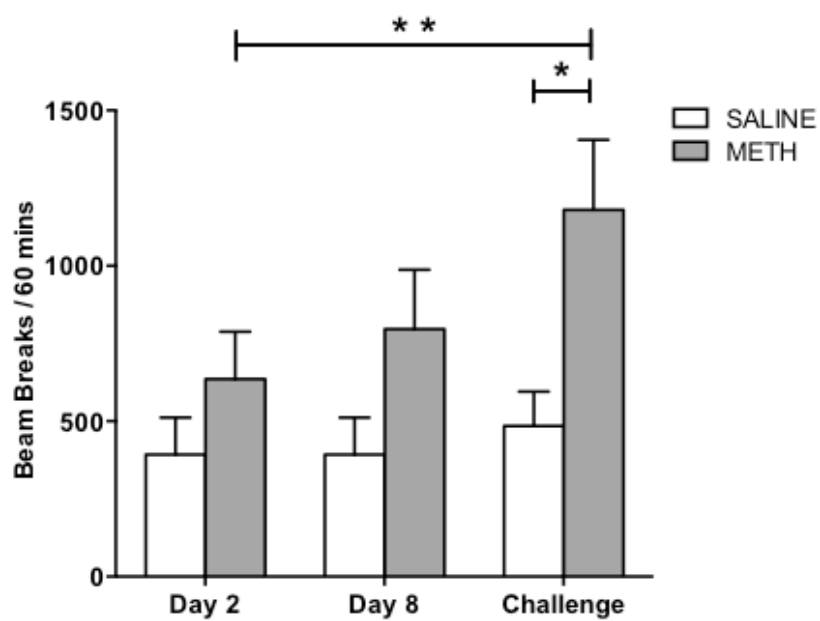


Figure 1. Locomotor sensitization to repeated METH administration. Rats were assigned to undergo either repeated METH (1ml/kg intraperitoneal (i.p.) days 1 & 7; 5mg/kg i.p. days 2 – 6, n=6) or saline (1 mg/kg i.p., n=6) treatment for 7 days. Following 14 days of withdrawal, both METH and saline rats were challenged with an acute methamphetamine (1mg/kg, i.p.) injection. Figure 1A represents the total beam breaks across days 2, 8 and challenge, respectively. Rats showed a progressively increased locomotor response to repeated METH administration, with a METH challenge resulting in a significant sensitized locomotor response in METH pre-treated animals when compared to saline controls*. METH treated rats also showed a significant increase in locomotor activity between Day 2 and challenge**. Figure 1B represents the timecourse of locomotor activity for METH and saline pre-treated rats over 60 minutes post challenge administration. Data represented as mean + SEM beambreak in each 5 minutes period. METH-treated rats displayed significant higher

Table 1. Summary of peptide and protein analysis for both control and methamphetamine groups

	Total	Control			Methamphetamine			Control	Methamphetamine	FDR (%)
		1	2	3	1	2	3	Average (± %RSD)		
Proteins	1317	1260	1261	1255	1255	1280	1274	1259 (± 0.26%)	1270 (± 1.03%)	0.0759
Peptides ^a	143185	24406	25200	23867	22189	23555	23968	24491 (± 1.58%)	23237 (± 2.31%)	0.0077

^a low stringency peptides

Table 2. Down-regulated proteins (fold change > 1.3) in the prefrontal cortex following behavioral sensitization to chronic methamphetamine exposure, presented by fold change.

ENSEMBL NUMBER	SYMBOL	ENTREZ GENE NAME	FOLD	P
ENSRNOG00000030628	EIF4A1	eukaryotic translation initiation factor 4A1	-10.242	0.0020
ENSRNOG00000000007	GAD1	glutamate decarboxylase 1 (brain, 67kDa)	-8.283	0.0000
ENSRNOG00000026705	DGKI	diacylglycerol kinase, iota	-5.615	0.0250
ENSRNOG00000008744	COPS2	COP9 signalosome subunit 2	-5.346	0.0180
ENSRNOG00000000841	DDX39B	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B	-4.396	0.0470
ENSRNOG00000002989	NMT1	N-myristoyltransferase 1	-4.191	0.0190
ENSRNOG00000009495	SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	-3.781	0.0180
ENSRNOG00000019298	DCTN4	dynactin 4 (p62)	-2.987	0.0290
ENSRNOG00000014984	DMWD	dystrophia myotonica, WD repeat containing	-2.985	0.0270
ENSRNOG00000017852	NARS	asparaginyl-tRNA synthetase	-2.787	0.0130
ENSRNOG00000023529	RPL5	ribosomal protein L5	-2.236	0.0210
ENSRNOG00000004806	STRN	striatin, calmodulin binding protein	-2.175	0.0290
ENSRNOG00000015430	NLGN2	neuroligin 2	-2.109	0.0040
ENSRNOG00000010042	WDFY3	WD repeat and FYVE domain containing 3	-1.939	0.0030
ENSRNOG00000014718	ACSL3	acyl-CoA synthetase long-chain family member 3	-1.88	0.0090
ENSRNOG00000018795	RPL18A	ribosomal protein L18a	-1.78	0.0100
ENSRNOG00000023373	SEC24B	SEC24 family, member B (S. cerevisiae)	-1.763	0.0380
ENSRNOG00000002339	MARK1	MAP/microtubule affinity-regulating kinase 1	-1.708	0.0450
ENSRNOG00000018326	PGLS	6-phosphogluconolactonase	-1.659	0.0420
ENSRNOT00000007554	VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	-1.644	0.0240
ENSRNOG00000007518	NCKAP1	NCK-associated protein 1	-1.628	0.0400
ENSRNOG00000003782	ACOT9	acyl-CoA thioesterase 9	-1.605	0.0390
ENSRNOG000000046502	LONP1	lon peptidase 1, mitochondrial	-1.558	0.0000
ENSRNOG00000026930	NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39kDa	-1.556	0.0060
ENSRNOG00000009155	NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa	-1.538	0.0410
ENSRNOG00000006471	PVALB	parvalbumin	-1.52	0.0090
ENSRNOG00000005836	FAM49A	family with sequence similarity 49, member A	-1.499	0.0220
ENSRNOG00000008996	DPYSL5	dihydropyrimidinase-like 5	-1.43	0.0440
ENSRNOG00000030371	MT-CO2	cytochrome c oxidase subunit II	-1.419	0.0050
ENSRNOG00000008961	MAPRE3	microtubule-associated protein, RP/EB family, member 3	-1.401	0.0100
ENSRNOG00000011142	CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	-1.366	0.0040
ENSRNOG00000017428	MAP1B	microtubule-associated protein 1B	-1.335	0.0310

Table 3. Up-regulated proteins (fold change > 1.3) in the prefrontal cortex following behavioral sensitization to chronic methamphetamine exposure, presented by fold change.

ENSEMBL NUMBER	SYMBOL	ENTREZ GENE NAME	FOLD	P
ENSRNOG00000015182	PPP2CB	protein phosphatase 2, catalytic subunit, beta isozyme	32.57	0.0000
ENSRNOG00000009760	PALM	Paralemmmin	7.774	0.0122
ENSRNOG00000018700	MOBP	myelin-associated oligodendrocyte basic protein	6.392	0.0001
ENSRNOG00000008203	SYNPR	Synaptoporin	5.059	0.0035
ENSRNOG000000025715	DYNLRB1	dynein, light chain, roadblock-type 1	5.043	0.0000
ENSRNOG00000025539	VPS13A	vacuolar protein sorting 13 homolog A (S. cerevisiae)	4.327	0.0452
ENSRNOG00000019740	HDGFRP3	hepatoma-derived growth factor, related protein 3	4.289	0.0161
ENSRNOG00000014109	PSMD13	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	4.289	0.0161
ENSRNOG00000015320	ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex, C2 (subunit 9)	3.875	0.0157
ENSRNOG00000028366	GPHN	Gephyrin	3.786	0.0209
ENSRNOT00000006542	SEC14L2	SEC14-like 2 (S. cerevisiae)	3.437	0.0230
ENSRNOG00000012724	C1orf123	chromosome 1 open reading frame 123	3.428	0.0260
ENSRNOG00000004862	MRPS36	mitochondrial ribosomal protein S36	3.382	0.0265
ENSRNOG00000027408	PPID	peptidylprolyl isomerase D	3.344	0.0110
ENSRNOG00000014635	CLTA	clathrin, light chain A	3.295	0.0480
ENSRNOG00000010807	COX6C	cytochrome c oxidase subunit VIc	3.054	0.0250
ENSRNOG00000036835	COPZ1	coatamer protein complex, subunit zeta 1	3.027	0.0270
ENSRNOG00000018556	TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)	2.998	0.0460
ENSRNOT00000012915	TST	thiosulfate sulfurtransferase (rhodanese)	2.84	0.0070
ENSRNOG00000018457	PPP2R4	protein phosphatase 2A activator, regulatory subunit 4	2.795	0.0350
ENSRNOG00000013300	ATPIF1	ATPase inhibitory factor 1	2.739	0.0450
ENSRNOG000000049075	FABP5	fatty acid binding protein 5, epidermal	2.633	0.0170
ENSRNOG000000043210	PPP3R1	protein phosphatase 3, regulatory subunit B, alpha	2.382	0.0020
ENSRNOG00000003975	PFN1	profilin 1	2.341	0.0410
ENSRNOG00000012084	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	2.328	0.0430
ENSRNOG000000027149	RPL10	ribosomal protein L10	2.298	0.0230
ENSRNOG000000045928	MYL6	myosin, light chain 6, alkali, smooth muscle and non-muscle	2.284	0.0270
ENSRNOG000000004146	CORO7	coronin 7	2.125	0.0210
ENSRNOG00000007134	STRAP	serine/threonine kinase receptor associated protein	2.112	0.0160
ENSRNOG000000016580	RPS23	ribosomal protein S23	2.094	0.0110
ENSRNOG000000046705	Snx3	sorting nexin 3	2.085	0.0150
ENSRNOG00000020715	DDB1	damage-specific DNA binding protein 1, 127kDa	2.073	0.0110
ENSRNOG00000002440	RALB	v-ral simian leukemia viral oncogene homolog B	1.983	0.0030
ENSRNOG00000010434	DYNC1LI1	dynein, cytoplasmic 1, light intermediate chain 1	1.971	0.0490
ENSRNOG00000002642	PTGES3	prostaglandin E synthase 3 (cytosolic)	1.969	0.0400
ENSRNOG00000005924	DSTN	destrin (actin depolymerizing factor)	1.909	0.0460
ENSRNOG00000002693	NME1	NME/NM23 nucleoside diphosphate kinase 1	1.887	0.0210
ENSRNOG00000003990	GRB2	growth factor receptor-bound protein 2	1.876	0.0490
ENSRNOG000000019189	ACAT2	acetyl-CoA acetyltransferase 2	1.84	0.0360
ENSRNOG00000017446	NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa	1.803	0.0440
ENSRNOG00000007806	ARF5	ADP-ribosylation factor 5	1.759	0.0210
ENSRNOG00000011550	KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	1.744	0.0500
ENSRNOG000000012879	FABP3	fatty acid binding protein 3, muscle and heart	1.71	0.0120
ENSRNOG00000011857	MTPN	myotrophin	1.701	0.0230
ENSRNOG00000027006	HNRNPA3	heterogeneous nuclear ribonucleoprotein A3	1.696	0.0110
ENSRNOG00000016257	COTL1	coactosin-like 1 (Dictyostelium)	1.693	0.0300
ENSRNOG000000004494	LTA4H	leukotriene A4 hydrolase	1.685	0.0360
ENSRNOG00000016507	SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	1.682	0.0040
ENSRNOG000000047247	PTPRS	protein tyrosine phosphatase, receptor type, S	1.674	0.0210
ENSRNOG00000014868	HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	1.644	0.0390
ENSRNOG00000001559	MTX2	metaxin 2	1.633	0.0180
ENSRNOG00000016251	DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2	1.545	0.0060
ENSRNOG000000000840	ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2	1.505	0.0240
ENSRNOG00000005345	VSNL1	visinin-like 1	1.499	0.0010
ENSRNOG00000006947	PDHX	pyruvate dehydrogenase complex, component X	1.487	0.0330
ENSRNOG00000018680	RPL17	ribosomal protein L17	1.487	0.0310
ENSRNOG00000007895	PDHB	pyruvate dehydrogenase (lipoamide) beta	1.483	0.0150
ENSRNOG00000000805	GJA1	gap junction protein, alpha 1, 43kDa	1.481	0.0340
ENSRNOG00000012999	PHB2	prohibitin 2	1.478	0.0170
ENSRNOG000000012594	SUGT1	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	1.412	0.0000
ENSRNOG00000008569	NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	1.405	0.0430
ENSRNOG00000003365	CADM3	cell adhesion molecule 3	1.396	0.0430
ENSRNOG000000047374	GNAS	GNAS complex locus	1.367	0.0080
ENSRNOG00000018282	GDA	guanine deaminase	1.334	0.0140

Table 4. Ingenuity pathway analysis of the PFC following methamphetamine sensitization

Top Networks		
Network	Associated Network Functions	Score
	Cardiovascular Disease, Genetic Disorder, Metabolic Disease	53
	Organismal Survival, Cellular Assembly and Organization, Cellular Function and Maintenance	39
	Cellular Compromise, Cell Death and Survival, Nervous System Development and Function	37
	Neurological Disease, Cell Death and Survival, Nervous System Development and Function	30
	Cardiac Arteriopathy, Cardiovascular Disease, Gene Expression	30
Top Disease and Disorders		
	<i>p</i> -value	no.
Neurological Disease	3.88×10^{-6} - 4.17×10^{-2}	36
Psychological Disorders	3.88×10^{-6} - 4.56×10^{-2}	29
Skeletal and Muscular Disorders	2.18×10^{-5} - 4.75×10^{-2}	24
Hereditary Disorder	3.95×10^{-5} - 4.17×10^{-2}	31
Inflammatory Response	9.35×10^{-5} - 4.11×10^{-2}	6
Top Canonical Pathways		
	<i>p</i> -value	Ratio
Cellular Assembly and Organization	4.03×10^{-5} - 4.69×10^{-2}	32
Cell-to-Cell Interaction	9.35×10^{-5} - 4.75×10^{-2}	16
Cellular Function and Maintenance	9.35×10^{-5} - 4.43×10^{-2}	34
Small Molecule Biochemistry	1.96×10^{-4} - 4.75×10^{-2}	30
Cell Morphology	4.81×10^{-4} - 4.43×10^{-2}	26
Top Tox Lists		
	<i>p</i> -value	Ratio
Mitochondrial Dysfunction	5.06×10^{-6}	0.051
Decreased Permeability of Mitochondria	4.17×10^{-2}	0.143
Xenobiotic Metabolism Signaling	6.31×10^{-2}	0.014
Hypoxia-Inducible Factor Signaling	6.74×10^{-2}	0.029
Aryl Hydrocarbon Receptor Signaling	7.35×10^{-2}	0.019

Table 5. Proteins associated with the top molecular and cellular functions altered in the PFC following methamphetamine sensitization

Proteins Associated with Top Molecular and Cellular Functions			
Function	<i>p</i> -value	Up-regulated Proteins	Down-regulated Proteins
Cellular Assembly and Organization	4.03×10^{-5} - 4.69×10^{-2}	PPID, NME1, GJA1, PFN1, MYL6, RAB3A, GRB2, RALB, SNX3, PALM, GPHN, VAMP2, TST, DYNC1L1, GNAS, DSTN, SNRPD1, GFAP, HDGFRP3	MYO6, SRC, STRN, NLGN2, MAP1B, MARK1, DPYSL5, TUBA1A, DDX39B, SYNJ1, LONP1, MAPRE3, NCKAP1
Cell-To-Cell Signaling and Interaction	9.35×10^{-5} - 4.75×10^{-2}	GJA1, NME1, RAB3A, GRB2, RALB, PALM, GPHN, KCNAB2, VAMP2, PPP3R1, GFAP, FABP3, VSNL1	MYO6, SRC, SYNJ1, NLGN2
Cellular Function and Maintenance	9.35×10^{-5} - 4.43×10^{-2}	NME1, GJA1, PFN1, RAB3A, GRB2, RALB, PALM, VAMP2, DYNC1L1, HSP90AB1, PPP3R1, GNAS, DSTN, HSPE1, GFAP, HDGFRP3, NAPB	MYO6, PVALB, SRC, SYNJ1, NLGN2, MAP1B, MARK1, DPYSL5, LONP1, MAPRE3, NCKAP1
Small Molecule Biochemistry	1.96×10^{-4} - 4.75×10^{-2}	LTA4H, NME1, GJA1, ACAT2, COTL1, VAMP2, TST, ATP1F1, FABP5, HSP90AB1, PPP2R4, HSPE1, SEC14L2, FABP3, ATP6V1G2, PDHB	MYO6, SRC, PVALB, ACSL3, CYB5B, PGLS, MAP1B, ACOT9, SYNJ1, GAD1, SLC6A1, OGDH, LONP1
Cell Morphology	4.81×10^{-4} - 4.43×10^{-2}	PPID, GJA1, NDUFS8, NDUFA6, PPP3R1, DSTN, RALB, TST	SRC, STRN, TUBA1A, MAP1B, DPYSL5, NCKAP1

representing mitochondrial dysfunction. The proteins associated with these pathways are in Table 5.

In addition, neurological and psychological disorders were the top disease and disorders identified through the IPA analysis. As such, we show here that METH sensitization shares many of the similar molecular changes in the PFC as reported in schizophrenia. Specifically, 20% of differentially expressed proteins identified in the current study have previously been implicated in schizophrenia pathology (Figure 2) (Ayalew, 2012; Benitez-King, Ramirez-Rodriguez, Ortiz, & Meza, 2004; Halim et al., 0000; Martins-de-Souza, Gattaz, Schmitt, Rewerts, Maccarrone, et al., 2009; Martins-de-Souza, Gattaz, Schmitt, Rewerts, Marangoni, et al., 2009; Martins-de-Souza et al., 2010; Minoretti et al., 2006; Moskvina et al., 2009; Shao et al., 2008; Shimamoto et al., 2014; Smutzer, Lee, Trojanowski, & Arnold, 1998; Sun et al., 2011). Using ontology classification and categorization of biological functions and pathway membership of the differentially expressed proteins from the IPA analysis, we identified biological and functional categories that appear to be commonly altered across both METH sensitization and schizophrenia, and include mitochondrial function, synaptic proteins, protein phosphatase signaling and alteration to the ‘inhibitory GABAergic network’.

3.4 Top Canonical Pathway: Mitochondrial Dysfunction

A total of 8 proteins related to mitochondrial function and energy expenditure were differentially expressed in the PFC of METH sensitized rats, with mitochondrial dysfunction isolated as the top canonical pathway altered in our experimental paradigm (Table 5). Specifically, proteins involved in oxidative phosphorylation were differentially expressed, with decreases in the expression of two NADH dehydrogenase subunit proteins (NDUFA9, NDUF3) while two subunits were up-regulated (NDUFA6, NDUF8). Specifically,

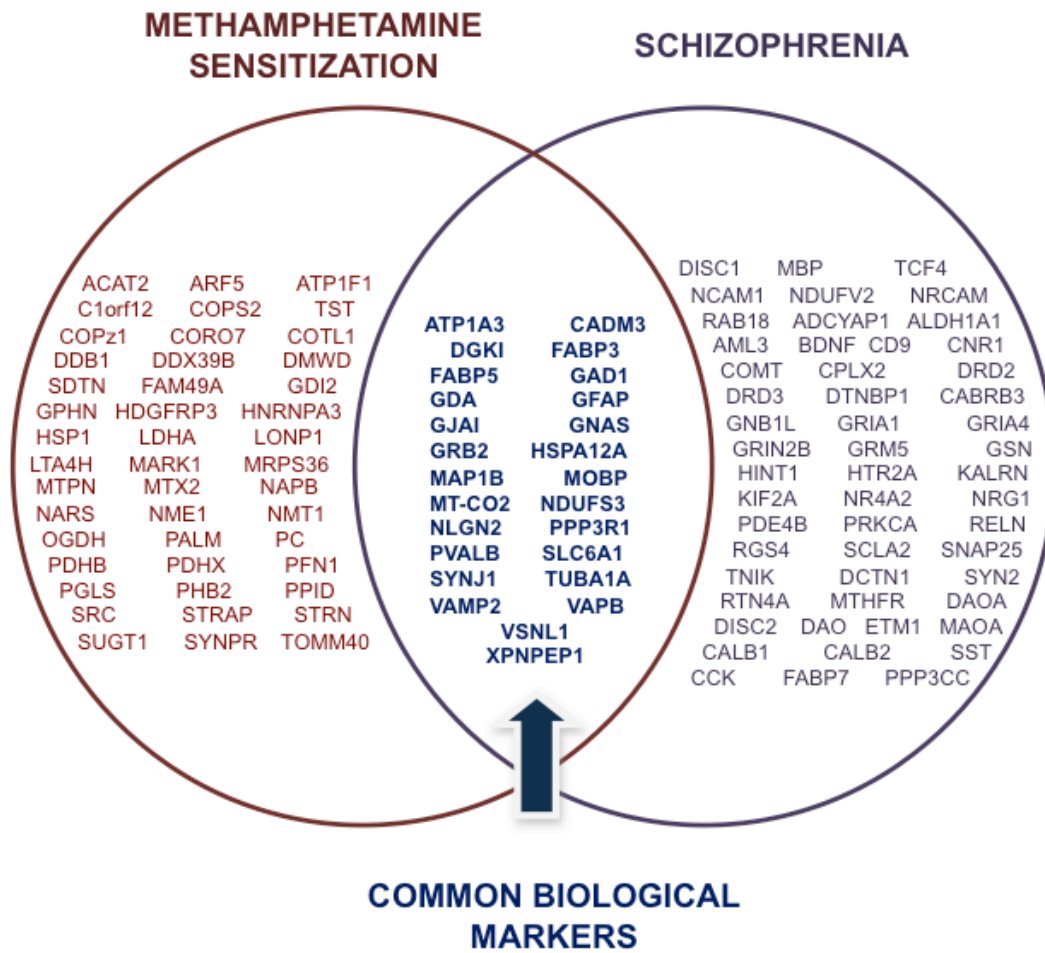


Figure 2. Venn diagram of the overlap between proteins involved in the neurobiology of methamphetamine sensitization and schizophrenia. The left represents proteins that were differentially expressed in the current study that had not been previously implicated in the neurobiology of schizophrenia and therefore reflect the effects of METH. The right represents proteins that have previously been implicated in the neurobiology of schizophrenia and are regarded as markers of dysfunction in the disorder (not measured in the current study)(Ayalew, 2012). Proteins that are altered in both disorders are shown in the overlap and are in bold.

NDUFS3 is important in the assembly and enzymatic activity of Complex 1 (Taurino et al., 2012), suggesting that a down-regulation of this enzyme likely contributes to the overall reduced activity of the complex and possibly oxidative phosphorylation. We also found cytochrome oxidase II (MT-CO2) was down-regulated and cytochrome oxidase VI (COX6C) was up-regulated in the PFC of rats sensitized to METH, suggesting such changes could have salient consequences on the energy production of the mitochondrial cellular environment within the PFC, particularly given that Complex IV (COX) synthesizes over 80% of the ATP required for cellular processes (Senior, 1988).

Accumulating morphological (Uranova et al., 2001), imaging (Buchsbaum & Hazlett, 1998; Steen, Hamer, & Lieberman, 2005) and genetic (James et al., 2004; Prabakaran et al., 2004) evidence has suggested a critical and prominent role of mitochondrial dysfunction in the pathogenesis and/or progression of schizophrenia, with alterations of subunits within ATPases and within complexes I-IV of the electron transport chain (ETC) differentially expressed in the PFC of schizophrenia patients (Karry et al., 2004; Prabakaran et al., 2004). Interestingly, mitochondrial diseases are frequently comorbid with psychotic symptoms (Fattal, Budur, Vaughan, & Franco, 2006) and can be misdiagnosed as schizophrenia (Mancuso et al., 2008), while mitochondrial conditions such as myopathy and lactic acidosis have been observed in patients with schizophrenia (Prayson & Wang, 1998). Given that the etiological cause for these disorders are mutations of mitochondrial DNA and mRNA coding for mitochondrial proteins, these observations increase the probability that psychotic symptoms are either the cause or secondary to mitochondrial dysfunction. As such, transgenic mice that have mitochondrial DNA deletions display dysfunctional PFC-mediated behavior that is also altered in schizophrenia, such as startle response (Kasahara et al., 2006). Thus, the changes in oxidative phosphorylation and energy production detected here may underlie certain behavioral changes associated with sensitization to METH and schizophrenia, such as

psychotic symptoms or cognition (Figure 3).

3.5 Molecular and Cellular Functions

3.5.1 Cell-to-cell Signaling and Interaction: Synaptic Proteins

A total of 15 proteins were differentially expressed in the PFC following sensitization to METH that could be involved in vesicle trafficking, synaptogenesis, gap junctions and neurotransmitter release, with many of these proteins previously implicated in the pathophysiology of schizophrenia. Specifically, gap Junction Protein 1, 43 kDa (GJA1; Connexin43) was up-regulated in the PFC of METH sensitized rats. GJA1 mediates gap-junction communication between glial cells and neurons, and safeguards against propagation of neuronal inactivation by reuptake of glutamate and potassium (Theis, Speidel, & Willecke, 2004). Interestingly, knock-out mice for the GJA1 gene display increased locomotor activity and exploratory behavior, suggesting an anxiolytic effect of GJA1 deletion (Frisch et al., 2003). It has also been hypothesized that changes to the function of gap junctions between astrocytes and neurons could contribute to the cognitive dysfunction reported in schizophrenia (Mitterauer, 2009).

Furthermore, 3 proteins (VSNL1, FABP3, GPHN) were up-regulated in the PFC of METH sensitized rats that are involved in the regulation of synaptic receptors. VSNL1 is a neuronal calcium sensor protein that binds to synaptic receptors in a calcium-dependent manner (Wang et al., 2011), leading to increased signaling, internalization and/or surface expression of acetylcholine and glutamate receptors. Further, FABP3 has been shown to regulate dopamine D2 receptors (Shioda, Yamamoto, Watanabe, Owada, & Fukunaga, 2010), while GPHN is specifically involved in the scaffolding and clustering of GABA receptors at post-synaptic sites (Fritschy, Panzanelli, & Tyagarajan, 2012). These findings likely suggest an increased density of post-synaptic receptor types, potentially as a

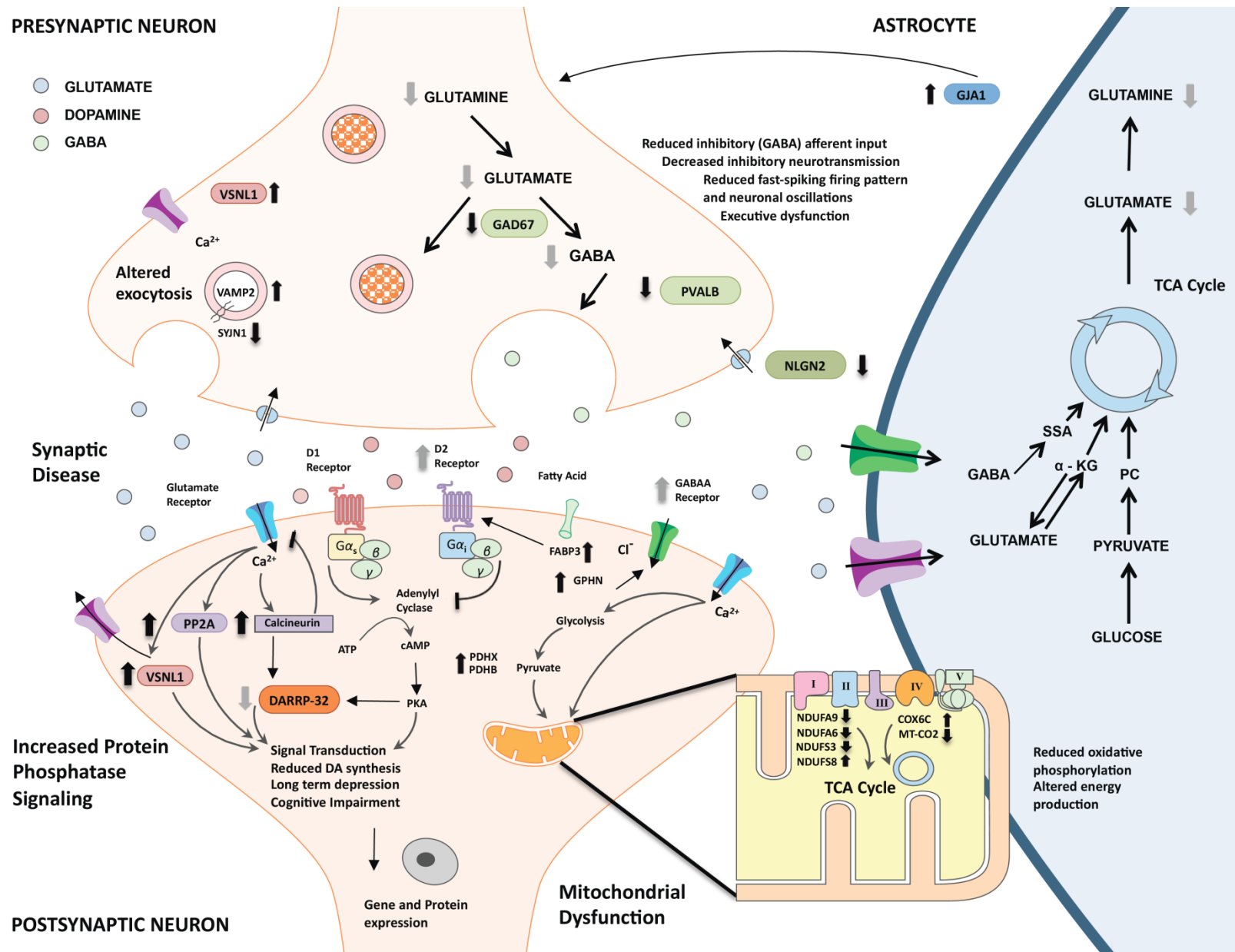


Figure 3. Depiction of the proposed common neurobiological mechanisms underlying the vulnerability to psychosis in the prefrontal cortex (PFC). Black arrows adjacent to protein names represent proteins that were differentially expressed in the current study, with the direction of the arrow representing whether the proteins was down- or up-regulated following METH sensitization. Grey arrows represent hypothesized changes based on previous research on METH sensitization and schizophrenia. GPHN and FABP3, which were increased in the current study, regulate the expression of GABA_A receptors and Dopamine D2 receptors, respectively, with D2 receptors negatively involved in the regulation of cAMP and corresponding intracellular downstream regulators such as PKA and DARPP-32. The NMDA and AMPA glutamate receptors allow influx of calcium into the cell, with altered calcium levels induced by METH treatment leading to increased expression of VSNL1, PP2A and PPP3R1 (calcineurin), which also negatively regulate the expression of DARPP-32. Collectively, alterations to these proteins are thought to lead to changes in signal transduction, dopamine synthesis, long-term depression and cognitive dysfunction. The disturbed intracellular calcium levels also affect energy pathways via modulation of pyruvate metabolism and oxidative phosphorylation, ultimately leading to changes in TCA activity and energy output. The decreased expression of GAD₆₇ results in reduced production of GABA and decreased inhibitory neurotransmission within the PFC. This most likely occurs in parvalbumin (PVALB) expressing interneurons, which together with neuroligin 2 (NLGN2) regulate interneuronal firing patterns and the oscillations requisite for cognitive tasks. As such, the down-regulation of PVALB and NLGN2 is hypothesized to result in reduced oscillatory power and executive dysfunction. Drawing designed and created by adapting various slides purchased from Motifolio (<http://motifolio.com/>).

compensatory mechanism for decreased neurotransmitter levels at afferent sites. VSNL1 has previously been shown to be up-regulated in the PFC of schizophrenia (Wesseling et al., 2013) and linked to both functional and morphological deficits in the disorder (Hong et al., 2009), particularly in pyramidal cells (Bernstein et al., 2003). Single-nucleotide polymorphisms VSNL1 have also been linked to schizophrenia cognitive impairments (Braunewell et al., 2011). Further, increased expression of dopamine D2 (Seeman & Kapur, 2000) and GABA receptors are reported in the PFC of schizophrenia, which could be linked to an up-regulation of FABP3 and GPHN, respectively. Overall, these findings suggest that changes to pre- and post-synaptic proteins may be a potential contributor to the pathogenesis and maintenance of these psychotic conditions (Figure 3).

3.5.2 Cellular Function and Maintenance: Protein Phosphatase Signaling

Three specific subunits (PPP2CB, PPP2R4, PPP3R1) of serine/threonine phosphoprotein phosphatases (PPPs) were up-regulated in the PFC of rats sensitized to METH. PPPs mediate intracellular signaling and dephosphorylation (Gee & Mansuy, 2005) and have been implicated in a range of synaptic functions (Mumby, 2007), with PPP3 (calcineurin) a key regulator of axonal guidance, endocytosis, exocytosis and signal transduction (Mansuy, 2003; Perrotti & Neviani, 2008). Importantly, calcineurin regulates intracellular calcium levels, making it a mediator of NMDA receptor function and long-term depression (LTD). As such, an up-regulation of calcineurin is negatively correlated with cognitive performance (Foster, Sharrow, Masse, Norris, & Kumar, 2001), while forebrain-specific PP2(B1)-knockout mice exhibit decreased working memory, latent and prepulse inhibition (Miyakawa et al., 2003; Zeng et al., 2001). Furthermore, regulatory subunits of the PPP2A complex are negatively implicated in the phosphorylation of tyrosine hydroxylase, the rate-limiting precursor in dopamine synthesis (Saraf, 2008), while elevated PPPs regulate the

dephosphorylation of DARPP-32 (Nishi, Snyder, & Greengard, 1997), particularly following activation of dopamine D2 or NMDA receptors. As such, changes in both dopaminergic and glutamatergic signaling within the mesolimbic system are regarded as critically involved in the development of locomotor sensitization (Karler, Calder, Thai, & Bedingfield, 1994; Wolf, 1998), which according to the current results, may be secondary to elevated PPPs.

Importantly, calcineurin is increased in the PFC of schizophrenia (Hakak et al., 2001) and there is a significant association between single nucleotide polymorphisms of calcineurin γ catalytic subunit (PPP3CC) and the disorder (Gerber et al., 2003). Furthermore, the PPP3CC gene is significantly associated with poor performance on PFC-mediated cognitive tasks such as the Wisconsin Card Sorting Test (Liu et al., 2007). Schizophrenia is also characterized by reduced dopamine and over-active D2 receptors (Seeman & Kapur, 2000) in the PFC, which may be the result or secondary to elevated PP2A subunits, particularly given that DARPP-32 is also down-regulated in the PFC in schizophrenia (Albert et al., 2002). Overall, the PPP pathway may provide a biological mechanism that mediates the changes between monoamine systems and the phosphorylation status of downstream mediators not only in schizophrenia but also in METH sensitization, leading to long-term depression and cognitive dysfunction (Figure 3).

3.5.3 Small Molecule Biochemistry: Inhibitory GABAergic Network

In light that GABA is synthesized from the metabolism of glutamate, together with the changes to the glutamine-glutamate network described above, it serves that several alterations to the GABAergic inhibitory network were identified following sensitization to METH in the PFC. Indeed, glutamate dehydrogenase 1 (GAD₆₇), which is involved in the synthesis of GABA from glutamate, was significantly down-regulated in METH sensitized rats. Given that over 90% of GABA production is derived from GAD₆₇ activity (Asada et al., 1997), and that

GAD₆₇ and the concentration of GABA in interneurons are activity-dependent (Lau & Murthy, 2012), the reduced GAD₆₇ expression likely reflects decreased GABA within the PFC, and indeed, GABA is reduced in the PFC following METH sensitization (Bu et al., 2013). Alterations to GABAergic neurotransmission are the most consistent findings in the PFC of schizophrenic brains (Lewis, 2012), with the mRNA and protein expression of GAD₆₇ (Guidotti et al., 2000; Hashimoto et al., 2003) consistently reported as down-regulated.

Neurologin2 (NLGN2), a cell adhesion molecule that is specifically found at inhibitory synapses (Varoqueaux, Jamain, & Brose, 2004), was down-regulated in the PFC of METH-sensitized rats. Alterations to NLGN2 expression affects GABAergic synaptogenesis (Levinson et al., 2005) and the pool of vesicular reserve in frontal cortex synapses (Hines et al., 2008), suggesting a key role for NLGN2 in synaptic signaling at inhibitory junctions in the PFC. Importantly, over expression or knockout of NLGN2 increases (Hines et al., 2008) and decreases (Blundell et al., 2009) GABAergic neurotransmission, respectively, and a mutations of NLGN2 have previously been found in schizophrenia (Sun et al., 2011). Deletion of NLGN2 has also been found to selectively decrease GABAergic synaptic transmission within fast-spiking cells (Gibson, Huber, & Sudhof, 2009), suggesting that changes to NLGN2 expression following sensitization to METH may mediate reduced activity of GABAergic circuits within the PFC specifically in fast-spiking interneurons.

Indeed, parvalbumin (PVALB), a calcium-binding protein that is associated with a fast-spiking firing pattern (Sohal et al., 2009), was down-regulated in the PFC of METH sensitized rats. Not only is the expression of parvalbumin down-regulated in the PFC of schizophrenia (Beasley & Reynolds, 1997; Hashimoto et al., 2003), but the down-regulation of GAD₆₇ is most prominent in parvalbumin-expressing interneurons (Curley et al., 2011; Hashimoto et al., 2003). Parvalbumin-expressing GABAergic neurons regulate the temporal organization of cortical networks through control of pyramidal cells and neuronal oscillations

(Massi et al., 2012; Volman et al., 2011). Interestingly, fast-spiking parvalbumin-GABA cells are believed to mediate oscillations and neural synchrony during cognitive tasks (Başar et al., 2001), particularly those mediated by the PFC (Benchenane et al., 2011), with schizophrenia characterized by significant alterations to PFC oscillatory patterns (Minzenberg et al., 2010). These findings have ultimately led to the hypothesis that dysfunctional inhibitory control of the PFC could underlie the executive deficits inherent to schizophrenia (Akbarian & Huang, 2006). As such, METH sensitization is also associated with global changes to the inhibitory GABAergic network within the PFC, with changes possibly localized to parvalbumin-containing fast-spiking cells. In light of the significant overlap in both the executive dysfunction and changes to the GABAergic system between METH sensitization and schizophrenia, alterations to the GABAergic network may present a common biological substrate that could underpin the executive dysfunction seen across psychoses (Figure 3).

4. Concluding Remarks

This is the first shotgun proteomics study to examine the differential protein expression of the PFC of adult rats sensitized to METH. We found multiple proteins that were differentially expressed following sensitization, which collectively form an integrated signaling network whose dysfunction shares many of the same biological changes commonly observed in schizophrenia. While original research on METH sensitization placed particular salience on the dopaminergic network, much like schizophrenia, the current results suggest an increasingly clear role for mitochondrial proteins, synaptic proteins, protein phosphatases and inhibitory GABAergic proteins in the neurobiology of both schizophrenia and METH sensitization within the PFC. Specifically, alterations to the protein phosphatase or GABAergic networks may subserve common clinical symptoms between METH sensitization and schizophrenia, particularly executive dysfunction. As such, this research should serve as an important step for further investigation with particular proteins of interest, to not only further understand how these changes could mediate clinical symptoms, but to also inform the development of new therapeutic strategies.

Chapter Three

GABAergic mRNA Expression is Upregulated in the Prefrontal Cortex of Rats Sensitized to Methamphetamine

Chapter published as:

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See Appendix B for a copy

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Total 8%

1. Introduction

Methamphetamine (METH) is a potent psychostimulant that can induce psychosis among recreational and chronic users (Chen et al., 2005; Farrell et al., 2002; McKetin, McLaren, Lubman, et al., 2006). However, while METH psychosis is typically transient, some users may develop a persistent psychotic syndrome that is indistinguishable to schizophrenia, with positive, negative, and cognitive deficits that persist after long periods of abstinence from drug use (Jacobs et al., 2008; Lecomte et al., 2013; Sato, 1992; Srisurapanont et al., 2011). Previous research has interpreted the significant similarities between chronic METH psychosis and schizophrenia in the context of behavioral sensitization (Ujike, 2002; Yui, Goto, et al., 1999), a phenomenon whereby repeated exposure to a stimulus results in a progressively increased sensitivity to that stimulus following a period of abstinence (Pierce & Kalivas, 1997). Indeed, both chronic METH psychosis and schizophrenia are characterized by psychotic relapse even during abstinence from METH use or if neuroleptic medication is discontinued (Akiyama et al., 2011; Ohmori et al., 1999; Sato et al., 1983), while patients with schizophrenia can experience a psychotic relapse following exposure to METH at a dose that does not induce psychosis in healthy controls (Lieberman et al., 1987). These findings suggest that chronic METH psychosis and schizophrenia may be the result of overlapping neurobiological factors that mediate the expression of similar phenotypes together with a persistent sensitivity to psychotic relapse. As such, understanding the molecular mechanisms that mediate METH sensitization could help understand the neurobiology of schizophrenia.

Normal brain function is dependent on the delicate balance of excitatory and inhibitory neurotransmission predominantly mediated by glutamatergic and gamma-aminobutyric acid (GABA) signaling, respectively. GABA-mediated processes facilitate inhibitory control of both inhibitory interneurons and excitatory pyramidal cells, thus providing both direct and indirect mechanisms of altering the activity of neural networks

(Lewis et al., 2005). Furthermore, the GABAergic system contributes to the production of synchronized network oscillations during cognitive tasks (Başar et al., 2001; Gonzalez-Burgos et al., 2010), particularly those mediated by the prefrontal cortex (PFC), a brain region associated with higher-order cognitive and behavioral processes (Anderson et al., 1999; Başar et al., 2001; Ridderinkhof et al., 2004). GABAergic disturbances are some of the most consistent findings in post-mortem analyses of the PFC in schizophrenic brains, with reduced protein and mRNA expressions of glutamate decarboxylase 1 (GAD₆₇) and GABA transporter 1 (GAT₁) routinely found in the PFC in schizophrenia (Lewis & Moghaddam, 2006). Given that executive dysfunction is considered a core feature of the disorder (Keefe & Harvey, 2012), dysfunctional GABAergic signaling of the PFC has been proposed to underlie the altered gamma oscillatory patterns and executive deficits inherent to schizophrenia (Keefe & Harvey, 2012; Lewis, 2012; Minzenberg et al., 2010).

Previous studies have shown that METH administration changes GABAergic function. Specifically, METH sensitization increases GAD₆₇ and GABA_Aα2 protein expression in the caudate and decreases the same protein in the nucleus accumbens core and shell (Zhang et al., 2006). Furthermore, METH sensitization has been shown to decrease the concentration of GABA in the striatum (Pereira et al., 2012) and PFC (Bu et al., 2013). METH sensitization is also associated with PFC-mediated cognitive dysfunction (Featherstone et al., 2007), with gamma oscillation disturbances reported in both chronic METH users (Newton et al., 2004) and METH sensitized animals (Janetsian, Linsenbardt, & Lapish, 2015). As such, GABAergic dysfunction may be a common biological substrate that underlies the PFC-mediated behavioral and cognitive dysfunction that appears to be conserved across psychotic disorders. While PFC GABAergic disturbances in schizophrenia have been well documented, GABAergic dysfunction in the PFC following METH sensitization has yet to be described.

Sensitization to psychostimulants can be reliably induced in experimental animals,

with METH sensitized locomotor activity regarded as an animal model of the neurobiological changes associated with METH-induced psychosis (Featherstone et al., 2007; Ujike, 2002). We have previously found that protein expression for GAD₆₇ and gephyrin - a scaffolding protein involved in the clustering of GABA_A and glycine receptors at postsynaptic sites (Tyagarajan & Fritschy, 2014) – were down-regulated and upregulated in the PFC following METH sensitization, respectively (Wearne et al., 2014). As an extension of these findings, the primary aim of the current study was to investigate changes to gene expression of GABA related proteins (including enzymes, transporters, and receptor subunits) in the PFC of rats sensitized to METH, with particular focus on mRNA that was both expressed within the PFC and that had previously been implicated in the pathophysiology of schizophrenia. Based on our previous findings, it was hypothesized that GAD₆₇ mRNA would be downregulated while GABA_A receptor mRNA would be upregulated in the PFC of METH sensitized rats compared with controls.

2. Materials and Methods

2.1 Animals

All experimental procedures were approved by the Macquarie University Animal Ethics Committee (reference number ARA 2010/045) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 8th Edition, 2013). Twelve experimentally naïve male Sprague-Dawley rats (Animal Resource Centre, WA, Australia), weighing an average of 261 ± 5 g at the start of testing, were used. Animals were housed in groups of four in plastic high top cages [(64 cm (L) x 40 cm (W) x 20 cm (H)] that were kept in a humidity- and temperature-controlled room (21 ± 2 °C, 60% humidity) on a 12 h light: 12 h dark cycle (lights on at 0600 h). All experimentation was carried out during the light period. The rats were given *ad libitum* access to food and water in their home cages for the duration of the experiment.

2.2 Methamphetamine-induced Behavioral Sensitization

Methamphetamine-induced sensitization was performed as previously described (Wearne et al., 2014). Briefly, on Day 1, all rats received a saline injection (0.9%, 1 mg/kg, i.p.) and were allocated to treatment groups such that there was no significant difference in baseline locomotor activity between METH and saline-treated rats prior to drug administration ($p = .997$). Rats were then assigned to undergo repeated METH (once daily 1mg/kg intraperitoneal (i.p.) on days 2 & 8; 5mg/kg i.p. days 3 – 7) or saline (1mg/kg i.p.) injections for 7 days, consistent with previously published methods (Iwazaki et al., 2008; Morshedi & Meredith, 2008). Following a 14-day withdrawal period (Days 9-22), rats were injected with 1 mg/kg saline (i.p.) to test for conditioned baseline locomotor responding (Day 23) before being assessed for locomotor sensitization via a challenge dose of 1mg/kg METH (i.p.) on Day 24. Locomotor activity was recorded on Days 1, 2, 8, 23 and 24 using standard

Med Associates chambers equipped with 4 infrared photobeam detectors (Quantum PIR motion sensor, part no. 890-087-2, NESS Security Products, Australia) 30mm above the floor on the front and back panels 50 mm apart. Locomotor activity was quantified as the number of photobeam interruptions recorded via a computer equipped with Med-IV PC software (Med Associates, St Albans, VT, USA). Rats were placed in the test chamber 15 minutes prior to drug injection to reduce novelty-induced increases in activity before locomotor activity was recorded for 1 hour following treatment. Immediately following the challenge locomotor session, rats were euthanized via rapid decapitation and the PFC was dissected out on dry ice as previously described (Harkin et al., 2001; Wearne et al., 2014).

2.2.1 Drugs

Methamphetamine hydrochloride was purchased from the Australian Government Analytical Laboratories (Pymble, NSW) and was dissolved in 0.9% saline. Intraperitoneal injections (i.p.) were delivered at a volume of 1 ml/kg.

2.3 Statistical Analysis for Methamphetamine-induced Sensitization

Mean locomotor activity following METH challenge was compared between saline and METH treated rats using an independent t-test. A paired t-test was used to compare mean baseline locomotor activity on Day 2 for METH pretreated rats to the mean locomotor activity in response to METH challenge. Results are reported as mean \pm SEM. Analyses were performed using SPSS version 21 and the significance level was set at $p < .05$.

2.4 Quantitative Polymerase Chain Reaction (qPCR)

2.4.1 Gene choice, Primer Design and Validation

As indicated above, the focus of the current study was on mRNA of GABA related

proteins that were both expressed within the PFC and that had previously been implicated in the pathophysiology of schizophrenia. Thus, the expression of GABA enzymes, GAD₆₇ and GAD₆₅ (Dracheva, Elhakem, McGurk, Davis, & Haroutunian, 2004; Lewis et al., 2005), GABA transporters, GAT₁ and GAT₃, (Schleimer et al., 2004), together with metabotropic GABA_B receptors, GABA_{B1} and GABA_{B2} (Ishikawa et al., 2005), were examined in the current study. While GABA_A receptors are composed from a pool of 19 distinct subunits, combinations of the α 1, α 2, α 3, α 5, β 2, β 3 and γ 2 subunits represent over 80% of benzodiazepine sensitive GABA_A receptors in the adult brain (Pirker et al., 2000b). However, given that the β 3 subunit is predominantly found in the striatum (Pirker et al., 2000b) and that β 1 and δ have also been implicated in schizophrenia (Fatemi, Folsom, Rooney, & Thuras, 2013a, 2013b; Vawter et al., 2002), the expression of α 1, α 2, α 3, α 5, β 1, β 2, γ 2 and δ subunits were examined in the current study.

Table 1 summarizes the primer sequences, GeneBank accession numbers, PCR product sizes and efficiencies for each gene. Primers were designed using NCBI Nucleotide Primer Design software with parameters: 18-22 nucleotides length, 70-150 base pair product size, 55-60°C melting point and 50% GC content. Primers did not distinguish between splice variants. PCR products were sequenced at the Macquarie University DNA Analysis Facility and specificity was verified through the use of a BLAST search against the rat genome. A four-point standard curve was measured for each primer set and the primer efficiencies were calculated using MxPro software (Stratagene). Similar primer efficiencies are necessary to compare genes and all were shown to have $r^2 > 0.975$ (Table 1).

2.4.2 Tissue Extraction, RNA isolation and Reverse Transcription

RNA was extracted from PFC tissue using the SV total RNA isolation system according to the manufacturer's protocol (Promega SV Total RNA Isolation Kit, Promega,

Madison, Wisconsin, USA). The concentration and integrity of total RNA was measured using a Nanodrop 5000 spectrophotometer (Beckman-Coulter DU-800; Fullerton, California, USA) before 500 µg of RNA was reverse transcribed with oligo-dT primers according to the manufacturer's protocol (Promega Improm II kit). All samples were reverse transcribed simultaneously.

2.4.3 Quantitative Real Time PCR

Template cDNA from each sample was used for quantitative real-time PCR using the DNA-binding dye SYBR Green (Stratagene Brilliant II Mastermix). Each reaction consisted of 12.5 µl of Mastermix (with 2.5 mM/L MgCl₂), 1.0 µl forward primer (300 nmol), 1.0 µl reverse primer (300 nmol), 1 µl (500 ng) cDNA and 9.5 µl nuclease-free PCR-grade water were combined in a 25 µl reaction. Forty-five cycles of real time PCR was carried out on a Stratagene MX3000P Real Time PCR machine (Agilent Biosciences) with an initial 10 min denaturing step at 95°C. Each cycle consisted of 30 seconds at 95°C (denaturation), 60 seconds at 60°C (annealing) followed by 60 seconds at 72°C (extension). Fluorescent data was acquired at the end of each extension cycle at 85°C for 10 seconds to eliminate primer dimer artefact. Following the 45 cycles, a melt curve was performed to determine amplification of all primer sets and to ensure that a single PCR product was formed in the reaction.

2.4.4 Normalization and Data Analysis for Real Time PCR

Measurements from each sample were performed in duplicate and 'no template control' samples did not produce any product. Cycle threshold (Ct) values were obtained for each target using the MxPro software and normalized via a housekeeping gene, GAPDH. GAPDH protein was unchanged in the PFC following METH sensitization (Wearne et al.,

2014) and initial analyses revealed GAPDH mRNA was also unaltered in the current study ($p = .68$). Expression levels were calculated relative to controls using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). For statistical analysis, outliers (± 2 SD from the mean) were removed from the control and METH sensitized conditions (1% of values). All genes were normally distributed except for 3 genes in the saline group (GAT_1 , $\alpha 2$, $\alpha 5$) and two genes in the METH sensitized condition ($\gamma 2$ and δ), although further analysis revealed that the violations against normality did not alter the interpretation of the results. Independent t-tests were consequently used to determine significant changes in gene expression between METH-sensitized rats and saline controls. All analyses were performed using SPSS version 21 and the significance level was set at $p < .05$.

Table 1. Summary of primers, Genebank ascension numbers, forward and reverse sequences and PCR-product sizes for the 14 GABA related genes analyzed in the current study. The relative expressions of GABAergic genes were normalized to the housekeeping gene, GAPDH. Genes were chosen based on abundance in the PFC and whether they had been previously implicated in the neurobiology of schizophrenia (Dracheva et al., 2004; Fatemi et al., 2013a; Ishikawa et al., 2005; Lewis et al., 2005; Pirker et al., 2000b; Schleimer et al., 2004; Vawter et al., 2002)

Primer	GeneBank ID	Forward (5' – 3')	Reverse (5' – 3')	Product Size
GAD ₆₇	NM_017007.1	<i>ACAAATGCCTGGAGCTGGCTGAAT</i>	<i>TTGTGTGCTCAGGCTCACCATTGA</i>	93
GAD ₆₅	NM_012563.1	<i>TCTCAAAGGTGGCGCCAGTGATTA</i>	<i>TTGGTGAGTTGCTGCAGGGTTTGA</i>	131
GAT ₁	NM_02437.1	<i>GCGCAACATGCACCAAATGACA</i>	<i>AGACCACCTTTCCAGTCCATCCAA</i>	140
GAT ₃	NM_024372.2	<i>TGGGCATGAGTGGAACACAGAGAA</i>	<i>AGGTTCCCGATGTGTTCAATGCCA</i>	159
GABA _A α_1	NM_183326.2	<i>TGTCTTTGGAGTGACGACCGTTCT</i>	<i>ACACGAAGGCATAGCACACTGCAA</i>	125
GABA _A α_2	NM_001135779.4	<i>TCCAGGATGACGGAACATTGCTGT</i>	<i>TTCGGCTTGGACTGTAAGCCTCAT</i>	53
GABA _A α_3	NM_017069.2	<i>TCTGGATGGCTATGACAACCGACT</i>	<i>ACTTCAGTCACTGCATCTCCAAGC</i>	57
GABA _A α_5	NM_017295.1	<i>AACATCAGCACCAGCACAGGTGAA</i>	<i>TGACTGTCATGATGCAGGGAAGGT</i>	112
GABA _A β_1	NM_012956.1	<i>TTGTGTTTCGTGTTCTGGCTCTAC</i>	<i>GGGCATCAACCTGGACTTTGTTCA</i>	150
GABA _A β_2	NM_012957.2	<i>GCTGTCTGTGCTCAGAGTGTCAT</i>	<i>CAGAAACCATATCGATGCTGGCGA</i>	166
GABA _A γ_2	NM_183327.1	<i>TCGCCAAATACATGGAGCACTGGA</i>	<i>TTTGGCTAGTGAAGCCTGGGTTAGA</i>	112
GABA _A δ	NM_017289.1	<i>ATGGCGCCAGAGCAATGAATGA</i>	<i>TTCTGAGATGTGGTCAATGCTGGC</i>	182
GABA _{B1}	NM_031028.3	<i>ACCTGAAGCGTCAAGATGCTCGAA</i>	<i>AGTTGTCAGCATACCACCCGATGA</i>	141
GABA _{B2}	NM_031802.1	<i>AATGATCCCTGCACCAGCGTCAAA</i>	<i>AACATGCTCTCCTCGAAGGCACAA</i>	119
GAPDH	NM_017008.4	<i>TGAAGGTCGGTGTGAACGGATTG</i>	<i>AGCCTTGACTGTGCCGTTGAACTT</i>	176

3 Results

3.1 Sensitization to Methamphetamine

There was no difference in the locomotor activity of METH- and saline-treated rats in response to saline challenge on Day 1 ($p = .997$) and on Day 23 ($p = .40$). METH challenge resulted in a significant increased locomotor response in METH pre-treated rats compared with saline controls, $p < .05$ (Figure 1A). METH pre-treated rats also showed a significantly increased locomotor response on challenge day when compared with their locomotor response on Day 1 of METH exposure, $p < .01$ (Figure 1A). Specifically, locomotor activity was significantly higher for METH pre-treated rats than saline pre-treated controls from 15 min to 30 min following challenge administration, $p < .001$ (Figure 1B).

3.2 Relative Expression of GABAergic mRNA in the Prefrontal Cortex following Methamphetamine Sensitization

3.2.1 GABA Related Enzymes

There was no significant difference in the expression of GAD₆₇ ($p = .68$) and GAD₆₅ ($p = .97$) mRNA in the PFC between saline and METH sensitized rats (Figure 2A).

3.2.2 GABA Transporters

GAT₁ (1.31-fold, $p < .01$) and GAT₃ (1.50-fold, $p < .0005$) were significantly upregulated in METH-treated rats compared with saline-treated controls (Figure 2B).

3.2.3 GABA_A Receptors

Ionotropic GABA_A receptor subunits α_3 (1.34-fold, $p < .0001$) and β_1 (1.26-fold, $p < .005$) were significantly upregulated in METH-treated rats compared with saline controls. There was no significant difference in the expression of subunits α_1 ($p = .24$), α_2 ($p = .41$), α_5

($p = .07$), $\beta 2$ ($p = .08$), $\gamma 2$ ($p = .47$) and δ ($p = .18$) in the PFC between METH-treated rats and saline-treated controls (Figures 3A & 3B).

3.2.4 *GABA_B Receptors*

GABA_{B1} (1.40-fold, $p < .0001$) mRNA was significantly upregulated in METH-treated rats compared with saline controls (Figure 3C). There was no significant difference in the expression of GABA_{B2} mRNA between METH-treated rats and saline-treated controls ($p = .16$).

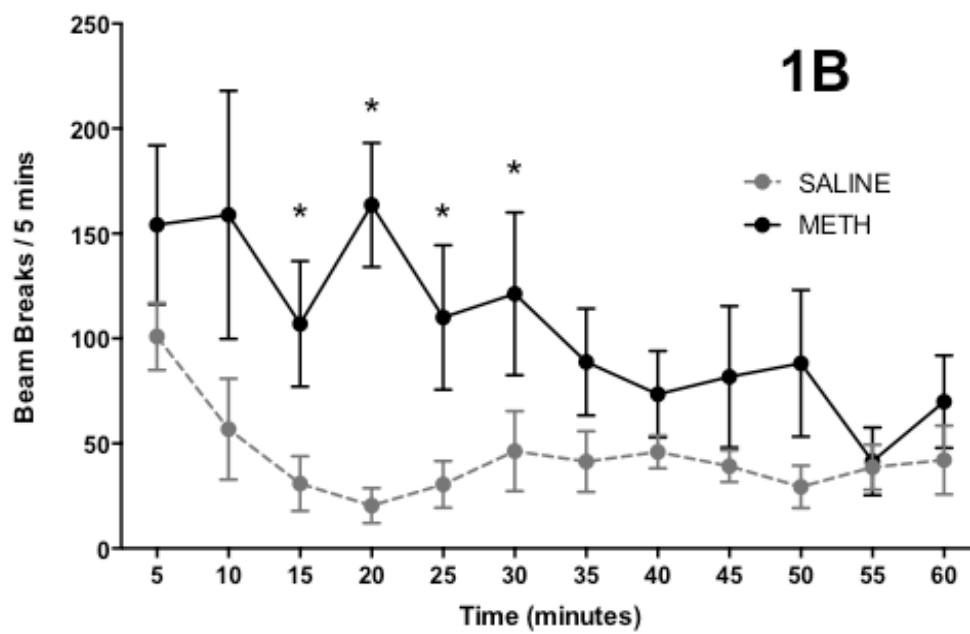
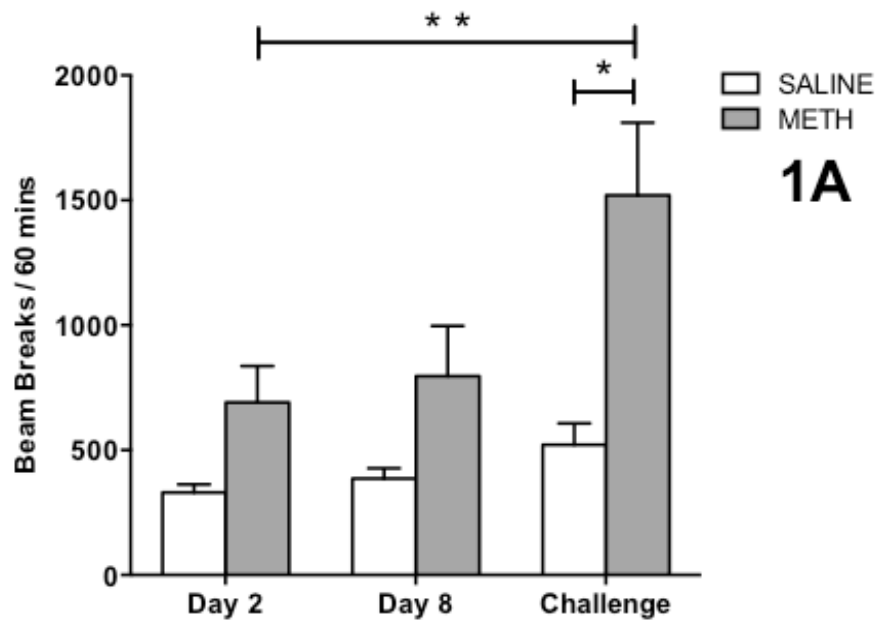


Figure 1. Locomotor sensitization to repeated METH administration. Rats were assigned to undergo either repeated METH (1mg/kg intraperitoneal (i.p.) days 2 & 8; 5mg/kg i.p. days 3 – 7, n=6) or saline (1 mg/kg i.p. n=6) treatment for 7 days. Following 14 days of withdrawal, both METH and saline rats were challenged with an acute METH (1mg/kg, i.p.) injection. Figure 1A represents the total beam breaks across days 2, 8 and challenge, respectively. Rats showed a progressively increased locomotor response to repeated METH administration, with a METH challenge resulting in a significant sensitized locomotor response in METH pre-treated animals when compared with saline controls*. METH treated rats also showed a significant increase in locomotor activity between Day 2 and challenge**. Figure 1B represents the time-course of locomotor activity for METH and saline pre-treated rats over 60 minutes post METH challenge administration. METH-treated rats displayed significantly higher locomotor activity 15 to 30 mins after challenge injection*. * and ** denotes $p < .05$.

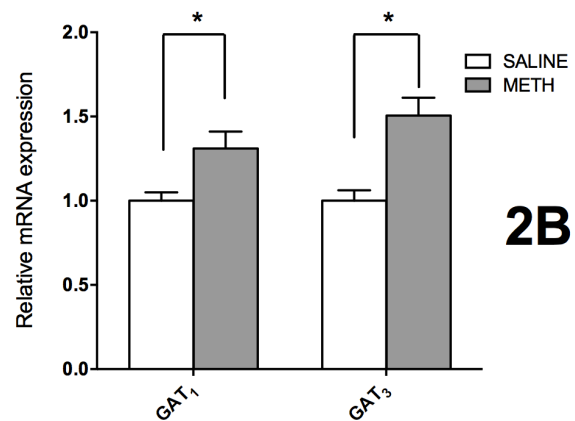
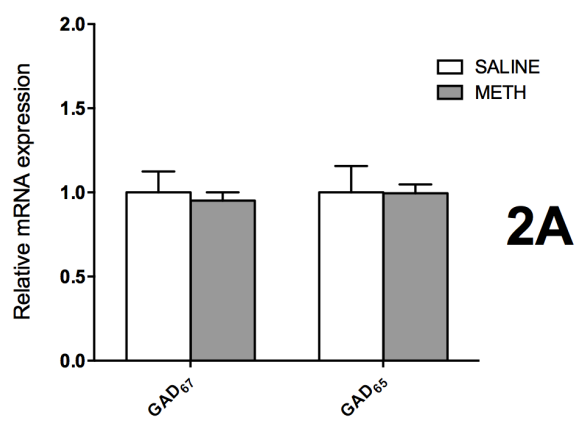


Figure 2. GABA enzymes and transporter mRNA expression after saline and METH sensitization (n = 6 per group) as determined using quantitative RT-PCR. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 2A shows no significant difference in the expression of GAD₆₅ and GAD₆₇ between METH sensitization and saline controls. Figure 2B shows that GAT₁ and GAT where both significantly upregulated following sensitization to METH. *Indicates GABA mRNA was significantly different ($p < .05$) when compared with saline controls. All error bars were produced with consideration of the exponential nature of qPCR using the $2^{-\Delta\Delta C_t}$ method.

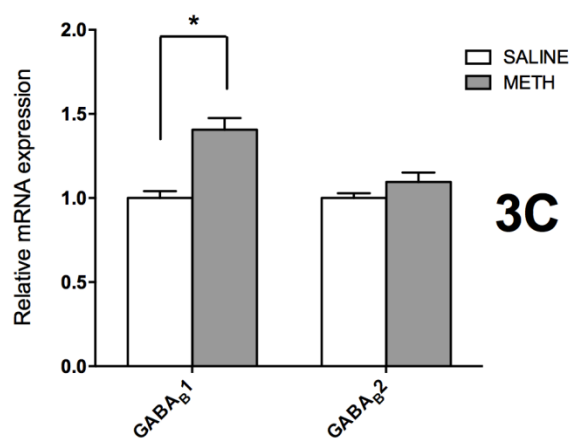
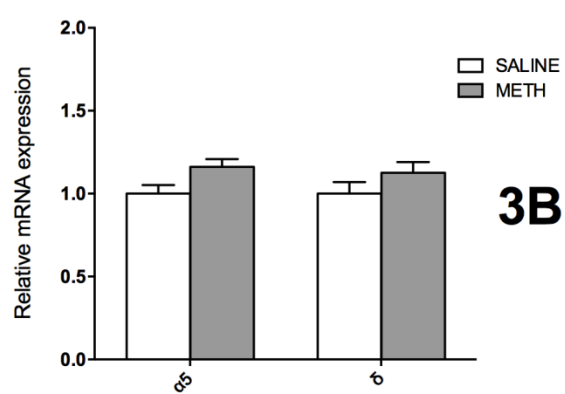
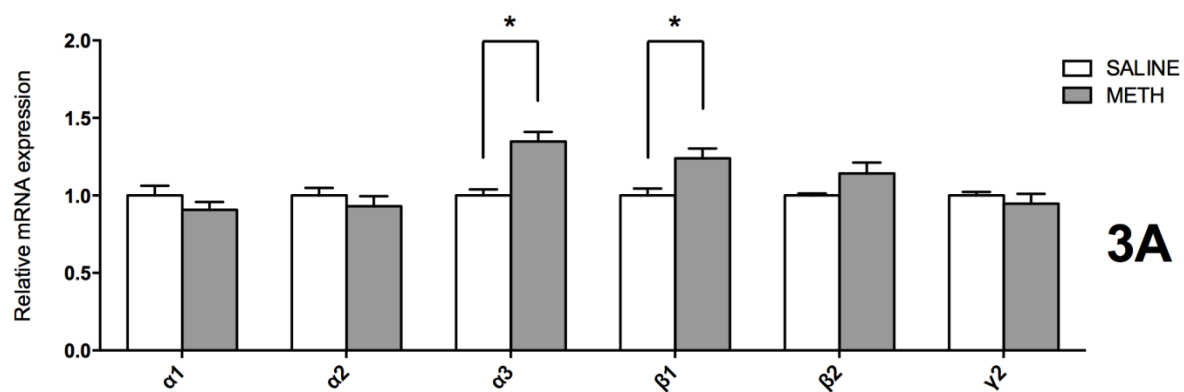


Figure 3. GABA receptor subunit mRNA expression after saline and METH sensitization (n = 6 per group) as determined using quantitative RT-PCR. Figure 3A shows that two GABA_A receptor subunits, $\alpha 3$ and $\beta 1$, were upregulated following sensitization to METH. Figure 3B depicts that the expression of extrasynaptic GABA_A subunits were unchanged following METH. Figure 3C shows that the expression of metabotropic GABA_B receptors, GABA_B, was upregulated following METH sensitization. *Indicates GABA mRNA was significantly different ($p < .05$) when compared with saline controls. All error bars were produced with consideration of the exponential nature of qPCR using the $2^{-\Delta\Delta C_t}$ method.

4. Discussion

The main findings of the current study were that a METH challenge produced a significant sensitized locomotor response in METH pre-treated rats compared to saline controls and that the mRNA expression of GAT₁, GAT₃, GABA_Aα3, GABA_A β1 and GABA_B1 were significantly upregulated in the PFC of rats sensitized to METH. The behavioral findings confirm the expression of METH-induced behavioral sensitization and are consistent with a large body of literature that has shown increased locomotor activity following a chronic psychostimulant administration and withdrawal regime (Ago et al., 2012; Pierce & Kalivas, 1997; Wearne et al., 2014). Furthermore, the clear lack of behavioral activation following saline challenge on Day 23 confirms that the rats were neither conditioned to the locomotor cages nor to the injection, suggesting that the change in behavior can be attributed to the sensitizing effects of METH.

The current findings suggest that METH sensitization is associated with multiple presynaptic and postsynaptic changes to the GABAergic system in the PFC. The fact that multiple GABAergic genes were unaltered following METH sensitization indicates that changes to the GABAergic system are subtype specific, and therefore reflect particular changes to GABAergic neurotransmission and inhibitory regulation secondary to the functional requirements of the cellular environment and/or PFC tissue. As such, these findings confirm that the GABAergic network plays an adaptive role in the PFC once the system becomes sensitized to the effects of METH. As sensitization has been traditionally associated with changes to the dopamine and glutamate systems (Cornish & Kalivas, 2001; Pierce & Kalivas, 1997), the current findings extend this body of literature by providing evidence of alterations to the PFC inhibitory GABAergic network following METH sensitization.

Two GABA_A receptor subunits, α3 and β1, were upregulated in the PFC of METH sensitized rats, partially supporting the hypothesis that GABA_A receptor mRNA expression

would be increased in the PFC following METH sensitization. The increased expression of these subunits may correspond with increased clustering of GABA_A receptors, as we have previously found an upregulation of gephyrin protein in the PFC following METH sensitization (Wearne et al., 2014). While the functional significance of these specific subunit changes is not clear, different combinations of receptor subtypes likely reflect altered postsynaptic inhibitory signaling, particularly given that GABA_A receptors containing the $\alpha 3$ and $\beta 1$ subunits mediate postsynaptic phasic inhibition and the hyperpolarization of efferent projections (Hines, Davies, Moss, & Maguire, 2012). Furthermore, in light of the fact that METH sensitization is associated with a reduced level of GABA in the PFC (Bu et al., 2013), these receptor changes may represent a compensatory mechanism that attempts to restore and/or maintain GABAergic mediated inhibition of efferent projections. Importantly, locomotor sensitization is augmented following microinjection of the GABA_A receptor antagonist, dicentrine, into the PFC (Enomoto, Tse, & Floresco, 2011) whereas total knock-out of the $\alpha 3$ subunit gene results in elevated locomotor activity and sensorimotor gating deficits but does not appear to alter amphetamine-induced locomotor activity (Yee et al., 2005). These findings suggest that while GABA_A receptors are important in mediating cognitive function and locomotor activity, GABA_A receptor activation in the PFC may be specifically involved in mediating sensitization to amphetamine type psychostimulants.

The finding that GAT₁ and GAT₃ mRNA expression was upregulated in the PFC following METH sensitization indicates increased reuptake of GABA at inhibitory synapses (Conti et al., 2004) and may explain the postsynaptic GABA_A receptor changes in the PFC described above. There are several possible reasons for increased mRNA expression of GAT₁ and GAT₃ in the PFC following METH sensitization. Firstly, GAT₁ and GAT₃ are believed to regulate the tonic inhibition of pyramidal neurons (Kinney, 2005), suggesting that their increased expression may modify inhibitory modulation of pyramidal cells in the PFC

following METH sensitization. Additionally, given that GAT₁ is predominantly localized to presynaptic terminals and astrocytes (Conti et al., 2004; Gadea & Lopez-Colome, 2001), while GAT₃ is exclusively found in astrocytes (Conti et al., 2004), the increased expression of GABA transporters may relate to increased reuptake of GABA to the glutamate/glutamine cycle in astrocytes, where GABA is metabolized into succinate (Bak, Schousboe, & Waagepetersen, 2006). Indeed, succinic acid semialdehyde, an intermediate in the catabolism of GABA, is increased following METH sensitization in the PFC (Bu et al., 2013), adding further support that accelerated removal and metabolism may be responsible for reduced GABA in the PFC. An additional explanation may be that increased GAT₁ and GAT₃ mRNA expression may be compensatory to decreased protein expression. In support for this hypothesis, we have previously found that GAT₁ protein levels were down-regulated in the PFC following sensitization to METH (Wearne et al., 2014), although the magnitude of the fold-change (-1.23 fold) failed to reach the threshold cutoff.

Based on our previous work (Wearne et al., 2014), we expected to find reduced mRNA expression for GAD₆₇ in the PFC, however we found no differential expression of GAD₆₇ mRNA following METH sensitization. While previous studies have found reduced mRNA and protein expression of GAD₆₇ following sensitization to METH (Pereira et al., 2012; Zhang et al., 2006), these findings have been localized to the striatum, which may be differentially affected following METH sensitization. As such, it is possible that GAD₆₇ protein may be downregulated in our previous work due to either post-translational modifications or increased GAD₆₇ protein metabolism. Alternatively, GAD₆₇ protein expression represents the protein found in soma, axons and terminals while GAD₆₇ mRNA expression likely consists mainly of transcripts localized to GABAergic cell bodies (Carr & Sesack, 2000; Gritti, Mainville, Mancina, & Jones, 1997), suggesting that reduced GAD₆₇ protein following METH sensitization may be due to reduced afferent inhibitory drive to the

PFC. In keeping with this idea, this potential decrease in inhibitory input due to lack of GAD₆₇ protein from distant sources could explain the upregulated expression of GAT₁ mRNA, as elevated reuptake of GABA to the presynaptic neuron may counteract disinhibited afferent projections by facilitating GABA exocytosis from recycled GABA stores.

While locomotor sensitization is regarded as an animal model of human psychostimulant-induced psychosis (Featherstone et al., 2007; Ujike, 2002), many of the transcriptional changes identified in the current study do not reflect the expression typically observed in schizophrenia. For example, compensatory changes to GABA_A receptors have been associated with upregulated expression of GABA_Aα2 mRNA (Lewis et al., 2005) while the expression of GABA_A3 is unchanged in the PFC of schizophrenia (Beneyto, Abbott, Hashimoto, & Lewis, 2011; Duncan et al., 2010). Additionally, analyses consistently indicate that the mRNA expressions for GAD₆₇ and GAT₁ are down-regulated in the PFC in schizophrenia (Guidotti et al., 2000; Volk et al., 2001). As such, while both METH sensitization and schizophrenia are characterized by changes to the GABAergic network, there appears to be both qualitative and quantitative differences in the expression of specific GABAergic genes between the two conditions. While the differences detected may be due to species-specific effects, an alternative explanation may be due to the global approach used in the current study. That is, the PFC is a heterogeneous structure, with schizophrenia research showing differential inhibitory changes across multiple areas of the PFC, including the dorsolateral prefrontal cortex (Schleimer et al., 2004) and orbitofrontal cortex (Joshi et al., 2012), while amphetamine sensitization is blocked by ibotenic acid lesion of the medial prefrontal cortex (Cador et al., 1999) but not the total PFC (Li & Wolf, 1997). Thus, the regional expression patterns of GABAergic mRNA may have escaped detection with the global approach used in the current study. Consequently, localized analysis of the PFC will be needed to determine the expression of GABAergic genes with respect to METH sensitization.

5. Conclusions

GABAergic mRNA expression is significantly altered at the pre and postsynaptic level in the PFC of rats sensitized to METH, with sensitization resulting in the transcriptional upregulation of several GABAergic markers. Even though GABAergic alterations could underlie certain aspects of psychosis symptomatology, it is unclear whether the changes identified here represent primary changes that mediate vulnerability to psychosis (i.e. sensitization) or secondary compensatory changes due to an unknown primary etiological cause (e.g. dopamine or glutamate dysregulation). While the results of the current study clearly implicate GABAergic dysfunction of the PFC in METH sensitization – suggesting that GABAergic disturbances may be common across psychotic syndromes - the underlying mechanisms mediating these inhibitory disturbances may not be consistent across conditions. As such, future research should determine the underlying nature of these changes in relation to regional and cellular expression profiles together with psychotic and cognitive phenotypes.

Chapter Four

GABAergic mRNA Expression is Differentially Expressed across the Prelimbic and
Orbitofrontal Cortices of Rats Sensitized to Methamphetamine

Co-Author Contribution

Cornish, J. L.

Contributed to research design and manuscript editing 2%

Total 2%

1. Introduction

Methamphetamine (METH) is a highly addictive and potent psychostimulant, with an estimated 34 million users worldwide (UNODC, 2013b). While the health consequences associated with chronic METH consumption include behavioral, physical, cognitive and neural changes (Meredith et al., 2005), an increasingly prominent health concern are psychiatric disturbances, with the prevalence of psychosis varying between 10% and 60% among recreational and chronic METH users (Chen et al., 2005; Farrell et al., 2002; McKetin, McLaren, Lubman, et al., 2006). Although METH psychosis is typically transient, with psychosis subsiding once the drug has been metabolized by the body, some users may develop a persistent psychotic syndrome that parallels the clinical profile of schizophrenia, with both chronic METH psychosis and schizophrenia characterized by positive, negative and cognitive symptoms (Chen et al., 2015; Ezzatpanah et al., 2014; Jacobs et al., 2008; Lecomte et al., 2013; Sato, 1992; Srisurapanont et al., 2011). As such, the distinction between schizophrenia and chronic METH psychosis can be clinically and diagnostically challenging (Grant et al., 2012). Indeed, those with METH psychosis are more likely to receive a schizophrenia diagnosis (Callaghan et al., 2012; Kittirattanapaiboon et al., 2010).

The neurobiological underpinnings of behavioral sensitization to psychostimulants are used to model the changes associated with psychoses and schizophrenia (Ujike, 2002; Yui, Goto, et al., 1999). Behavioral sensitization is a phenomenon whereby repeated exposure to a stimulus results in a progressively increased behavioral response to that stimulus following a period of abstinence (Pierce & Kalivas, 1997). Indeed, both METH psychosis and schizophrenia are characterized by a persistent vulnerability to psychotic relapse, particularly following re-exposure to METH (Sato et al., 1983), the onset of a significant life stressor (Olivares et al., 2013) or if medication is discontinued (Ohmori et al., 1999). Importantly, patients with schizophrenia are more susceptible to the psychotomimetic effects of

amphetamines than the general population (Lieberman, Kane, & Alvir, 1987), suggesting that psychostimulants target neural networks that are specifically sensitive in the schizophrenic brain. Sensitization can therefore be regarded as a common neuronal mechanism that initiates and maintains a persistent vulnerability to psychotic relapse across chronic METH psychosis and schizophrenia. As such, understanding the mechanisms that mediate METH sensitization could enhance our knowledge concerning the biological underpinnings of the persistent vulnerability to psychotic relapse that appears to be conserved across psychoses.

Recent advances in the psychiatric field have placed the underlying biology of schizophrenia in the context of dysfunctional inhibitory processing of the prefrontal cortex (PFC). For example, reduced protein and mRNA expressions of glutamate decarboxylase 1 (GAD₆₇) and GABA transporter 1 (GAT₁) are regarded as some of the most consistent findings in post-mortem analyses of the PFC in schizophrenia (Lewis & Moghaddam, 2006). Furthermore, given that the GABAergic system is heavily involved in the production of synchronized network oscillations during cognitive tasks (Başar et al., 2001; Gonzalez-Burgos et al., 2010), dysfunctional GABAergic signaling of the PFC is believed to mediate the altered gamma oscillatory patterns and executive deficits inherent to schizophrenia (Keefe & Harvey, 2012; Lewis, 2012; Minzenberg et al., 2010). METH sensitization induces PFC-mediated cognitive deficits that are consistent with the profile of schizophrenia (Featherstone et al., 2007), with gamma oscillation disturbances evident in chronic METH users (Newton et al., 2004) and METH- sensitized animals (Janetsian et al., 2015). Furthermore, a GABA deficit induced by the benzodiazepine inhibitor, iomanzenil predisposes healthy subjects to the psychotic effects of amphetamine at a dose that does not produce any psychosis-related behaviors when administered in isolation (Ahn et al., 2015), suggesting that the GABAergic system may mediate sensitivity to the psychotomimetic effects of amphetamines. In light of the significant overlap in executive dysfunction and oscillatory disturbances between METH

sensitization and schizophrenia, alterations to the GABAergic network may present a common biological substrate that could be similarly perturbed across psychotic conditions. The disturbances to the GABAergic system in the PFC of schizophrenics has been well documented, however, GABAergic dysfunction in chronic METH psychosis and/or METH sensitization has received considerably less attention.

Using label-free shotgun proteomics, we have previously found that several GABAergic protein markers, such as GAD₆₇, parvalbumin, neuroligin2 and gephyrin were differentially expressed in the PFC following sensitization to METH (Wearne et al., 2014). We have also found that several GABAergic genes were transcriptionally upregulated in the PFC following METH sensitization, such as GAT₁, GAT₃, GABA_Aα3, GABA_A β1 and GABA_B1 (Wearne et al., submitted). Overall, these mRNA and protein changes suggest that METH sensitization is associated with multiple inhibitory alterations to the PFC that could have profound consequences on inhibitory neurotransmission, neuronal synchronization and executive function. However, given that the PFC is a heterogeneous structure, with anatomical variability in morphology and cellular architecture across regions of the PFC, a significant limitation of these previous findings was the global approach used. The PFC consists of many subregions that appear to be dissociated with respect to functional output, with the dorsolateral prefrontal cortex (DLPFC) associated with planning, initiation, sequencing and working memory (Barone & Joseph, 1989; Curtis & D'Esposito, 2003; Kaller, Rahm, Spreer, Weiller, & Unterrainer, 2011); the orbitofrontal cortex (OFC) with emotion, reward and decision-making (Bechara, Damasio, & Damasio, 2000; Rolls, 2000); and the anterior cingulate cortex with autonomic function, empathy and impulse control (Critchley et al., 2003; Marsh et al., 2013; Pardo, Pardo, Janer, & Raichle, 1990; Paus & sbreve, 2001). Indeed, schizophrenia research has shown differential inhibitory changes across areas of the PFC, including the DLPFC (Curley et al., 2011; Dean et al., 1999; Hashimoto et al., 2008;

Schleimer et al., 2004) and the OFC (Joshi et al., 2012; Thompson, Weickert, Wyatt, & Webster, 2009). Furthermore, even though sensitized behavior has traditionally been associated with dopaminergic activity in the striatum (for review see Pierce & Kalivas, 1997), several studies have suggested an important and prominent role of the rat medial PFC and the OFC in sensitized behavior. For example, the expression of amphetamine sensitization is blocked by ibotenic acid lesions of the medial PFC (Cador et al., 1999) but not the total PFC (Li & Wolf, 1997), and viral-vector mediated overexpression of the transcription factor Δ FosB in the OFC can induce sensitization in drug naïve animals (Winstanley et al., 2009). Therefore, the role of the GABAergic system in sensitized behavior may be functionally and biologically dissociated across subregions of the PFC, suggesting that regional patterns of GABAergic mRNA expression may have been summated and escaped detection with the global approach employed in our previous studies.

The aim of the current study was to extend our previous findings by examining changes in GABAergic mRNA expression across subregions of the PFC following METH sensitization. Given that there is some evidence that the prelimbic cortex (PRL) of the rat is analogous to the DLPFC in mammals (Granon & Pucet, 2000), together with strong evidence that the PRL and the OFC are differentially affected following sensitization, we examined changes to GABAergic mRNA expression across the PRL and OFC of rats sensitized to METH.

2. Materials and Methods

2.1 Animals

All experiments were approved by the Macquarie University Animal Ethics Committee (ARA 2012/047; Appendix D) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 8th Edition, 2013). Twelve experimentally naïve male Sprague-Dawley rats (Animal Resource Centre, WA, Australia), weighing 397 ± 6 g at the start of testing, were used. Animals were housed in groups of four in plastic high top cages [(64 cm (L) x 40 cm (W) x 20 cm (H)] in a humidity- and temperature-controlled room (21 ± 2 °C, 60% humidity) and were provided with *ad libitum* access to food and water for the duration of the experiment.

2.2 Methamphetamine-induced Behavioral Sensitization

Methamphetamine-induced sensitization was performed as previously described (Wearne et al., 2014). Briefly, on Day 1, all rats received a saline injection (0.9%, 1 mL/kg, i.p.) and were allocated to treatment groups such that there was no significant difference in baseline locomotor activity between METH and saline-treated rats prior to drug administration ($p = 46$). Rats were then assigned to undergo repeated METH (once daily 1mg/kg i.p. on days 2 & 8; 5mg/kg i.p. days 3 to 7) or saline (1mL/kg i.p.) injections for 7 days. Following 14 days of withdrawal, rats were tested for conditioned baseline responding before being assessed for sensitization via a challenge dose of 1mg/kg METH (i.p.). Locomotor activity was recorded on Days 1, 2, 8, 23 and 24 using standard Med Associates chambers equipped with infrared photobeam detectors (NESS Security Products) placed 50 mm apart and 30mm above the floor on the front and back panels. Activity was quantified as the number of photobeam interruptions (Med Associates, VT, USA). Rats were placed in the test chamber for 15 minutes prior to drug injection. Locomotor activity was recorded for 1

hour following drug administration.

2.3 Drugs

Methamphetamine hydrochloride (METH) was purchased from the Australian Government Analytical Laboratories (Pymble, NSW) and was dissolved in 0.9% saline. Intraperitoneal injections (i.p.) were made at a volume of 1 mL/kg with control rats treated with saline (0.9%).

2.4 Statistical Analysis for Methamphetamine-induced Sensitization

Results are reported as mean \pm SEM. Mean locomotor activity following METH challenge was compared between saline and METH rats using an independent t-test. A paired t-test was used to compare mean baseline locomotor activity on Day1 for METH pretreated rats to the mean locomotor activity in response to METH challenge. Analyses were performed using SPSS version 17 and the significance level was set at $p < .05$.

2.5 Quantitative Polymerase Chain Reaction (qPCR)

2.5.1 Primer Design and Validation

In order to provide a detailed analysis of changes to the GABAergic system following METH sensitization in the PRL and OFC, we examined the mRNA expression of GABA enzymes (GAD₆₇ and GAD₆₅), transporters (GAT₁ and GAT₃), ionotropic receptor subunits (α 1, α 2, α 3, α 5, β 1, β 2, γ 2 and δ) and metabotropic receptors (GABA_B1 and GABA_B2), consistent with our previous analysis of the global PFC region (Wearne et al., 2014). Furthermore, as we previously found that mRNA expression for GABA transporters were increased in the PFC, together with the fact that elevated GAT₃ mRNA expression could reflect increased uptake and metabolism of GABA in astrocytes, we also extended the study

to examine whether altered GABAergic metabolism and GABA transport may be involved in mediating the altered GABAergic mRNA expression in the PRL and OFC. Consequently, GABA catalytic enzyme, 4-aminobutyrate transaminase (GABA_T) and vesicular GABA transporter (VGAT) were also designed for analysis in the current study. Table 1 summarizes the primer sequences, GeneBank accession numbers, PCR product sizes and efficiencies for each gene. Primers were designed using NCBI Nucleotide Primer Design software as previously described (Wearne et al., submitted to *Behavioural Brain Research*). PCR products were sequenced at the Macquarie University DNA Analysis Facility and specificity was verified through the use of a BLAST search against the rat genome. All genes were shown to have similar primer efficiencies, $r^2 > 0.975$.

2.5.2 Tissue Extraction, RNA isolation and Reverse Transcription

One hour following the METH challenge, the rats were euthanized with an i.p. injection of pentobarbitone sodium (1ml lethobarb 325 mg/mL; diluted in 1mL saline) once non-responsive to tail pinch, decapitated by guillotine. The brains were rapidly removed, snap frozen in liquid nitrogen then stored at -80°C. A brain matrix was used to remove 1 mm thick coronal sections of the PRL (3.2 mm rostral to bregma, 1 mm lateral to midline and 3.5 mm to 4.5 mm from dura, Paxinos & Watson, 2005) and OFC (4.2 mm rostral to bregma, 4 mm to 5 mm from dura, Paxinos & Watson, 2005). The medial, ventral lateral and dorsolateral orbitofrontal cortices were removed as a single section to represent the OFC. All dissections were carried out on dry ice and stored at -80°C until analysis. RNA was extracted using the SV total RNA isolation system according to the manufacturer's protocol (Promega SV Total RNA Isolation Kit, Promega, Madison, Wisconsin, USA). The concentration and integrity of total RNA was measured on a Nanodrop 5000 spectrophotometer (Beckman-Coulter DU-800; Fullerton, California, USA) before 500 µg of RNA was reverse transcribed with random

primers according to the manufacturer's protocol (Promega Improm II kit, Wisconsin, USA).

2.5.3 Quantitative Real Time PCR

Real-Time PCR was performed as previously described (Parker, Tallapragada, Kumar, & Goodchild, 2012). Briefly, cDNA was combined with DNA binding dye SYBR Green (Stratagene Brilliant II Mastermix), forward and reverse primer (300 nmol), and nuclease-free water in a 25µl reaction. Forty-five cycles of real time PCR was performed on a Stratagene MX3000P PCR machine (Agilent Biosciences) with an initial 10 min denaturing step at 95°C. Each cycle consisted of 30 seconds at 95°C, 60 seconds at 60°C followed by 60 seconds at 72°C. Florescent data was acquired at the end of each extension cycle at 85°C for 10 seconds. Following the 45 cycles, a melt curve was performed to determine amplification of all primer sets and to ensure that a single PCR product was formed in the reaction.

2.5.4 Normalization and Data Analysis

Samples were analyzed in duplicate and 'no template controls' did not produce any signal. Cycle threshold (Ct) values were obtained for each gene using the MxPro software (Stratagene) and normalized via a housekeeping gene, GAPDH. GAPDH was chosen as it was unchanged at the protein and mRNA level within the global PFC following METH sensitization (Wearne et al., 2014) and initial analyses revealed it was unchanged at the mRNA level in the PRL ($p = .33$) and OFC ($p = .51$) following METH sensitization. Expression levels were calculated relative to controls using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Outliers (± 2 SD from the mean) were removed from the control and METH sensitized conditions ($< 1\%$ of values across PRL and OFC). All genes were normally distributed except for one gene in the control group ($\alpha 2$) and two genes in the METH condition (GAD₆₅, GAT₃) in the PRL, while 2 genes in the saline group ($\alpha 1$, $\alpha 3$) and two

genes in the METH sensitized condition (GAD₆₅ and GABA_B1) were not normally distributed in the OFC. However, further analysis with non-parametric testing revealed that this did not alter the interpretation of the results. As such, independent t-tests were used to determine significant changes in gene expression between METH-sensitized rats and saline controls. All analyses were performed using SPSS version 21 and the significance level was set at $p < .05$.

Table 1. Summary of primers, Genebank ascension numbers, forward and reverse sequences and PCR-product sizes for the GABA related genes analyzed in the current study. The relative expressions of GABAergic genes were normalized to the housekeeping gene, GAPDH.

Primer	GeneBank ID	Forward (5' – 3')	Reverse (5' – 3')	Size
GAD ₆₇	NM_017007.1	<i>ACAAATGCCTGGAGCTGGCTGAAT</i>	<i>TTGTGTGCTCAGGCTCACCATTGA</i>	93
GAD ₆₅	NM_012563.1	<i>TCTCAAAGGTGGCGCCAGTGATTA</i>	<i>TTGGTGAGTTGCTGCAGGGTTTGA</i>	131
GAT ₁	NM_02437.1	<i>GCGCAACATGCACCAAATGACA</i>	<i>AGACCACCTTTCCAGTCCATCCAA</i>	140
GAT ₃	NM_024372.2	<i>TGGGCATGAGTGGAAACACAGAGAA</i>	<i>AGGTTCCCGATGTGTTCAATGCCA</i>	159
VGAT	NM_031782.1	<i>AAACGCCATTTCAGGGCATGTTC</i>	<i>CGTTAGCTATGGCCACATACGA</i>	197
GABA-T	NM_031003.2	<i>ACCGCATGTTGGACCTGTAT</i>	<i>GTTGATGAAAGTGCTCGCGT</i>	110
GABA _A α1	NM_183326.2	<i>TGTCTTTGGAGTGACGACCGTTCT</i>	<i>ACACGAAGGCATAGCACACTGCAA</i>	125
GABA _A α2	NM_001135779.4	<i>TCCAGGATGACGGAACATTGCTGT</i>	<i>TTCGGCTTGGACTGTAAGCCTCAT</i>	53
GABA _A α3	NM_017069.2	<i>TCTGGATGGCTATGACAACCGACT</i>	<i>ACTTCAGTCACTGCATCTCCAAGC</i>	57
GABA _A α5	NM_017295.1	<i>AACATCAGCACCAGCACAGGTGAA</i>	<i>TGACTGTCATGATGCAGGGAAGGT</i>	112
GABA _A β1	NM_012956.1	<i>TTGTGTTTCGTGTTTCCTGGCTCTAC</i>	<i>GGGCATCAACCTGGACTTTTGTTC</i>	150
GABA _A β2	NM_012957.2	<i>GCTGTCTGTGCTCAGAGTGTCAAT</i>	<i>CAGAAACCATATCGATGCTGGCGA</i>	166
GABA _A γ2	NM_183327.1	<i>TCGCCAAATACATGGAGCACTGGA</i>	<i>TTTGGCTAGTGAAGCCTGGGTAGA</i>	112
GABA _A δ	NM_017289.1	<i>ATGGCGCCAGAGCAATGAATGA</i>	<i>TTCTGAGATGTGGTCAATGCTGGC</i>	182
GABA _B 1	NM_031028.3	<i>ACCTGAAGCGTCAAGATGCTCGAA</i>	<i>AGTTGTCAGCATACCACCCGATGA</i>	141
GABA _B 2	NM_031802.1	<i>AATGATCCCTGCACCAGCGTCAAA</i>	<i>AACATGCTCTCCTCGAAGGCACAA</i>	119
GAPDH	NM_017008.4	<i>TGAAGGTCGGTGTGAACGGATTTG</i>	<i>AGCCTTGACTGTGCCGTTGAACTT</i>	176

3. Results

3.1 Sensitization to Methamphetamine

There was no difference in the locomotor activity of METH- and saline-treated rats in response to saline challenge on Day 1 ($p = .47$) and on Day 23 ($p = .58$). Methamphetamine challenge resulted in a significant increased locomotor response in METH pre-treated rats compared with saline controls, $p < .05$ (Figure 1A). METH pre-treated rats also showed a significant increased locomotor response on challenge when compared with their locomotor response on Day 1 of METH exposure, $p < .01$ (Figure 1A). Furthermore, as shown in Figure 1B, locomotor activity in response to METH challenge was significantly higher for METH pre-treated rats than saline pre-treated controls at 10, 15, 30, 45, 50 and 60 mins following challenge administration, $p < .05$.

3.2 GABAergic mRNA in the Prelimbic Cortex (PRL) following Methamphetamine Sensitization

3.2.1 GABA Enzymes and Metabolites

GAD₆₇ and GAD₆₅ were significantly 1.51-fold ($p < .001$) and 1.29-fold ($p < .05$) upregulated in the PRL of METH sensitized rats compared with saline-treated controls, respectively (Figure 2A). GABA-T was significantly 1.32-fold ($p < .01$) upregulated in the PRL in METH treated rats compared with saline controls (Figure 2A).

3.2.2 GABA Transporters

GAT₁ and GAT₃ were significantly 1.37-fold ($p < .001$) and 1.35-fold ($p < .05$) upregulated, in METH-treated rats compared to saline-treated controls, respectively (Figure 2B). VGAT was also 1.51-fold ($p < .005$) upregulated in the PRL when compared with saline controls (Figure 2B).

3.2.3 Synaptic Ionotropic GABA_A Receptors

Ionotropic GABA_A receptor subunit β_2 was significantly 1.24-fold ($p < .05$) upregulated in METH-treated rats compared with saline controls. There was no significant difference in the expression of subunits $\alpha 1$ ($p = 0.28$), $\alpha 2$ ($p = .19$), $\alpha 3$ ($p = .07$), $\alpha 5$ ($p = .22$), β_1 ($p = .09$) and $\gamma 2$ ($p = .48$) between METH-treated rats and saline-treated controls (Figure 3A).

3.2.4 Extrasynaptic Ionotropic GABA_A Receptors

There was no significant difference in the expression of subunits $\alpha 5$ ($p = .22$) and δ ($p = .34$) between METH-treated rats and saline-treated controls (Figure 3B).

3.2.5 Metabotropic GABA_B Receptors

GABA_{B1} mRNA was significantly 1.27-fold ($p < .05$) upregulated in METH-treated rats compared with saline controls (Figure 3C). There was no significant difference in the expression of GABA_{B2} mRNA between METH-treated rats and saline-treated controls ($p = .80$).

3.3 GABAergic mRNA in the Orbitofrontal Cortex (OFC) following Methamphetamine Sensitization

3.3.1 GABA Enzymes and Metabolites

There was no significant difference in the expression of GAD₆₇ ($p = .99$), GAD₆₅ ($p = .13$) and GABA-T ($p = .54$) mRNA in the OFC between saline and METH sensitized rats (Figure 4A).

3.3.2 GABA Transporters

There was no significant difference in the mRNA expression of GAT₁ ($p = .21$), GAT₃ ($p = .66$) and VGAT ($p = .15$) in the OFC between saline and METH sensitized rats (Figure 4A).

3.3.3 Synaptic Ionotropic GABA_A Receptors

Ionotropic GABA_A receptor subunits α_1 , α_3 and β_2 were significantly 1.57-fold ($p < .001$) 1.32-fold ($p < .005$) and 1.27-fold ($p < .0005$) upregulated in METH-treated rats compared with controls, respectively. There was no significant difference in the expression of subunits α_2 ($p = .27$), β_1 ($p = .76$) and γ_2 ($p = .52$) between METH-treated rats and controls (Figure 5A).

3.3.4 Extrasynaptic Ionotropic GABA_A Receptors

GABA_A α_5 was 1.40-fold ($p < .0005$) upregulated in the OFC in METH sensitized rats compared to saline controls. There was no significant difference in the expression of δ ($p = .34$) between METH-treated rats and saline-treated controls (Figure 5B).

3.3.5 Metabotropic GABA_B Receptors

GABA_B2 mRNA was significantly 1.21-fold ($p < .05$) upregulated in METH-treated rats compared to saline controls (Figure 5C). There was no significant difference in the expression of GABA_B1 mRNA between METH-treated rats and saline-treated controls ($p = .29$).

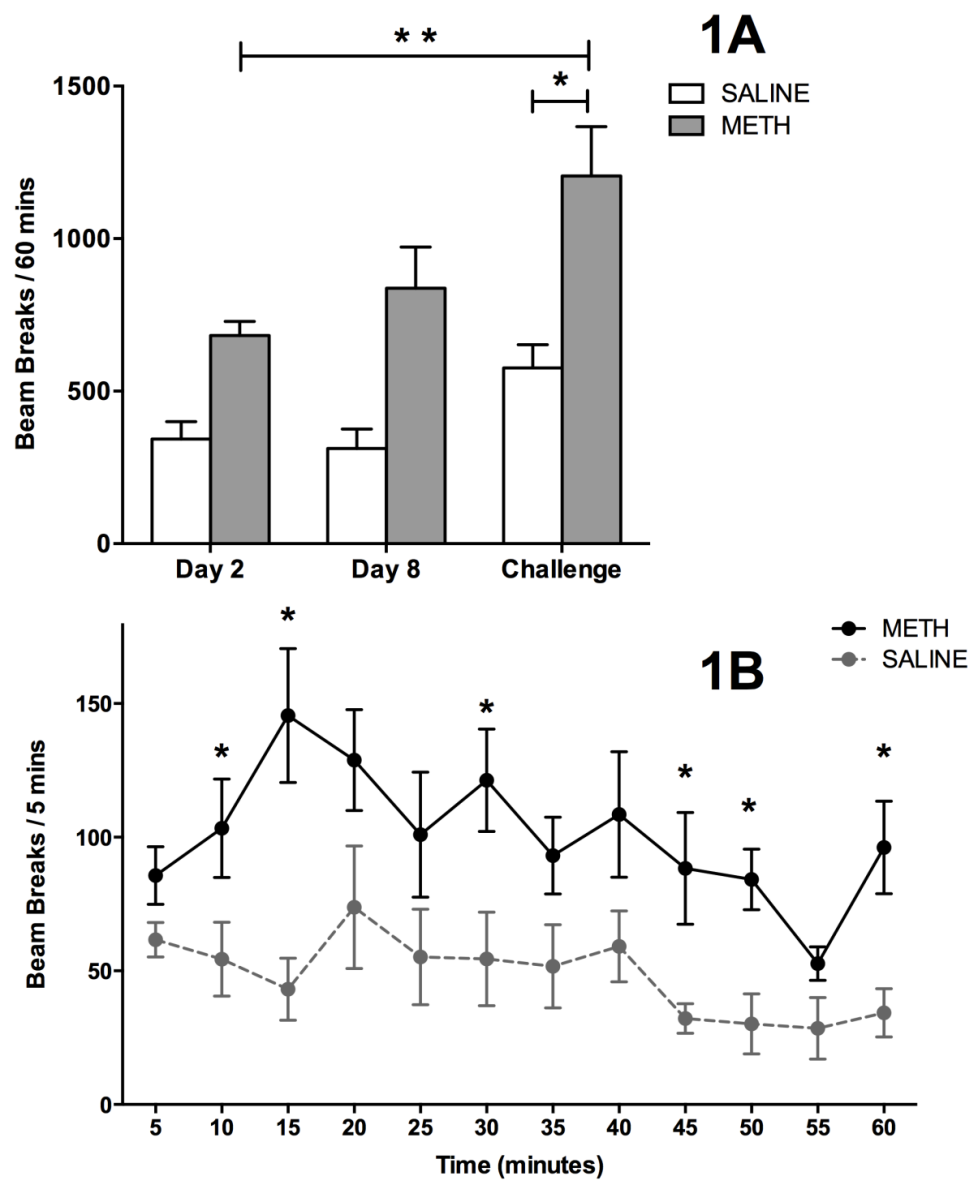
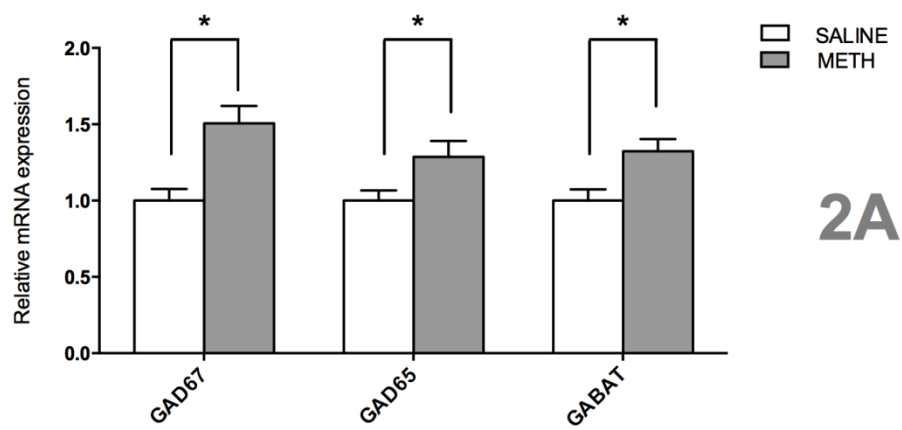
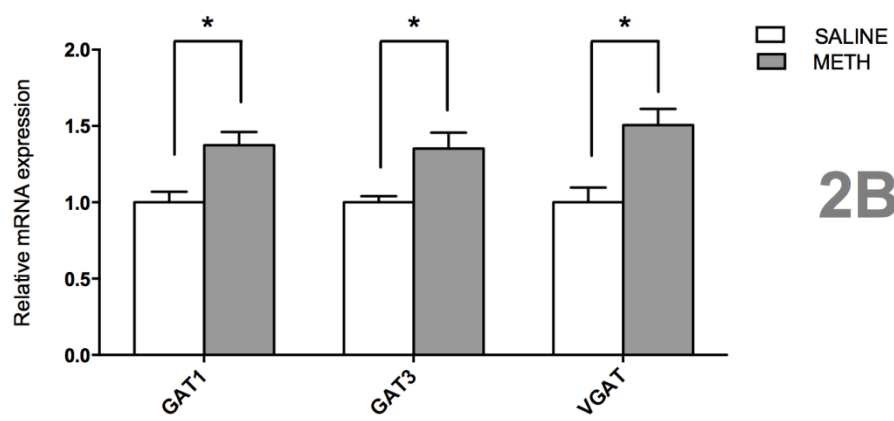


Figure 1. Locomotor sensitization to repeated METH administration. Rats were assigned to undergo either repeated METH (1ml/kg intraperitoneal (i.p.) days 1 & 7; 5mg/kg i.p. days 2 – 6, n=6) or saline (1 ml/kg i.p., n=6) treatment for 7 days. Following 14 days of withdrawal, both METH and saline rats were challenged with an acute METH (1mg/kg, i.p.) injection. Figure 1A represents the total beam breaks across days 2, 8 and challenge, respectively. Rats showed a progressively increased locomotor response to repeated METH administration, with a METH challenge resulting in a significant sensitized locomotor response in METH pre-treated animals when compared to saline controls*. METH treated rats also showed a significant increase in locomotor activity between acute METH exposure on Day 2 and challenge**. Figure 1B represents the time-course of locomotor activity for METH and saline pre-treated rats over 60 minutes post challenge administration. Data is represented as mean \pm SEM activity as beam breaks in each 5-minute period. METH-treated rats displayed significantly higher locomotor activity at 10, 15, 30, 45, 50 and 60 mins after challenge injection*. Solid lines represent chronic METH-treated animals while the dashed lines represent the saline condition.



2A



2B

Figure 2. The expression of GABAergic enzyme and transporter mRNA expression in the prelimbic cortex (PRL) after saline and METH sensitization (n = 6 per group) as determined using quantitative RT-PCR and normalized to housekeeping gene, GAPDH. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 2A shows that GAD₆₇, GAD₆₅ and GABA_T were all significantly upregulated in the PRL following sensitization to METH compared with saline-treated controls. Figure 2B shows that GAT₁, GAT₃ and VGAT were upregulated in the PRL of METH sensitized rats compared with saline treated controls. *Indicates GABA mRNA was significantly different when compared to saline controls.

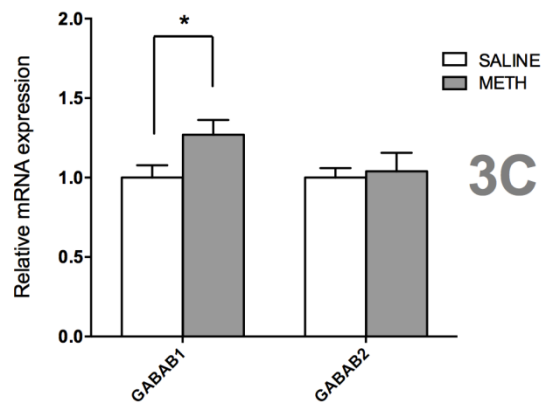
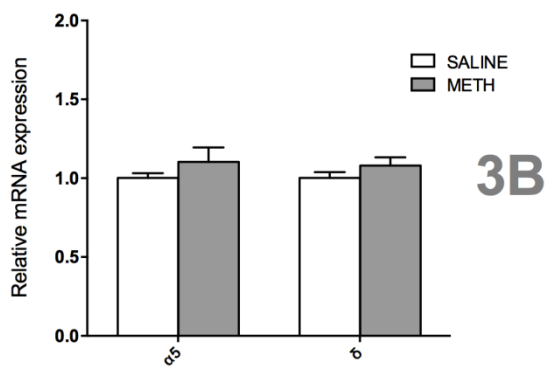
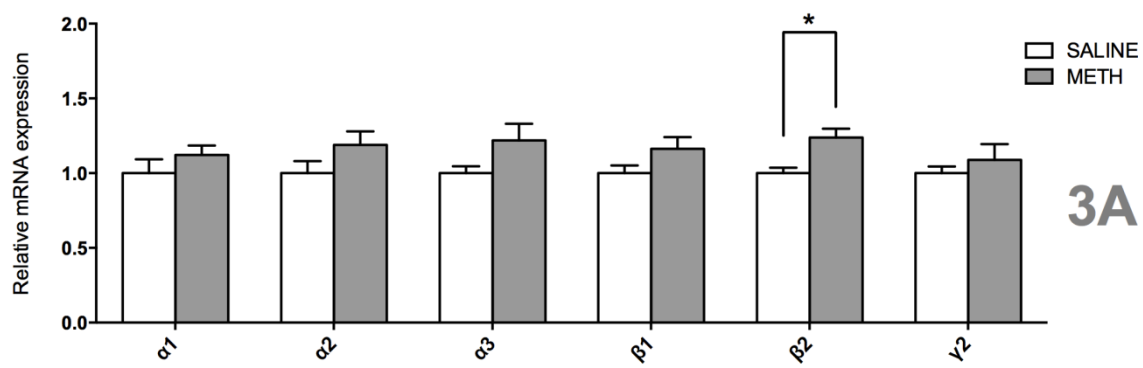
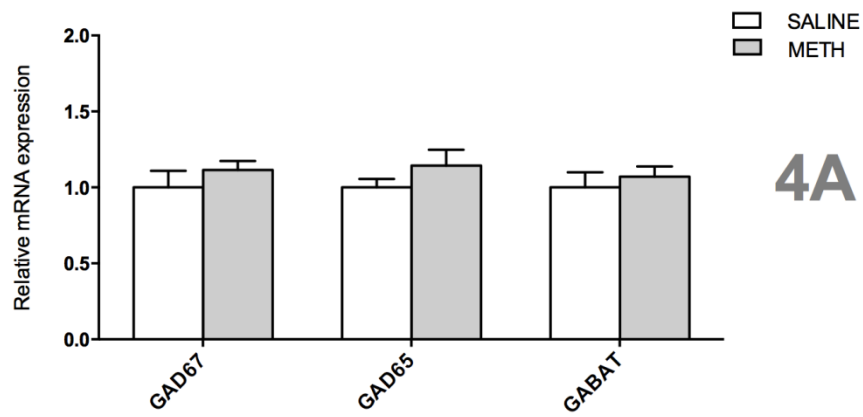
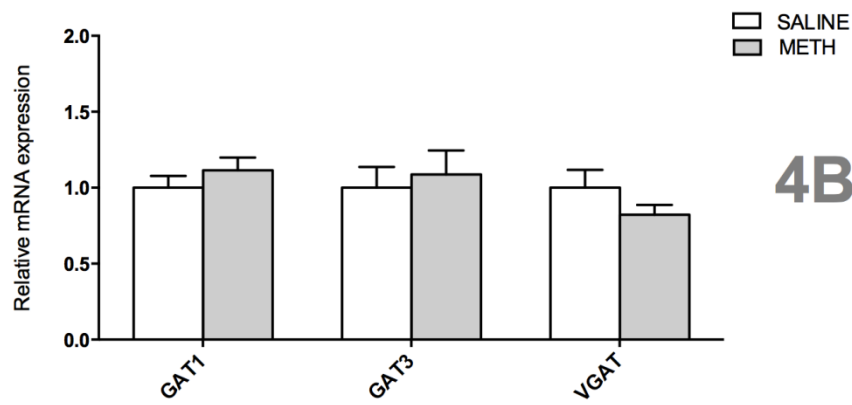


Figure 3. The expression of ionotropic and metabotropic GABA receptor mRNA expression in the prelimbic cortex (PRL) after saline and METH sensitization (n = 6 per group) as determined using quantitative RT-PCR and normalized to housekeeping gene, GAPDH. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 3A shows the relative expression of synaptic GABA_A receptors following METH sensitization whereby GABA_A β 2 was upregulated following sensitization to METH in the PRL. Figure 3B depicts the expression of extrasynaptic GABA_A subunits involved in tonic inhibitory neurotransmission, which were unchanged following METH treatment in the PRL. Figure 3C shows the expression of metabotropic GABA_B receptors, with the expression of GABA_B1 mRNA upregulated in the PRL following METH sensitization. *Indicates GABA mRNA was significantly different when compared to saline controls.



4A



4B

Figure 4. The expression of GABAergic enzyme and transporter mRNA expression in the orbitofrontal cortex (OFC) after saline and METH sensitization (n = 6 per group) as determined using quantitative RT-PCR and normalized to housekeeping gene, GAPDH. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 4A shows that there was no change in the mRNA expression of GAD₆₇, GAD₆₅ and GABA_T in the OFC following sensitization to METH compared with saline-treated controls. Similarly, Figure 4B shows that GAT₁, GAT₃ and VGAT mRNA expression was unchanged in the OFC of METH sensitized rats compared with saline treated controls. *Indicates GABA mRNA was significantly different when compared to saline controls.

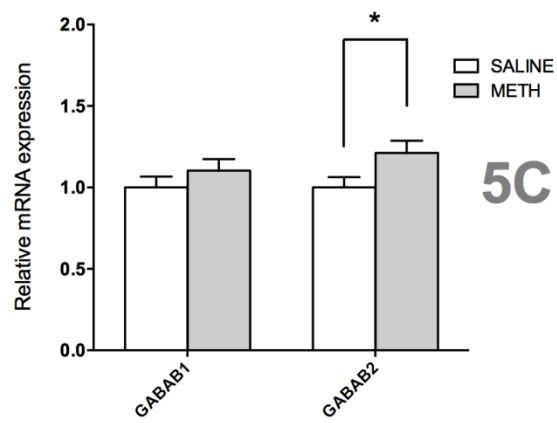
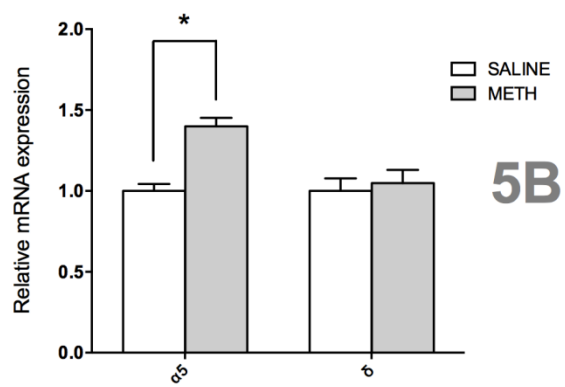
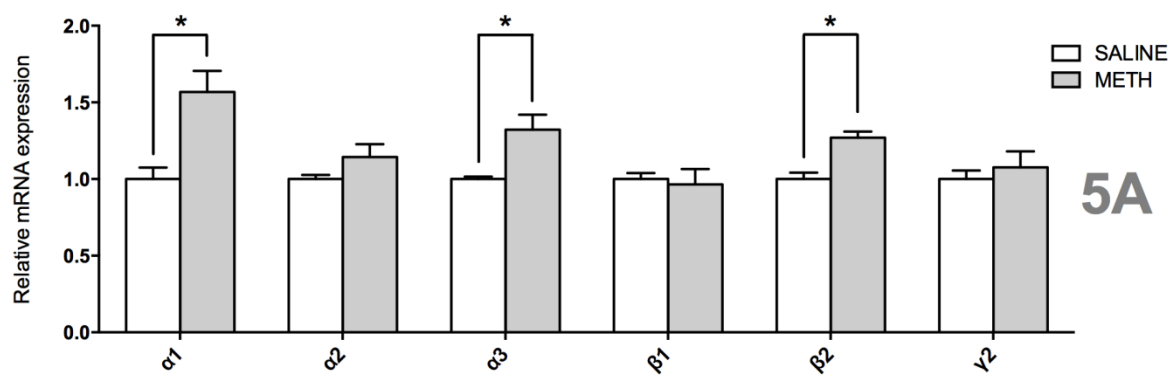


Figure 5. The expression of ionotropic and metabotropic GABA receptor mRNA expression in the orbitofrontal cortex (OFC) after saline and METH sensitization (n = 6 per group) as determined using quantitative RT-PCR and normalized to housekeeping gene, GAPDH. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 5A shows that GABA_A α 1, GABA_A α 3 and GABA_A β 2 subunit mRNA expression was upregulated following sensitization to METH in the OFC. Figure 3B depicts that the expression of extrasynaptic subunit GABA_A α 5 was upregulated following METH treatment in the OFC. Figure 3C shows the expression of metabotropic GABA_B receptors, with the expression of GABA_B2 mRNA upregulated in the OFC following METH sensitization. *Indicates GABA mRNA was significantly different when compared to saline controls.

4. Discussion

The main findings of this study were: (1) rats exposed to a chronic METH regime engaged in significantly more locomotor activity compared to saline pre-treated controls following a METH challenge; (2) GAD_{67} , GAD_{65} , GAT_1 , GAT_3 , $VGAT$, $GABA_T$, $GABA_{A\beta 2}$ and $GABA_B1$ mRNA expression was upregulated in the PRL of METH sensitized rats; and, (3) $GABA_{A\alpha 1}$, $GABA_{A\alpha 3}$, $GABA_{A\alpha 5}$ and $GABA_{A\beta 2}$ mRNA expression, together with $GABA_B2$, were significantly upregulated in the OFC following sensitization to METH.

The behavioral findings confirm the expression of METH-induced behavioral sensitization and are consistent with a large body of literature that has shown increased locomotor activity following chronic psychostimulant administration, including our own previous results (Ago et al., 2012; Pierce & Kalivas, 1997; Wearne et al., 2014). The clear lack of locomotor response of METH-exposed rats to saline challenge on Day 23 also confirms that the observed changes were independent of conditioned responding and attributable to the sensitizing effects of repeated METH administration. As sensitization is regarded as a model of psychostimulant-induced psychosis (Featherstone et al., 2007; Ujike, 2002), with sensitized behavior observed in METH psychosis (Akiyama et al., 2011; Sato et al., 1983) and schizophrenia (Ohmori et al., 1999; Olivares et al., 2013), the sensitized response to METH indicates that any GABAergic changes in the PRL and the OFC may represent biological factors that may contribute to both the vulnerability to psychotic relapse and the shared symptoms between METH psychosis and schizophrenia.

All GABA-related genes examined were detected in the PRL and the OFC, suggesting that under normal conditions, all are involved in the functioning and coordination of inhibitory neurotransmission across subregions of the PFC. However, multiple inhibitory transcripts were upregulated following sensitization to METH across both the PRL and the OFC, with all presynaptic GABAergic markers upregulated following METH sensitization in

the PRL, including enzymes and transporters, while the changes to the OFC were predominantly associated with increased expression of ionotropic GABA_A receptors. These findings extend our previous work by demonstrating that GABAergic gene expression is significantly altered following METH sensitization in a brain-region and GABA-specific manner. It is also important to note the lack of convergence in GABAergic mRNAs that were changed following METH sensitization between the PRL and OFC, with the exception of an upregulation of GABA_Aβ2 in each region. These findings suggest that changes to the GABAergic system are biologically dissociated between the OFC and PRL following sensitization to METH, and are consistent with research that has shown dissociated changes in the PRL and OFC following sensitization and repeated drug administration (Crombag, Gorny, Li, Kolb, & Robinson, 2005; Homayoun & Moghaddam, 2006; Moghaddam & Homayoun, 2008). As such, regional alterations to inhibitory neurotransmission following METH sensitization likely reflect cellular demands that subserve functionally distinct inhibitory cellular environments. These findings are therefore the first to provide evidence of molecular mechanisms, at least at the transcriptional level, which can lead to GABAergic dysfunction across the PRL and OFC following sensitization to repeated METH administration.

All GABAergic presynaptic markers were upregulated in the PRL following sensitization to METH, indicating that METH sensitization is associated with increased synthesis, release, reuptake and metabolism of GABA in this region. While these findings may suggest increased inhibition of the PRL, several lines of evidence suggest that this region is actually excitable during the expression of sensitization. For example, lidocaine-induced blockade of the mPFC potentiates locomotor response to acute amphetamine administration while the same blocks locomotor sensitization (Degoulet et al., 2009), indicating that the mPFC is inhibitory to acute psychostimulant administration and has an excitatory role in the expression of sensitization to amphetamine. Consistent with this, Aguilar-Rivera et al. (2015)

found that sensitization to amphetamine resulted in increased neuronal burst firing rate in the PRL while previous studies have reported increased synapses (Morshedi et al., 2009), dendritic spine density (Robinson & Kolb, 1997) and upregulated expression of the immediate early gene, *fos*, in the PRL of sensitized animals (Fanous, Lacagnina, Nikulina, & Hammer, 2011; Morshedi & Meredith, 2008). These findings suggest that PRL neurons are hyperexcitable following sensitization and places glutamatergic networks in the PRL as salient in the expression of sensitization. Indeed, extracellular glutamate is increased in the mPFC following amphetamine and METH exposure (Del Arco, Martínez, & Mora, 1998; Stephans & Yamamoto, 1995) while mPFC neurons are hypersensitive to glutamate following sensitization to amphetamine (Peterson, Wolf, & White, 2000).

The increased expression of GAD₆₇ and GAD₆₅ indicates increased production of GABA in the PRL following METH sensitization, however, it is possible that these changes are in response to a hyperglutamatergic state in the PRL. GAD catalyzes the decarboxylation of GABA from glutamate, with the expression of GAD₆₇ and GABA being activity dependent (Lau & Murthy, 2012), suggesting that the increased GAD₆₇ expression identified in the current study likely reflects increased synthesis of interneuronal GABA within the PRL due to elevated and subsequent degradation of glutamate. Previous studies have shown that GAD₆₇ protein expression is down-regulated in the PFC following sensitization to METH (Wearne et al., 2014) and amphetamine (Peleg-Raibstein, Knuesel, & Feldon, 2008), raising the possibility that GAD₆₇ mRNA expression is upregulated due to decreased protein expression. However, these findings are derived from global protein analyses of the PFC and may not be a reflective of the status of GAD₆₇ protein expression specifically in the PRL. Furthermore, as GAD₆₅ is only increased under periods of elevated synaptic activity (Choi et al., 2002; Hensch et al., 1998; Kash et al., 1997; Patel et al., 2006; Stork et al., 2000; Tian et al., 1999), the increased expression of GAD₆₅ adds further support for elevated activity at the synapse.

Taken together, the increased transcriptional upregulation of GAD enzymatic activity may be in response to elevated activity of the PRL. In keeping with this idea, VGAT and glutamate vesicular transporter 1 (VGlut1) are important in maintaining neurotransmission such that if the excitatory/inhibitory homeostasis is disrupted for a prolonged period of time, VGlut1 and VGAT are oppositely regulated to avoid over-excitation or over-inhibition (Turrigiano & Nelson, 2004). For example, the GABA_A receptor antagonist bicuculline - which hyperexcites neurons - reduces VGlut1 and increases VGAT mRNA expression in primary cortical cultures (De Gois et al., 2005). Therefore, the finding that VGAT mRNA expression is upregulated in the PRL also provides evidence of hyperexcited pyramidal neurons, and may consequently serve to maintain balanced excitatory and inhibitory circuits. Furthermore, the combined upregulated expression of GAD₆₇, GAD₆₅ and VGAT create a coupling for the degradation of glutamate and the subsequent synthesis and packaging of GABA levels into the synapse, potentially as a way of compensating for excess glutamate and subsequently minimizing glutamatergic neurotoxicity.

Consistent with our previous findings of the global PFC mRNA levels (Wearne et al., 2014), GAT₁ and GAT₃ mRNA were upregulated in the PRL following sensitization to METH. Given that GAT₁ is predominantly localized to both neurons and astrocytes (Conti et al., 2004; Gadea & Lopez-Colome, 2001) and GAT₃ is exclusively found in astrocytes (Conti et al., 2004), the increased expression of GABA transporters may indicate increased reuptake of GABA to the astrocytic glutamate/glutamine cycle, where GABA is metabolized to glutamate (Bak et al., 2006). In order to test this hypothesis, we extended our previous findings by examining the relative mRNA expression of GABA aminotransferase, GABA_T, the enzyme that catalyzes the degradation of GABA (van der Laan et al., 1979). Indeed, mRNA expression of GABA_T was increased in the PRL, suggesting that increased expression of GABA transporters may facilitate increased GABA metabolism as a positive feedback

mechanism to reduce the amount of GABA in the synapse and to avail a constant supply of glutamate in the PRL. These findings, therefore, provide molecular evidence that the increased excitation of the PRL may be driven by the increased metabolism of GABA. From the current results, it cannot be determined if the changes in mRNA expression are due to increased re-uptake and metabolism or increased production of GABA. Increased expression of GAT₁, GAT₃ and GABA_T may imply increased production and endocytosis of GABA from the presynaptic neuron, which therefore require increased metabolism. Alternatively, the increase in GAD₆₇ or GAD₆₅ mRNA expression may reflect increased production of GABA, and therefore glutamate, due to increased GABA metabolism and requires more mRNA to support a constant level of GAD and GABA protein in the PRL. As all these markers were similarly perturbed, it is possible that these changes may attempt to maintain a constant level of inhibitory neurotransmission in light of the increased excitable tone of PRL pyramidal cells produced by METH sensitization.

The findings of the current study also suggest transcriptional upregulation for several ionotropic GABA_A receptors in the OFC following sensitization to METH. However, the functional significance of these receptor subunit changes are difficult to interpret, particularly given that GABA_A receptors are hetero-oligomeric membrane proteins composed of five receptor subunits and that GABA_A receptors have distinct functional and spatio-temporal profiles (Boileau et al., 2005; Olsen & Sieghart, 2009). Nevertheless, variations of the GABA_A receptor subunit mediate both postsynaptic phasic and extrasynaptic tonic inhibition, with GABA_A receptors containing the α 1, α 3 and β 2 subunits mediating phasic inhibition, and therefore the hyperpolarization of efferent neurons (Hines et al., 2012), while GABA_A receptors containing the α 5 subunit are located extrasynaptically and control the tonic inhibition of postsynaptic cells (Fritschy & Mohler, 1995; Pirker et al., 2000b). Therefore, the up-regulation of α 1, α 3, α 5 and β 2 may reflect increased tonic and phasic GABAergic

inhibition of efferent pyramidal cells. While these receptor changes may represent a compensatory mechanism due to reduced GABA in the OFC, previous studies have shown that the OFC is underactive (Rita Z. Goldstein & Volkow, 2011; R. Z. Goldstein, Volkow, Wang, Fowler, & Rajaram, 2001) and that dendritic branching is decreased (Crombag et al., 2005) during abstinence following chronic exposure to psychostimulants. As such, these current results suggest that the decrease in excitatory output may be secondary to elevated inhibition of glutamatergic circuits, and may provide evidence that the GABAergic system promotes an inactive OFC. Likewise, as postsynaptic GABA_B receptors alter multiple cellular processes via slow synaptic transmission and the inhibition of intracellular signaling pathways, the increased mRNA expression of GABA_{B2} in the OFC following METH sensitization may represent increased inhibitory metabotropic receptor signaling. While these findings are the first to implicate the GABAergic system of the OFC in the neurobiology of METH sensitization, there is a paucity of research on the role of the OFC, and its neurotransmitter systems, in mediating sensitized behavior or its relationship amongst the known circuitry of sensitized behavior.

Changes to the GABA network identified throughout this study may also have direct relevance to chronic METH psychosis and schizophrenia. For example, deletion of the GABA_Aα3 subunit induces a hyperdopaminergic state and sensorimotor deficits that are consistent with schizophrenia (Yee et al., 2005), placing the α3 subunit as indirectly involved in the maintenance of psychosis-related behavior and psychosis pathology. Studies have also reported genetic associations between schizophrenia and GABA_Aα1 (Petryshen et al., 2005) and α5 subunits (Papadimitriou et al., 2001), while GABA_Aα1 mRNA expression is increased in the PFC of schizophrenic patients (Ohnuma et al., 1999). Furthermore, given that METH sensitization is associated with altered gamma band oscillations in the PFC (Ahn, Rubchinsky, & Lapish, 2013; Janetsian et al., 2015), together with the important role of the

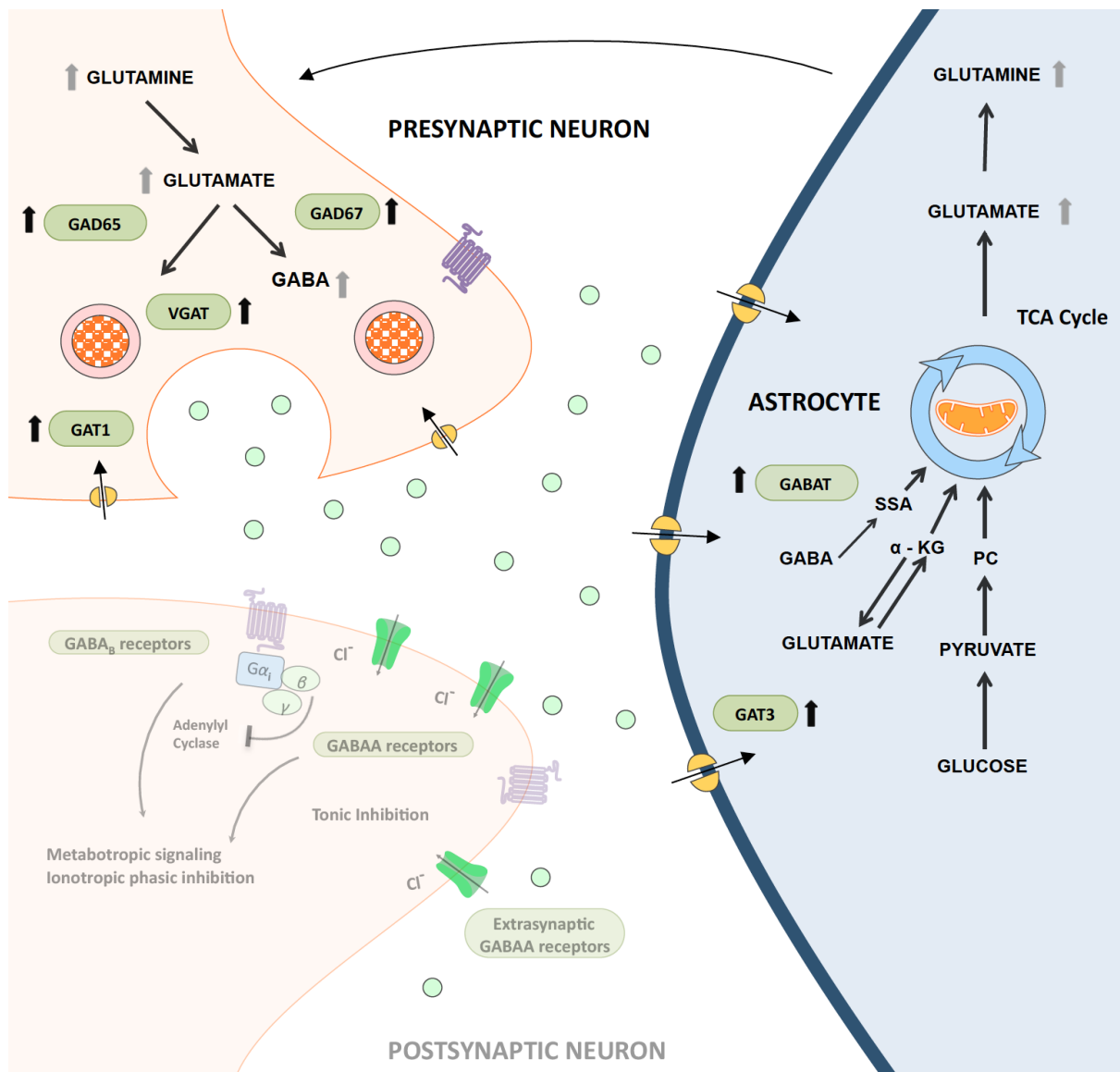


Figure 6. A diagrammatic depiction of the proposed changes to the GABAergic system in the prelimbic cortex (PRL) following methamphetamine sensitization. Grey arrows adjacent to various components of the GABAergic network represent mRNA expressions that were upregulated in the PRL. All presynaptic inhibitory markers were upregulated in the PRL following sensitization to METH. GABA is produced by the α -decarboxylation of glutamate by the rate-limiting enzyme, glutamate-decarboxylase (GAD), suggesting that the upregulated expression of the two GAD isoforms, GAD₆₇ and GAD₆₅, reflects increased production of the cytoplasmic and synaptic stores of GABA. Increased VGAT expression reflects increased transport and exocytosis of GABA into the synapse. GABAergic signaling is terminated through the reuptake of GABA to either the presynaptic membrane, via the GABA transporter 1 (GAT₁), or to astrocytes through the action of GABA transporter 3 (GAT₃). Therefore, increased GAT1 and GAT3 mRNA expression could indicate increased endocytosis to astrocytes. As GABA is metabolized to glutamate and succinic-semialdehyde via the action of GABA transaminase (GABA_T), the increased mRNA expression of GABA_T could mediate increased GABAergic metabolism and elevated production of glutamate in the PRL. Drawing designed and created by adapting various slides purchased from Motifolio (<http://motifolio.com/>).

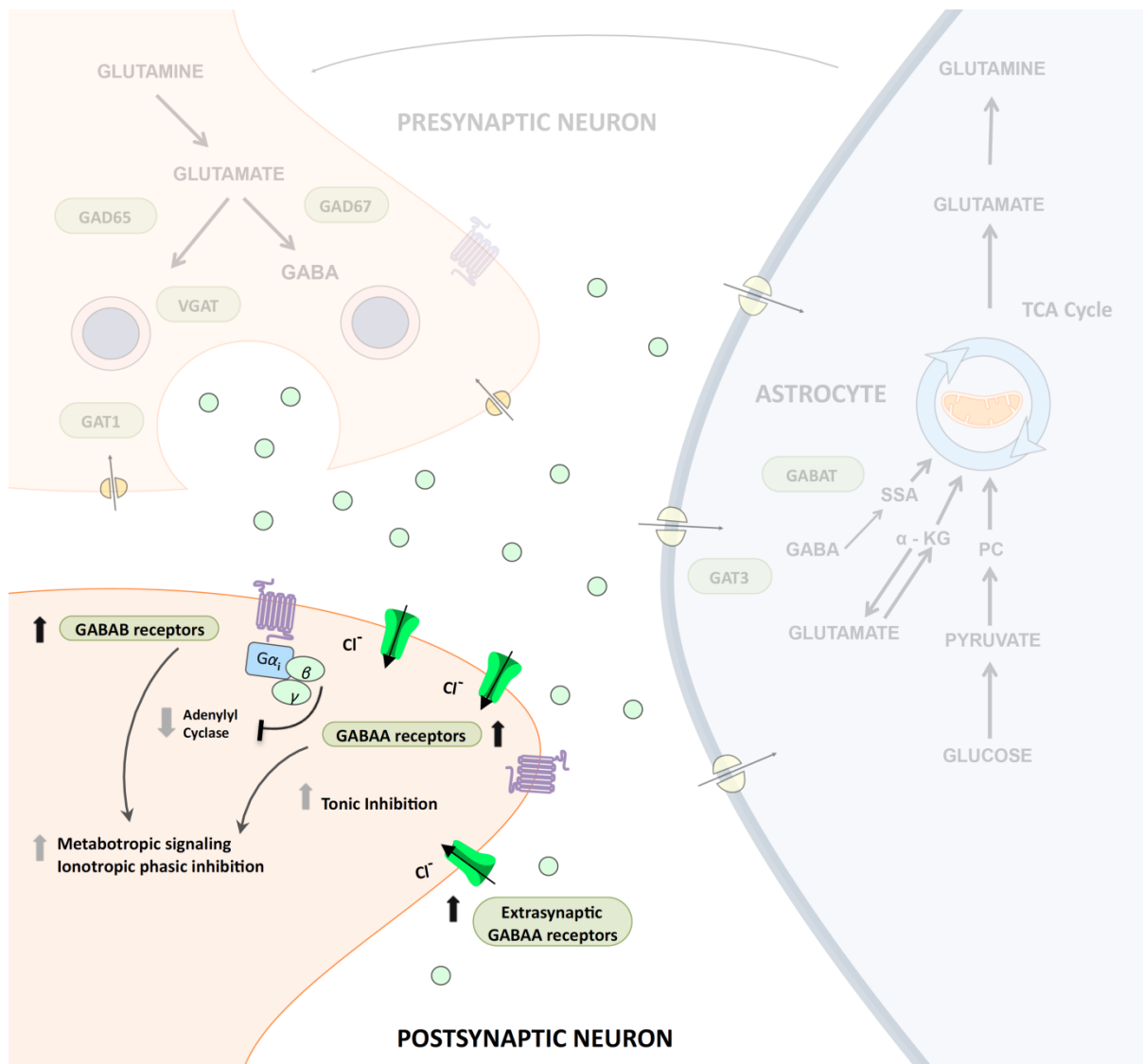


Figure 7. A diagrammatic depiction of the proposed changes to the GABAergic system in the orbitofrontal cortex (OFC) following methamphetamine sensitization. Grey arrows adjacent to various components of the GABAergic network represent mRNA expressions that were upregulated in the OFC. The OFC was associated with multiple changes to postsynaptic GABA receptors. Exocytosis of GABA into the synapse activates a constellation of postsynaptic ionotropic (GABA_A) and metabotropic (GABA_B) receptors that mediate phasic inhibition and slow synaptic inhibitory neurotransmission, respectively. GABA spillover can also activate extrasynaptic GABA_A receptors that are responsible for the tonic inhibition of postsynaptic neurons. As the mRNA expression of $\alpha 1$, $\alpha 3$, $\alpha 5$, β_2 and GABA_{B2} were upregulated in the OFC, these changes likely reflect increased phasic, tonic and metabotropic inhibitory signaling in the OFC following sensitization to METH. Drawing designed and created by adapting various slides purchased from Motifolio (<http://motifolio.com/>).

PFC in cognition, changes to the GABAergic system may play an important role in underlying altered cognitive phenotypes elicited by METH sensitization.

Many of the upregulated changes to GABA system mRNA identified in the PRL in the current study do not typically reflect the directional changes reported in schizophrenia. For example, multiple studies have found that mRNA expression of GAD₆₇ is reduced in the DLPFC in schizophrenia (Akbarian et al., 1995; Blum & Mann, 2002; Curley et al., 2011; Gonzalez-Burgos et al., 2010; Guidotti et al., 2000; Hashimoto, Bazmi, et al., 2008; Joshi et al., 2012; Straub et al., 2007; Volk et al., 2000), although there are reports that GAD₆₇ and GAD₆₅ mRNA expression is upregulated (Dracheva et al., 2004). VGAT mRNA expression is also decreased in the DLPFC of schizophrenia (Hoftman et al., 2015) while GABA transporters, particularly GAT₁, are also reduced (Volk et al., 2001; Woo et al., 1998). While the findings reported here demonstrate quantitatively differences to those reported in primary psychotic disorders, supporting that METH sensitization and schizophrenia are associated with distinct inhibitory pathologies, they do suggest that both METH sensitization and schizophrenia are characterized by changes at inhibitory synapses.

5. Conclusions

METH sensitization resulted in the transcriptional upregulation of multiple inhibitory GABAergic markers across subregions of the PFC, with alterations to GABAergic mRNA expression biologically dissociated between the orbitofrontal and prelimbic cortices following sensitization to METH. These changes extend our previous global analysis of the PFC, by providing evidence that GABAergic gene expression is significantly altered in a brain-region and GABA-specific manner following sensitization. These changes could have a profound influence on central inhibitory mechanisms of these localized regions and may mediate both excitatory (PRL) and inhibitory (OFC) output of the PFC. Furthermore, many of the changes identified in the current study were diametrically opposed to the direction typically reported in the PFC in schizophrenia, particularly in the PRL. As such, the results of the current study provide preliminary evidence that METH sensitization may be associated with a distinct inhibitory environment of the PFC compared to schizophrenia.

Chapter Five

Interneuronal mRNA Expression across the Prelimbic and Orbitofrontal Cortices following
Methamphetamine Sensitization: Do GABAergic changes correlate to a specific cellular
phenotype?

Co-Author Contribution

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Contributed to research design and manuscript editing 2%

Total 2%

1. Introduction

GABA (γ -aminobutyric acid) is the most ubiquitous inhibitory neurotransmitter in the central nervous system, with GABA-mediated processing modulating both inhibitory and excitatory networks via interneurons and glutamatergic pyramidal cells, respectively, in cortical networks. Interneurons constitute 20-30% of neuronal cells of the cortex and are traditionally categorized into distinct subtypes based on the expression of neuropeptides and calcium binding proteins that correspond to particular synaptic and electrophysiological characteristics (Markram et al., 2004). Interneurons arborize on pyramidal cell bodies and/or axon initial segments (containing cholecystokinin or parvalbumin), synapse onto superficial or deep pyramidal dendrites (containing somatostatin or neuropeptide Y) (Markram et al., 2004) or can indirectly modulate excitatory output through the disinhibition of interneurons (containing vasoactive intestinal peptide) (Pi et al., 2013). Interneurons also demonstrate distinct electrophysiological properties, including irregular spiking (calretinin), bursting (calbindin) and fast-spiking patterns (parvalbumin) (Chow et al., 1999; Toledo-Rodriguez et al., 2004). This variability in peptide colocalization within GABA interneurons ultimately allows for specific temporal-regulation of the inhibitory and excitatory signaling required for the fine-tuning and synchronization of network oscillations (Volman et al., 2011). Therefore, altered inhibitory GABAergic processing is hypothesized to mediate dysfunctional neuronal oscillations and cognitive impairments (Başar et al., 2001; Gonzalez-Burgos et al., 2010), particularly those mediated by the prefrontal cortex (PFC) (Anderson et al., 1999; Başar et al., 2001; Ridderinkhof et al., 2004).

Schizophrenia is associated with significant interneuron pathology of the PFC. Parvalbumin protein and mRNA expression is downregulated in the PFC of schizophrenia (Gonzalez-Burgos et al., 2010; Takanori Hashimoto et al., 2003) while mRNA expressions of cholecystokinin (Hashimoto et al., 2008), somatostatin (Fung et al., 2010; Hashimoto et al.,

2008) and neuropeptide Y (Hashimoto et al., 2008) are also reduced. Calretinin appears to be unaltered (Hashimoto et al., 2008), and given that 40-50% of interneurons express calretinin, this finding suggests that schizophrenia is associated with a selective inhibitory deficit in the PFC. There is also significant evidence that changes to the GABAergic system in schizophrenia are localized to particular interneurons. For example, GAD₆₇ mRNA expression is reduced in 50% of parvalbumin mRNA positive neurons (Hashimoto et al., 2003), and given that the decrease in parvalbumin mRNA expression is not caused by a reduced number of parvalbumin-expressing neurons in the PFC, the reduced expression of GAD₆₇ mRNA is not secondary to a loss of interneuron cells. Additionally, the expression of parvalbumin per neuron and the change in GAD₆₇ mRNA density in the PFC of schizophrenia are positively correlated (Hashimoto et al., 2008), supporting the conclusion that GAD₆₇ mRNA expression is reduced in parvalbumin-expressing neurons that also have reduced parvalbumin mRNA. Furthermore, reductions of GABA transporter 1 (GAT₁) is most prominent in parvalbumin-expressing chandelier interneurons (Curley et al., 2011; Hashimoto et al., 2003; Nakazawa et al., 2012); cells believed to mediate the inhibition of pyramidal neurons and neuronal oscillations during cognitive tasks (Başar et al., 2001; Gonzalez-Burgos et al., 2010). These findings indicate that interneurons of the PFC are specifically implicated in the neurobiology of schizophrenia and that GABAergic disturbances may localize to specific subsets of the inhibitory cells of this region.

Methamphetamine (METH; “ice”) is a commonly used psychostimulant that can induce an acute and chronic psychosis, with a growing body of literature showing that METH psychosis is associated with positive, negative and cognitive deficits that are qualitatively and quantitatively comparable to schizophrenia (Ezzatpanah et al., 2014; Medhus et al., 2013; Srisurapanont et al., 2011). These similar symptom profiles suggest that METH psychosis and schizophrenia may overlap in neurobiology and that both conditions may fall on a continuum

of brain pathology. Behavioral sensitization - a phenomenon whereby repeated exposure to a psychostimulant drug, such as METH, results in a progressively increased behavioral response to that stimulus following a period of abstinence (Pierce & Kalivas, 1997) - is believed to reproduce the behavioral and neurobiological changes driving psychoses (Featherstone et al., 2007; Ujike, 2002). Therefore, elucidating the molecular mechanisms that mediate METH sensitization could enable understanding of the neurobiological underpinnings that subserve psychoses. In light of the similarity in symptoms between METH psychosis and schizophrenia, METH sensitization may alter the GABAergic profile of the PFC similarly to that reported in psychotic disorders. Indeed, we have previously found that METH sensitization induces adaptive and molecular changes to various components of the GABAergic network across both global and localized regions of the PFC (Wearne et al., 2015; Wearne et al., in revision; Chapter 4). However, given the range of inhibitory interneurons present in the cortex, together with the interneuronal pathology reported in schizophrenia, it is possible that the changes to inhibitory neurotransmission following METH sensitization may also be evident at the cellular interneuronal level.

The primary aim of this study was to examine the relative mRNA expression of several inhibitory interneuron markers (parvalbumin, calbindin, calretinin, somatostatin, cholecystokinin, neuropeptide Y and vasoactive intestinal peptide) in two subregions of the PFC following METH sensitization: the prelimbic cortex (PRL) and orbitofrontal cortex (OFC). We have previously found that multiple genes associated with the GABA system are altered across the PRL and the OFC following METH sensitization (Chapter 4). To extend these findings using samples from the same cohort of rats, the second aim of this study examined the association between GABAergic and interneuronal mRNA expression across the PRL and OFC in METH sensitization. It was anticipated that these analyses would determine whether our previous findings correspond to a particular inhibitory cellular subtype

and whether the profile of interneuronal changes following METH sensitization is consistent with that typically reported in schizophrenia.

2. Materials and Methods

2.1 Animals

The analysis of this study was performed on RNA isolated from an already described cohort of METH-sensitized rats and saline-treated controls (Chapter 4). All experiments were approved by the Macquarie University Animal Ethics Committee (ARA 2012/047; Appendix D) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013).

2.2 Methamphetamine-induced Behavioral Sensitization

Methamphetamine behavioral sensitization was performed as previously described (Wearne et al., 2014; Wearne et al., submitted; Chapter 5). Briefly, rats were assigned to undergo repeated METH (once daily 1ml/kg i.p. on days 1 & 7; 5mg/kg i.p. days 2 to 6) or saline (1ml/kg i.p.) injections for 7 days. Following 14 days of withdrawal, changes to locomotor activity was tested via a challenge dose of 1mg/kg METH (i.p.). Rat locomotor activity was recorded on Days 2, 8 and 24 using standard Med Associates chambers equipped with infrared photobeam detectors (NESS Security Products), with activity quantified as the number of photobeam interruptions (Med Associates, VT, USA). Methamphetamine hydrochloride (METH) was purchased from the Australian Government Analytical Laboratories (Pymble, NSW), dissolved in saline and administered at a volume of 1ml/kg.

2.3 Quantitative Polymerase Chain Reaction (qPCR)

2.3.1 Primer Design and Validation

Table 1 summarizes the primer sequences, GeneBank accession numbers, PCR product sizes and efficiencies for each gene. Primers were designed using NCBI Nucleotide Primer Design software as previously described (Wearne et al., submitted). Primers did not

distinguish between splice variants. PCR products were sequenced and specificity was verified through the use of a BLAST search against the rat genome. A four-point standard curve was measured for each primer set and the primer efficiencies were calculated using MxPro software (Stratagene). All genes had similar primer efficiencies, $r^2 > 0.986$.

2.3.2 Quantitative Real Time PCR

Real-Time PCR was performed as previously described (Parker et al., 2012) on cDNA obtained from a previous cohort (Wearne et al., 2015). Samples were analyzed in duplicate and templates without product did not amplify any signal. Cycle threshold (Ct) values were obtained for each interneuronal mRNA using the MxPro software (Stratagene) and normalized to GAPDH. GAPDH was unchanged at the mRNA level in the PRL ($p = .14$) and OFC ($p = .47$) following METH sensitization.

2.2.3 Data Analysis

Expression levels were calculated relative to controls using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). All genes were normally distributed except for two genes in the METH condition (somatostatin & neuropeptide Y) in the PRL, while 1 gene in the saline group (calretinin) and METH sensitized conditions (neuropeptide Y) were not normally distributed in the OFC. However, non-parametric testing revealed that this did not alter the interpretation of the results. As such, independent t-tests were used to determine significant changes in gene expression between METH-sensitized rats and saline controls. Pearson's correlations within the same set of subjects were performed to determine the association between interneuronal gene expression and the GABAergic mRNA expression of upregulated genes previously identified in the PRL and OFC (Chapter 4). All analyses were performed using SPSS version 21 and the significance level was set at $p < .05$.

Table 1. Summary of primers, Genebank ascension numbers, forward and reverse sequences and PCR-product sizes for the gene analyzed in the current study

Primer	GeneBank ID	Forward (5' – 3')	Reverse (5' – 3')	Size
Parvalbumin	NM_022499.2	<i>ATAGGAGCCTTTACTGCTGC</i>	<i>CATCCTCCTCAATGAAGCCACT</i>	142
Cholecystokinin	NM_012829.2	<i>ATACATCCAGCAGGTCCGCAA</i>	<i>TGTAGTCCCGGTCACCTTATCCT</i>	101
Calbindin	NM_031984.2	<i>AAGGCTGGATTGGAGCTATCAC</i>	<i>TCAGTTGCTGGCATCGAAAG</i>	148
Calretinin	NM_053988.1	<i>ATGGAGACGGCAAATTGGGT</i>	<i>CCGCTTCCATCCTTGTCATAGA</i>	142
Somatostatin	NM_012659.1	<i>CCCAACCAGACAGAGAACGAT</i>	<i>GAAGTTCTTGCAGCCAGCTTTG</i>	147
Neuropeptide Y	NM_012614.2	<i>TGCTCGTGTGTTTGGGCATT</i>	<i>GGCTGGATCTCTTGCCATATCT</i>	156
Vasoactive Intestinal Peptide	NM_053991.1	<i>TCAGTGTGCTGTTCTCACAGTC</i>	<i>GTCATTCTCCGCTAAGGCATTC</i>	161
GAPDH	NM_017008.4	<i>TGAAGGTCGGTGTGAACGGATTTG</i>	<i>AGCCTTGACTGTGCCGTTGAACTT</i>	176

3. Results

3.1 Sensitization to Methamphetamine

Behavioral results regarding this cohort can also be found elsewhere (Chapter 4). Briefly, METH challenge resulted in a significant increased locomotor response in METH pre-treated rats compared to saline controls (Figure 1A), with METH pre-treated rats also showing a significant increased locomotor response on challenge when compared to their locomotor response on Day 1 of acute METH exposure.

3.2 Interneuronal mRNA Expression in the Prelimbic Cortex following Sensitization

3.2.1 Relative expression of Interneuron Markers in the Prelimbic Cortex of Methamphetamine Sensitized Rats

Calbindin (1.30-fold, $p < .005$) and calretinin (1.59-fold, $p < .01$) mRNA expression was significantly upregulated in the PRL of METH-sensitized rats compared with saline-treated controls (Figure 2A). Similarly, mRNA expression for somatostatin (1.30-fold, $p < .05$), cholecystokinin (1.32-fold, $p < .005$) and vasoactive intestinal peptide (1.33-fold, $p < .005$) were also significantly increased following sensitization to METH compared with saline controls. There was no change in the mRNA expression of parvalbumin ($p = .16$) or neuropeptide Y ($p = .58$) between METH sensitized rats and saline-treated controls.

3.2.2 Association between GABAergic and Interneuronal mRNA expression in the Prelimbic Cortex of Methamphetamine Sensitized Animals

GAD₆₇ ($r = .74$, $p < .05$) and GAT₁ ($r = .68$, $p < .01$) mRNA expression positively correlated with the mRNA expression of parvalbumin. Similarly, GAD₆₇ ($r = .66$, $p < .05$), GAD₆₅ ($r = .71$, $p < .01$) and GAT₁ ($r = .65$, $p < .05$) significantly correlated with the mRNA expression of cholecystokinin. Additionally, GABA β 2 correlated with the expression of

somatostatin ($r = .71, p < .01$), VGAT positively correlated with calretinin ($r = .71, p < .05$) and GABA_T was positively associated with the expression of neuropeptide Y ($r = .67, p < .05$) (Table 2).

3.3 Interneuronal mRNA Expression in the Orbitofrontal Cortex following Sensitization

3.3.1 Relative Expression of Interneuronal mRNA in the Orbitofrontal Cortex of Methamphetamine Sensitized Rats

Parvalbumin (1.40-fold, $p < .01$), somatostatin (1.21-fold, $p < .05$), cholecystokinin (1.24-fold, $p < .005$) and vasoactive intestinal peptide (1.48-fold, $p < .005$) mRNA expressions were significantly increased following sensitization to METH compared with saline controls (Figure 3A and 3B). There was no change in the mRNA expression of calbindin ($p = .19$), calretinin ($p = 0.54$) or neuropeptide Y ($p = .18$) between METH sensitized rats and saline-treated controls (Figure 3A and 3B).

3.3.2 Association between GABAergic and Interneuronal mRNA Expression in the Orbitofrontal Cortex of Methamphetamine Sensitized Animals

GABA_Aα1 was positively correlated with the mRNA expression of somatostatin ($r = .64, p < .05$). GABA_Aα5 was negatively correlated with the expression of somatostatin ($r = -.78, p < .005$) and calbindin ($r = -.60, p < .05$) (Table 3). All other correlations were not significant (all $ps > .05$).

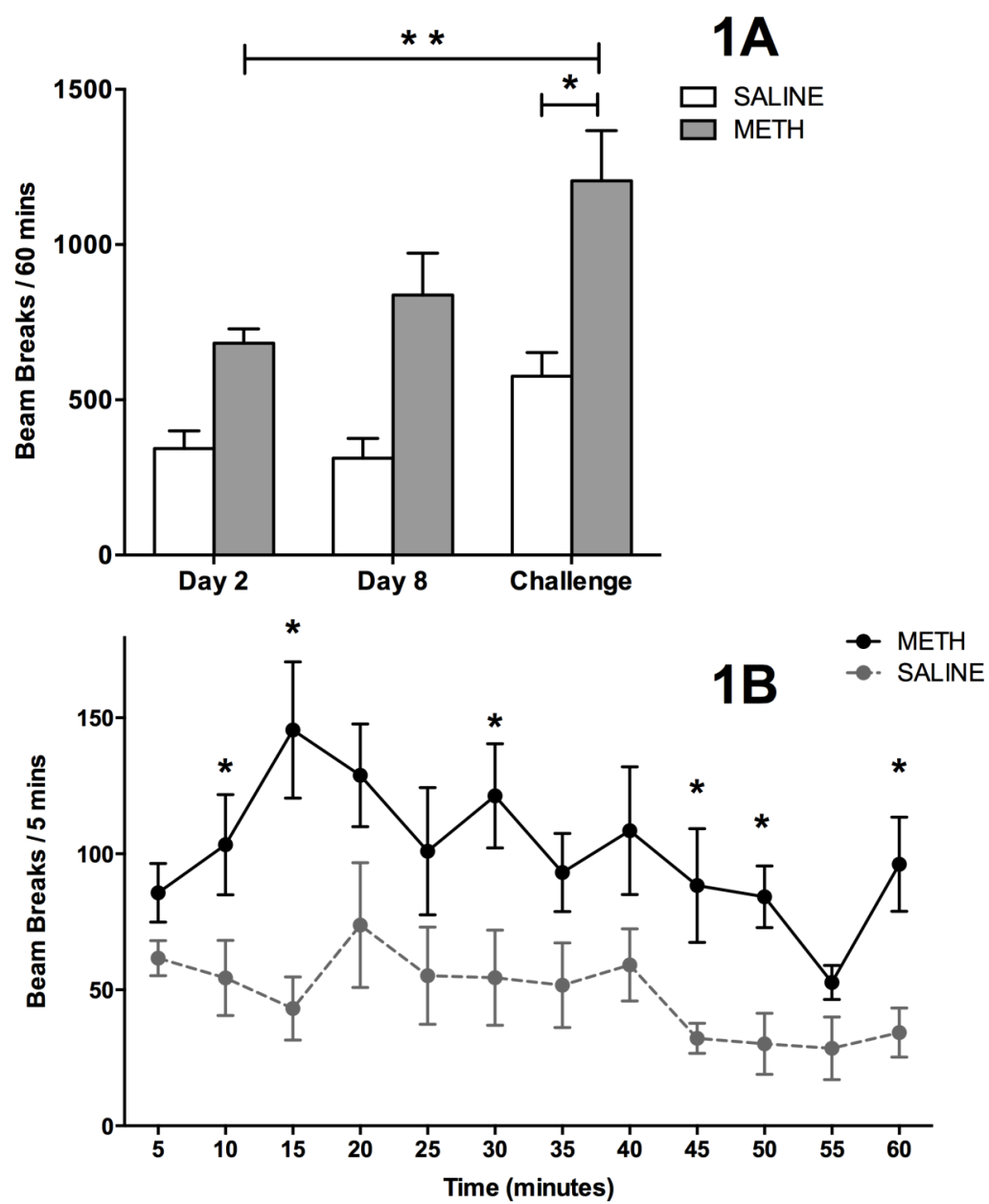
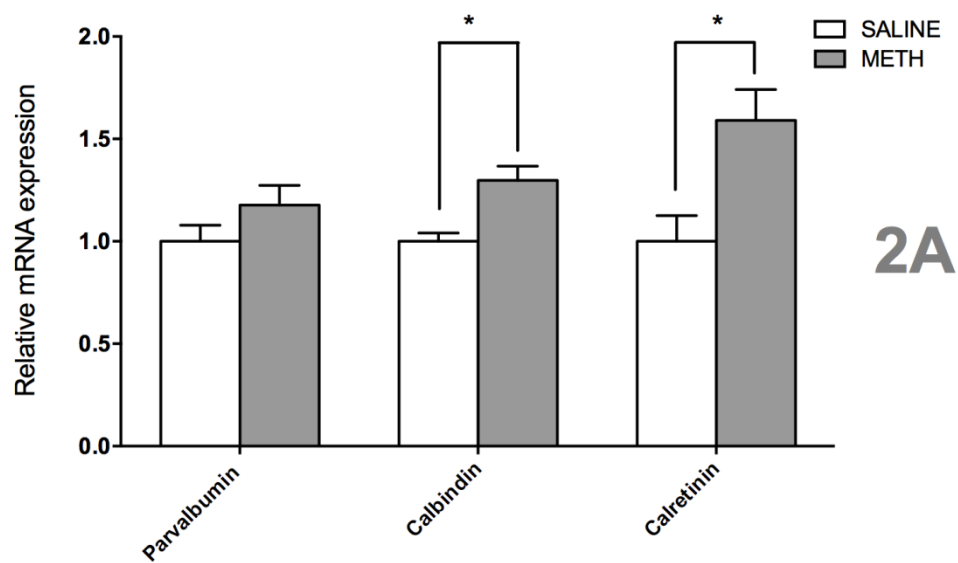
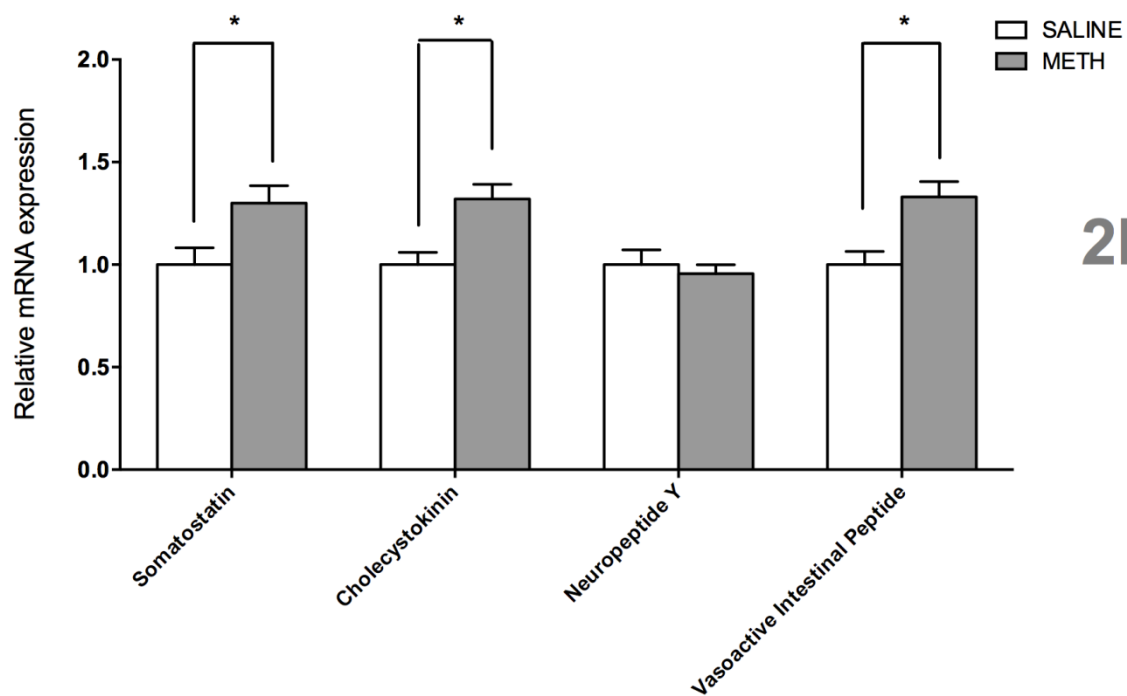


Figure 1. Locomotor sensitization to repeated METH administration. Rats were assigned to undergo either repeated METH (1ml/kg intraperitoneal (i.p.) days 1 & 7; 5mg/kg i.p. days 2 – 6, n=6) or saline (1 ml/kg i.p., n=6) treatment for 7 days. Figure 1A represents the total beam breaks across days 2, 8 and challenge, respectively. Rats showed a progressively increased locomotor response to repeated METH administration, with a METH challenge resulting in a significant sensitized locomotor response in METH pre-treated animals when compared with saline controls*. METH treated rats also showed a significant increase in locomotor activity between Day 2 and challenge**. Figure 1B represents the time-course of locomotor activity for METH and saline pre-treated rats over 60 minutes post challenge administration. Data represented as mean \pm SEM activity as beam breaks in each 5-minute period. METH-treated rats displayed significantly higher locomotor activity at 10, 15, 30, 45, 50 and 60 mins after challenge injection*. Solid lines represent METH while the dashed lines represent the saline condition.



2A



2B

Figure 2. The expression of inhibitory interneuronal mRNA in the prelimbic cortex (PRL) after saline and METH sensitization (n = 6 per group) as determined by quantitative RT-PCR normalized to housekeeping gene, GAPDH. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 2A shows that the calcium binding proteins, calbindin and calretinin were upregulated following METH sensitization in the PRL. Figure 2B shows that the neuropeptides somatostatin, cholecystokinin and vasoactive intestinal peptide were also significantly increased in the PRL following sensitization to METH compared with saline-treated controls. *Indicates GABA mRNA was significantly different when compared to saline controls at $p < .05$.

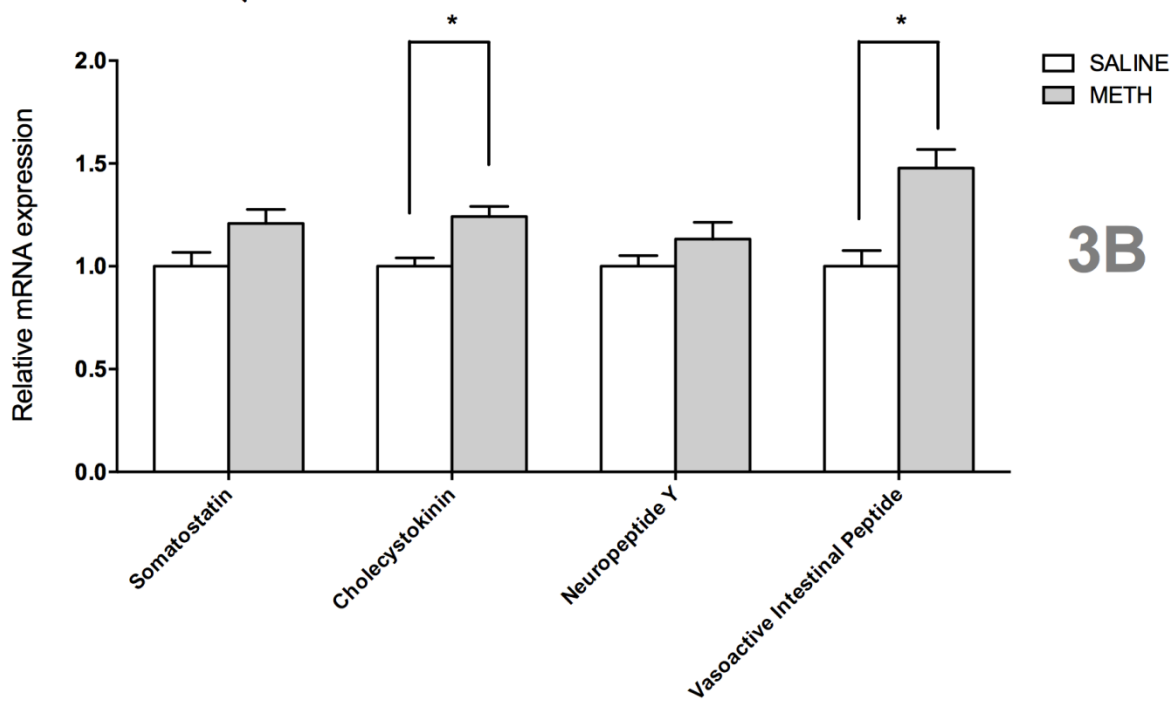
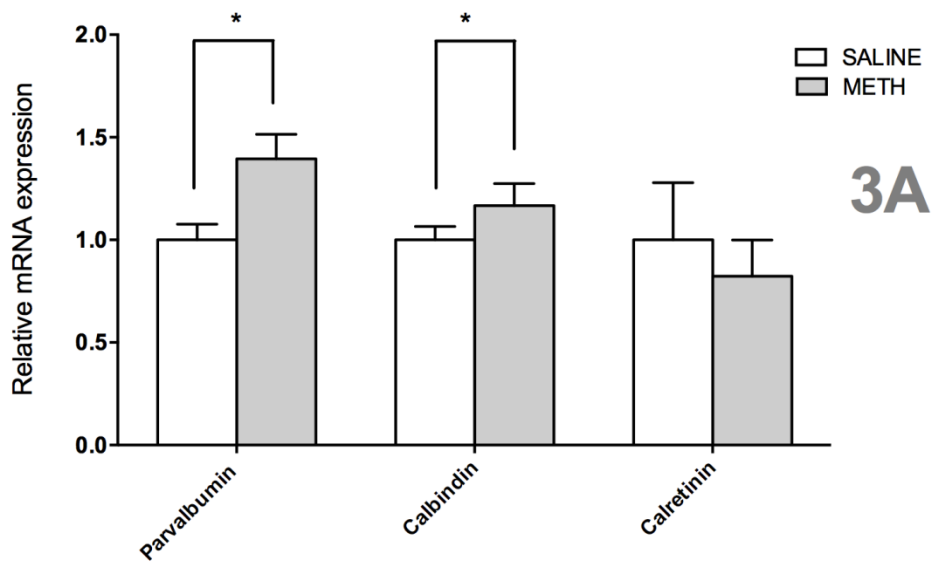


Figure 3. The relative mRNA expression of inhibitory interneuron markers in the orbitofrontal cortex (OFC) after saline and METH sensitization (n = 6 per group) using quantitative RT-PCR. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta C_t}$ method. Figure 2A shows that parvalbumin and calbindin (calcium binding proteins) mRNA expressions were significantly upregulated following METH sensitization in the OFC. Figure 2B depicts that the neuropeptides cholecystokinin and vasoactive intestinal peptide were also significantly upregulated following sensitization to METH. *Indicates GABA mRNA was significantly different when compared to saline controls at $p < .05$.

Table 2. Bivariate relationships between mRNA expression for upregulated GABAergic genes and interneuronal markers in the PRL for METH sensitized rats

	GAD ₆₇	GAD ₆₅	GAT ₁	GAT ₃	VGAT	GABA _T	β2	G _{B1}	PVALB	SST	CALB	CRET	CCK	NPY	VIP
GAD ₆₇	1														
GAD ₆₅	.803**	1													
GAT ₁	.920**	.798**	1												
GAT ₃	.634*	.694*	.555	1											
VGAT	-.392	-.132	-.328	-.153	1										
GABA _T	-.718**	-.620*	-.687*	-.271	.656*	1									
β2	.367	.271	.296	.626*	-.439	-.171	1								
G _{B1}	.567	.507	.609*	.303	-.585*	-.586*	.693*	1							
PVALB	.737**	.253	.681*	.344	-.407	-.364	.424	.496	1						
SST	.327	.130	.355	.339	-.366	.014	.707*	.560	.590*	1					
CALB	.340	.313	.168	.496	.435	-.006	.004	-.270	.167	-.301	1				
CRET	-.474	-.412	-.537	-.509	.712*	.559	-.386	-.489	-.359	-.315	.206	1			
CCK	.664*	.714**	.654*	.432	-.049	-.321	.063	.354	.397	.161	.194	-.469	1		
NPY	-.326	-.533	-.248	-.326	.297	.672*	.074	-.105	.247	.553	-.212	.361	-.197	1	
VIP	.486	.356	.349	.213	.193	-.324	.086	.195	.361	-.234	.763**	.162	.235	-.200	1

G_{B1} = GABA_{B1}, PVALB = parvalbumin, SST = somatostatin, CALB = calbindin, CRET = calretinin, CCK = cholecystokinin, NPY = Neuropeptide Y, VIP = vasoactive intestinal peptide

* $p < .05$

** $p < .01$

Table 3. Bivariate relationships between mRNA expression for upregulated GABAergic genes and interneuronal makers in the OFC for METH sensitized rats

	$\alpha 1$	$\alpha 3$	$\alpha 5$	$\beta 2$	G _B 2	PVALB	SST	CALB	CRET	CCK	NPY	VIP
$\alpha 1$	1											
$\alpha 3$.644*	1										
$\alpha 5$	-.581*	-.531	1									
$\beta 2$	-.107	-.451	.075	1								
G _B 2	.030	-.029	.343	-.412	1							
PVALB	.110	.373	-.191	-.549	.207	1						
SST	.643*	.522	-.775**	-.234	-.162	.406	1					
CALB	.448	.282	-.597*	.158	-.436	.136	.480	1				
CRET	.258	.046	-.239	.118	-.383	-.708**	-.029	.209	1			
CCK	.502	.313	-.252	-.573	.209	.530	.365	.240	-.024	1		
NPY	.322	-.099	-.314	-.078	-.089	-.488	.198	-.099	.637*	.160	1	
VIP	.197	.289	-.365	.230	-.260	.642*	.367	.350	-.489	-.216	-.453	1

GB2 = GABA_B2, PVALB = parvalbumin, SST = somatostatin, CALB = calbindin, CRET = calretinin, CCK = cholecystokinin, NPY = Neuropeptide Y, VIP = vasoactive intestinal peptide

* $p < .05$

** $p < .01$

4. Discussion

Behavioral sensitization is commonly used as an animal model of stimulant induced psychosis (Ujike, 2002), particularly given that sensitized behavior has been observed in both METH psychosis (Sato et al., 1992; Yui et al., 2003) and schizophrenia (Ohmori et al., 1999; Olivares et al., 2013). However, the involvement of the GABAergic system in the PFC following sensitization to psychostimulants has not been thoroughly examined. To this end, we have previously found that several molecular GABAergic transcripts were upregulated in both the global and localized regions of the PFC, including the PRL and OFC. Here, as an extension of these findings, we show that METH sensitization also changes the transcriptional expression of multiple GABAergic interneuronal markers (calcium binding proteins & neuropeptides), with sensitization increasing the expression of calbindin, calretinin, somatostatin, cholecystokinin and vasoactive intestinal peptide in the PRL while parvalbumin, calbindin, cholecystokinin and vasoactive intestinal peptide were transcriptionally upregulated in the OFC. These findings provide the first evidence that sensitization mediates differential interneuronal pathology across the PRL and OFC.

The upregulated expression of multiple mRNAs encoding for neuropeptides and calcium binding proteins in the PRL would suggest that a range of interneuron subtypes are affected following sensitization to METH. Calcium-binding proteins in the PFC are typically described as reflecting interneuronal subtypes with distinct electrophysiological profiles. As such, the increased expression of calretinin and calbindin may indicate that the PRL is associated with increased calcium influx that mediates elevated irregular spiking and bursting across distinct inhibitory cells, respectively. Furthermore, the increased mRNA expression of somatostatin and cholecystokinin suggests increased inhibitory drive from somatostatin-martinotti cells and cholecystokinin-basket cells to the perisomatic region (Somogyi et al., 2004) or the distal dendritic arbor of pyramidal cells (Yasuo et al., 2002), respectively (Figure

4). Given that the PRL is hyperexcitable during the expression of sensitization (Aguilar-Rivera et al., 2015; Degoulet et al., 2009), these changes may attempt to compensate for elevated glutamatergic output, particularly given that these neurons are in a position to modulate efferent excitatory outflow due to their positioning with pyramidal cells (Figure 4). Furthermore, the elevated expression of vasoactive intestinal peptide may be interpreted as increased activation of multipolar cells. Given that these directly inhibit other interneurons (Pi et al., 2013), the increased expression of vasoactive intestinal peptide may promote an excitable PRL through neuronal disinhibition.

Calretinin was also upregulated in the PRL following sensitization. Calretinin is expressed in almost half of interneurons and these cells do not overlap in the expression of parvalbumin or somatostatin mRNA (Conde, Lund, Jacobowitz, Baimbridge, & Lewis, 1994), supporting that METH sensitization altered distinct interneuronal subtypes in the PRL. As the expression of calretinin interneurons is unchanged in the PFC following schizophrenia (Hashimoto et al., 2008), the current results also provide preliminary evidence that METH sensitization may be associated with a distinct interneuronal pathology to the PFC compared to schizophrenia. Furthermore, while previous analyses of schizophrenia and sensitization have focused predominantly on the expression of parvalbumin, these results suggest that molecular changes in the PRL following sensitization may be more widespread or pronounced across different interneuron subtypes, particularly given that we observed no change in the expression of parvalbumin between METH-sensitized rats and saline controls in this subregion of the PFC.

Previous analyses, however, have reported reduced parvalbumin markers in the PRL following amphetamine sensitization (Morshedi & Meredith, 2007), although there are several possible reasons for the discrepancy with the current results. For example, this previous study focused on parvalbumin protein expression while the current analysis specifically examined

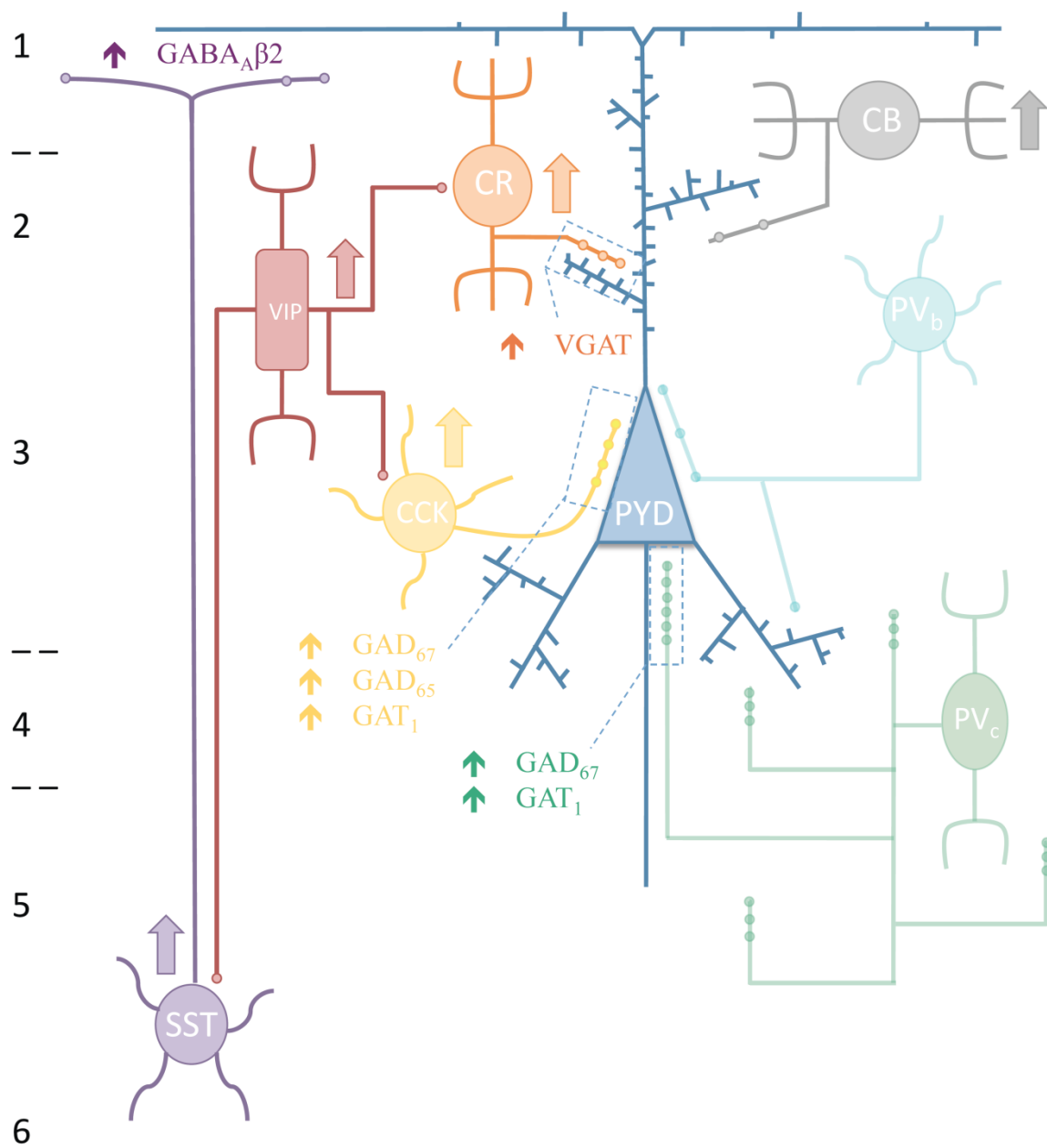


Figure 4. Depiction of the proposed inhibitory mechanisms underlying METH sensitization in the prelimbic cortex (PRL). Colored arrows adjacent to cells represent mRNA expressions that were upregulated in the PRL following sensitization to METH. mRNA expression for the neuropeptides, somatostatin (SST), vasoactive intestinal peptide (VIP) and cholecystikinin (CCK) were increased in the PRL, and suggest increased demand of SST-martinotti (purple), VIP-multipolar (red) and CCK-basket (yellow) cells in the PRL. Similarly, increased expression of mRNA encoding for calcium binding proteins calbindin (CB) and calretinin (CR) likely reflect increased bursting (orange) and irregular spiking (grey) of bouquet interneurons, respectively. Gene expression does not seem to be altered for parvalbumin-containing GABA neurons. However, altered GABA neurotransmission by parvalbumin (PV)-containing neurons, including basket (PVb; light blue) and chandelier (PVc; green) cells, is indicated by expression deficits in several gene products, including GAD₆₇ and GAT₁ mRNA that correlate with the expression of these neurons. The mRNA expressions of GAD₆₇, GAD₆₅ and GAT₁ also correlated with the expression of CCK, suggesting that these deficits are also occurring within basket cells that target the initial axon segment of the pyramidal (PYD) neuron (blue). GABA_Aβ also correlated with the expression of SST and VGAT with the expression of CR following sensitization to METH in the PRL.

mRNA, suggesting there may be differential expression of mRNA and protein expression in the PRL. Additionally, Morshedi & Meredith (2007) found reduced parvalbumin expression in layer 5 of the PRL, indicating potential lamina specific changes as a result of sensitization. As the current results were found using a tissue-level homogenate approach, it is possible that lamina differences in expression escaped detection in the current study. Given that schizophrenia is associated with reduced protein and mRNA expression in the dorsolateral PFC (Gonzalez-Burgos et al., 2010; Hashimoto et al., 2008; Takanori Hashimoto et al., 2003) - the mammal equivalent to the PRL in rats (Granon & Poucet, 2000) - it will be important for future studies to determine whether the protein expression of parvalbumin is altered following sensitization to METH in the PRL under similar parameters described here.

Differential expression of interneuronal mRNA between METH-sensitized rats and saline treated controls was also observed in the OFC. Cholecystokinin and vasoactive intestinal peptide were similarly perturbed in both direction and magnitude to the changes identified in the PRL, suggesting increased drive from cholecystokinin-basket cells and vasoactive-intestinal peptide-multipolar cells in the OFC following METH sensitization (Figure 5). Additionally, the increased expression of parvalbumin and calbindin may represent increased calcium influx in interneuronal subtypes that could lead to dysfunctional inhibitory control of localized networks. Specifically, the largest increase detected in the OFC was for mRNA encoding for parvalbumin, which likely suggests increased fast-spiking pattern of basket and chandelier cells (Figure 5), and may indicate increased inhibitory tone of the OFC during sensitization to METH. Indeed, previous findings have indicated that the OFC is underactive following sensitization to psychostimulants (Rita, Goldstein & Volkow, 2011; Goldenstein, Volkow, 2001), suggesting that the hypoexcitable OFC could derive from enhanced inhibition of fast-spiking interneurons. Furthermore, given that parvalbumin-containing neurons plays an important role in generating oscillations in the gamma band range

(30 – 80 Hz) (Bartos, Vida, & Jonas, 2007), the finding that parvalbumin was increased in the OFC following sensitization to METH could have significant consequences on the oscillatory power requisite for mediating OFC functions. While previous findings have implicated disturbed oscillatory patterns following sensitization, these have been specific to the medial PFC (Janetsian et al., 2015). Even though the role of the OFC in the circuitry of sensitization remains to be determined, the present findings place the OFC as an important area of future research with regard to altered gamma band oscillations and sensitized behavior.

We have previously shown that sensitization to METH induced a distinct pattern of GABAergic changes across the PRL and OFC, and the results of the current study demonstrate that these alterations are concomitant with changes to the expression of calcium binding proteins and neuropeptides that reflect different subsets of GABAergic interneurons. Together, these findings suggest that interneurons are likely associated with distinct inhibitory GABAergic changes and intrinsic pathology across the OFC and PRL following sensitization. Since the results of the current study were found using tissue from the same cohort of rats previously used to examine GABAergic changes across the OFC and the PRL (Chapter 4), we extended the study to determine correlation patterns between interneuronal and GABAergic mRNA expression. It was assumed that significant correlations could suggest that the GABAergic change may occur within the interneuronal subtypes and/or that the expression of one mRNA could underlie the change in the other.

Significant correlations patterns were found with GAD₆₇ and GAT₁ with the mRNA expression of parvalbumin, while GAD₆₇, GAD₆₅ and GAT₁ were positively associated with the expression of cholecystokinin in the PRL of METH sensitized rats. Additionally, GABA β 2 correlated with the expression of somatostatin, VGAT positively correlated with calretinin and GABA_T was positively associated with the expression of neuropeptide Y.

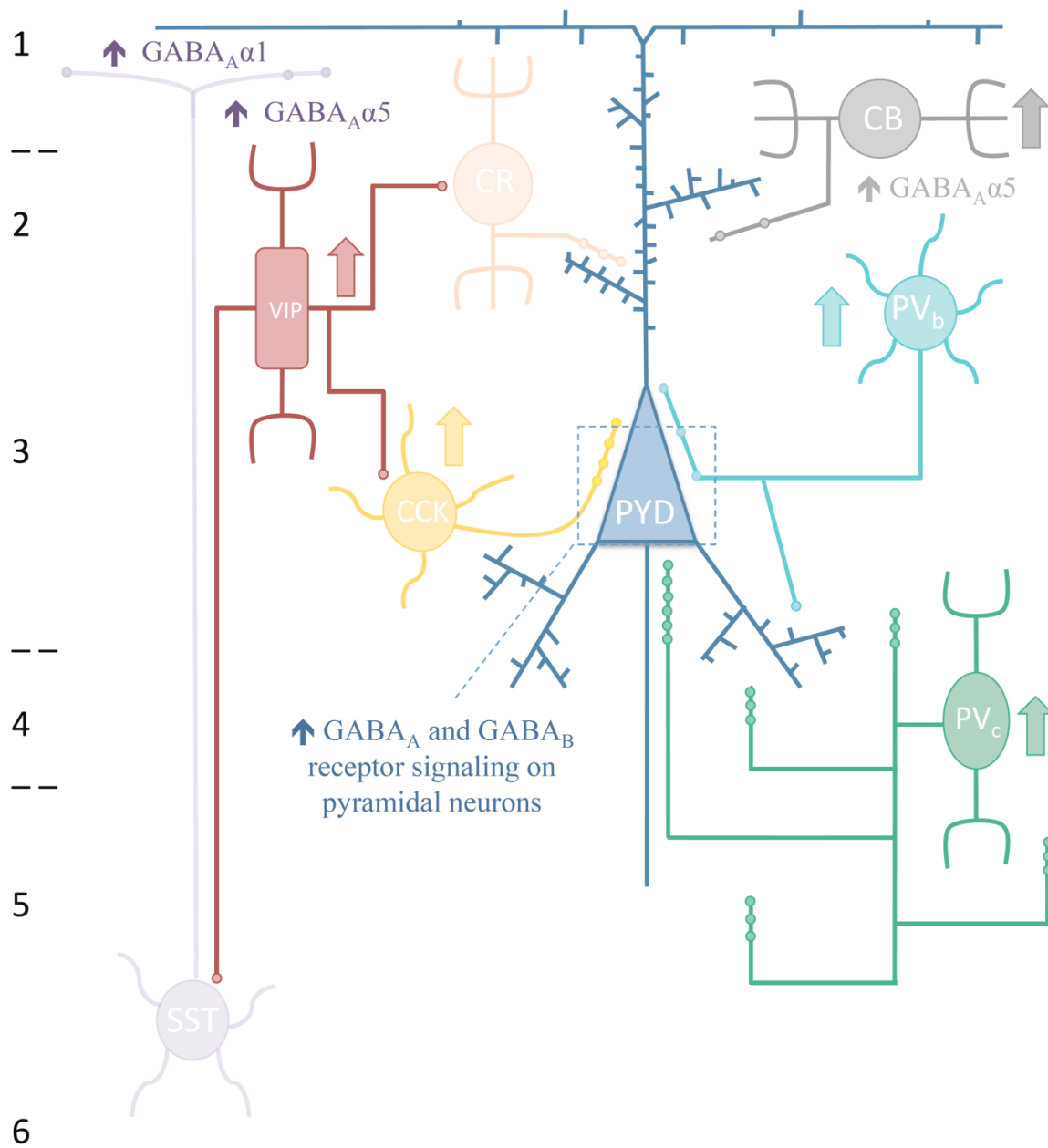


Figure 5. Depiction of the proposed inhibitory mechanisms underlying METH sensitization in the orbitofrontal cortex (OFC). Colored arrows adjacent to cells represent mRNA expressions that were upregulated in the OFC following sensitization to METH while written gene names correspond to GABAergic changes that correlated with the expression of interneuronal markers. Expression of the neuropeptides vasoactive intestinal peptide (VIP) and cholecystinin (CCK) were increased in GABA neurons that target other interneurons (multipolar cells; red) or the perisomatic region of pyramidal cells (basket cells; yellow). Increased expression of calbindin (CB) and parvalbumin (PV) suggests increased bursting and fast-spiking firing of double bouquet (grey), basket (light blue) and chandelier cells (green) in the OFC following sensitization to METH. While the expression of GABA_Aα1 and GABA_Aα5 both differentially correlated with the expression of SST, suggesting they are both implicated in the phasic and tonic inhibition of martinotti cells, altered GABA neurotransmission appears to be specific to both ionotropic (GABA_A) and metabotropic (GABA_B) receptors localized to pyramidal cells (blue). Collectively, these changes may result in reduced excitatory output of the OFC and may mediate an underactive OFC following sensitization to METH.

GAD₆₇ and GAT₁ are expressed by all GABAergic neurons. However, in light of the significant correlation with parvalbumin in the current study, these findings may indicate that GAD₆₇ and GAT₁ mRNA may be increased within fast-spiking chandelier cells following METH sensitization. Furthermore, the fact that parvalbumin mRNA expression was unchanged following sensitization suggests that these changes are not compensatory to the increased number of parvalbumin-expressing neurons in the PRL. Interestingly, the relationship between GAD₆₇, GAT₁ and parvalbumin is consistent with post-mortem analyses of the dorsolateral PFC in schizophrenia, although these findings typically indicate reduced GAT₁ and GAD₆₇ expression within parvalbumin-expressing neurons in the PFC (Curley et al., 2013). Furthermore, the significant correlation pattern between GAD₆₇, GAD₆₅ and GAT₁ with the mRNA expression of cholecystokinin indicates that changes to GABAergic mRNAs may occur across multiple cell types. However, as cholecystokinin mRNA expression was increased in the PRL, this could indicate increased GABAergic mRNA expression secondary to increased cholecystokinin neurons and/or activation. It is noteworthy that GAD₆₇ and GAT₁ specifically correlated with interneuron markers that arborize on the initial axon segments of pyramidal cells (Figure 4), and therefore these neuronal subtypes are positioned to significantly affect the glutamatergic output of pyramidal neurons in the PRL (Figure 4). Collectively, these findings suggest that cortical GABAergic neurotransmission in METH sensitization may involve specific changes within parvalbumin and cholecystokinin-containing GABA neurons.

Within the OFC, the mRNA expression of GABA_Aα1 was positively correlated with the expression of somatostatin while GABA_Aα5 was negatively associated with somatostatin and calbindin, suggesting that GABA_Aα1 and GABA_Aα5 may be localized to somatostatin-containing martinotti cells. The fact that the α1 and α5 subunits of the GABA_A receptor were inversely related may suggest that the inhibitory control of somatostatin-containing cells is

irrevocably dependent on the balance of phasic and tonic inhibitory signaling, as $\alpha 1$ and $\alpha 5$ are located both postsynaptically (Hines et al., 2012) and extrasynaptically (Fritschy & Mohler, 1995), respectively. With the exception of the relationship with somatostatin, there was no other association between GABAergic mRNA expression and interneuronal markers in the OFC following sensitization to METH. As GABA receptors are expressed on both interneurons and pyramidal cells (Fritschy & Mohler, 1995), the lack of correlations with interneuron subtypes may suggest that the changes to GABA_A and GABA_B receptors may be predominantly localized on glutamatergic pyramidal neurons. This suggests that these changes may mediate elevated inhibition of glutamatergic circuits of the OFC following sensitization to METH. Therefore, the simultaneous increase of phasic inhibition of pyramidal cells via the increased expression of GABA_A receptors, together with the increased firing of parvalbumin interneurons, may underlie the underactive tone of the OFC following chronic psychostimulant administration.

5. Conclusions

In summary, METH sensitization resulted in the upregulated expression of mRNAs encoding for multiple peptides and calcium binding proteins across the PRL and the OFC. Furthermore, while our previous findings suggest that GABAergic changes following sensitization are widespread and subregion specific, the current findings add further support that these changes are also likely occurring within specific cell types in a regional specific manner across the PFC. This is in agreement with a large body of literature that has shown the changes to the GABAergic system and cellular changes typically reported in schizophrenia, although the directional changes typically reported are opposite to the findings reported in the current study. As such, this study provides evidence that schizophrenia and METH sensitization may be associated with distinct inhibitory pathologies of the PFC. Nevertheless, the interneuronal changes identified here could still have profound consequences on behavior and cognitive output, particularly given that these changes are localized to regions that are markedly different in connectivity and function.

Chapter Six

General Discussion

6.1 Thesis Aims

Inhibitory GABA-mediated neurotransmission plays an important role in the regulation of the PFC, with increasing evidence suggesting that dysfunctional GABAergic processing of the PFC may underlie certain deficits reported across psychotic disorders. METH is a psychostimulant that can induce acute and chronic psychoses in a subset of users, with research showing that chronic psychosis can cause positive, negative and cognitive deficits that are comparable to schizophrenia (Ezzatpanah et al., 2014; Medhus et al., 2013; Srisurapanont et al., 2011). Behavioral sensitization to repeated psychostimulant use has been proposed to reflect many of the neurochemical and behavioral changes that are characteristic of psychoses, indicating that examination of the neurobiological changes subserving sensitization could enable understanding of the neural alterations that mediate and maintain different aspects of psychotic disease states. While previous studies have examined the role of dopamine and glutamate neurotransmission in mediating sensitization, particularly within the PFC, research on the role of the GABAergic system in sensitization is lacking. This is an important area of investigation as GABA-mediated processing is ubiquitous throughout the CNS and plays a salient role in the control of excitatory networks, the production of oscillations and in cognitive output. The aim of this thesis, therefore, was to determine the changes to the GABAergic system in the PFC following sensitization to METH.

6.2 Summary of Findings

6.2.1 Methamphetamine Sensitization Alters the Proteome of the Prefrontal Cortex

The effect of METH administration has been previously examined at the mRNA and protein level for multiple researcher-selected targets, while the proteome of the PFC has only been examined immediately following 8 days of METH exposure in adolescent rats (Faure et al., 2009) or at 24 hours after acute METH exposure (Kobeissy et al., 2008). Therefore, the

aim of the first experimental study of this thesis (Chapter 2) was to investigate changes to protein expression in the PFC in adult male rats sensitized to METH using quantitative label-free shotgun proteomics. Furthermore, no previous proteomic research on METH sensitization has placed their results in the context of psychotic disorders. As such, it was expected that, if particular biological factors subserve the shared symptoms and vulnerability to psychotic relapse across psychoses, then these proteins should be similarly perturbed across METH sensitization and schizophrenia.

Proteomic analysis revealed 96 proteins were differentially expressed in the PFC of METH treated rats, and via the review of the literature on schizophrenia, 20% of these had previously been implicated in the neurobiology of schizophrenia in the PFC. Multiple biological functions in the PFC appeared to be commonly altered across METH-induced sensitization and schizophrenia, including synaptic regulation, protein phosphatase signaling, mitochondrial function and alterations to the inhibitory GABAergic network. Specifically, METH sensitization upregulated the expression of gephyrin and down-regulated the expression of GAD₆₇, parvalbumin and neuroligin2, all of which are implicated in the regulation of inhibitory GABAergic neurotransmission, suggesting that METH sensitization is associated with alterations to GABA synthesis, GABA_A receptor clustering, inhibitory synapses and interneuronal subtypes. Indeed, in light of the relationship between neuroligin2 and parvalbumin in relation to fast-spiking firing patterns (Gibson et al., 2009; Basar et al., 2001), the changes to parvalbumin and neuroligin2 expression following sensitization to METH indicated reduced activity of GABAergic circuits within the PFC. Furthermore, given that fast-spiking parvalbumin-containing cells mediate neuronal oscillations and neural synchrony during cognitive tasks (Basar et al., 2001), particularly in the PFC (Benchenane et al., 2011), the findings of this Chapter suggest that alterations to the GABAergic network may present a common biological substrate that could underpin the executive dysfunction

observed across psychoses. While METH sensitization is usually placed in the context of dopamine dysregulation, the current results suggest an increasingly clear role for GABAergic dysfunction that could subserve common clinical symptoms between METH sensitization and schizophrenia.

6.2.2. Global GABAergic mRNA Expression is Upregulated in the PFC following Methamphetamine Sensitization

In light of the significant and numerous changes to the GABAergic proteomic network identified in the PFC in Chapter 2, the aim of Chapter 3 was to investigate changes to GABAergic mRNA expression in the PFC of rats sensitized to METH. Based on the results from Chapter 2, it was hypothesized that GAD₆₇ mRNA expression would be downregulated while GABA_A receptor mRNA expression would be upregulated in the PFC following sensitization to METH. To assess these hypotheses, a combination of behavioral sensitization and quantitative polymerase chain reaction (qPCR) was employed, whereby RNA was isolated from the PFC following sensitization and the relative mRNA expression of a range of GABA enzymes, transporters and receptors subunits were compared between METH-sensitized and saline-treated controls. Primers used for the PCR analyses were made in-house and were validated through gel electrophoresis and DNA sequencing.

The findings of Chapter 3 were that mRNA expression of transporters (GAT₁ and GAT₃), ionotropic GABA_A receptor subunits (α 3 and β 1), together with the metabotropic GABA_B1 receptor, were upregulated in the PFC of METH sensitized rats compared with saline controls. These findings indicated increased GABA reuptake to either presynaptic neurons or astrocytes, increased phasic inhibition of postsynaptic sites and elevated metabotropic GABA signaling in the PFC following sensitization to METH. While the hypothesis that GABA_A receptor mRNA expression would be increased was partially

supported, the expression of GAD₆₇ mRNA was unchanged in the PFC following sensitization. This unexpected finding indicated divergent expression between mRNA and protein expression in the PFC, and may suggest post-translational modifications, increased GAD₆₇ protein metabolism or reduced afferent inhibitory drive to the PFC in sensitization to METH. Overall, the findings of this chapter suggest that GABAergic mRNA expression is significantly altered at the pre and postsynaptic level following sensitization to METH, with sensitization resulting in the transcriptional upregulation of several inhibitory genes.

6.2.3 GABAergic mRNA Expression across the Prelimbic and Orbitofrontal Cortices following Methamphetamine Sensitization: Evidence of a Neurobiological Dissociation?

The PFC is a heterogeneous area of multiple brain regions, driving the aim of Chapter 4 to extend the findings of Chapter 3 by investigating changes to GABAergic mRNA expression in subregions of the PFC of rats sensitized to METH. As previous research had determined differential involvement of the prelimbic cortex (PRL) (Cador et al., 1999) and the orbitofrontal cortex (OFC) (Winstanley et al., 2009) in sensitization to psychostimulants, together with strong evidence of distinct inhibitory changes in schizophrenic brains across the OFC (Joshi et al., 2012; Thompson et al., 2009) and dorsolateral PFC (DLPFC) (Curley et al., 2011; Dean et al., 1999; Hashimoto et al., 2008; Schleimer et al., 2004) - the mammalian equivalent to the PRL in rodents (Granon & Poucet, 2000) – the focus of this Chapter was on the PFC subregional changes of GABAergic function in the PRL and OFC).

Many GABAergic mRNA markers were upregulated in the PRL: GAD₆₇, GAD₆₅, GAT₁, GAT₃, VGAT, GABA_T, GABA_Aβ2 and GABA_B1 mRNA expression, suggesting increased synthesis, release, reuptake and metabolism of GABA in this region following sensitization to METH. As the PRL appears to be hyperexcitable during the expression of sensitization (Aguilar-Rivera et al., 2015; Degoulet et al., 2009), these findings may be

secondary to increased glutamate efflux in the PRL in an attempt to counteract glutamatergic neurotoxicity. An important finding of this Chapter was the increased expression of GABA_T, the enzyme that catalyzes the degradation of GABA, as this provided evidence that the increased expression of GABA transporters may promote the reuptake of GABA to astrocytes where it is metabolized to glutamate. This finding provides molecular evidence to suggest how altered inhibitory transmission may promote a constant supply of glutamate in the PRL following sensitization to METH. An additional finding of Chapter 4 was that ionotropic GABA_A receptor subunits $\alpha 1$, $\alpha 3$, $\alpha 5$ and $\beta 2$ together with metabotropic GABA_{B2} mRNA expression were upregulated in the OFC. As the OFC is underactive in the withdrawal period from psychostimulants (Rita Z. Goldstein & Volkow, 2011; R. Z. Goldstein, Volkow, Wang, Fowler, & Rajaram, 2001), these findings suggest increased phasic, tonic and metabotropic inhibition of efferent pyramidal cells, and provide evidence that decreased excitatory output of the OFC may be secondary to elevated inhibition of glutamatergic circuits.

Overall, as all presynaptic markers were increased in the PRL, while ionotropic GABA_A and GABA_B receptors were differentially expressed in the OFC, the findings of Chapter 4 suggest that alterations to GABAergic mRNA expression following sensitization to METH are biologically dissociated between subregions of the PFC. This indicates that GABAergic gene expression is significantly altered following chronic METH exposure in a brain-region and GABA-specific manner.

6.2.4 Expression of Interneuronal Markers across the Prelimbic and Orbitofrontal Cortices following Methamphetamine Sensitization

The first aim of Chapter 5 was to determine the relative mRNA expression of calcium binding proteins (parvalbumin, calretinin & calbindin) and neuropeptides (somatostatin, cholecystokinin, neuropeptide Y and vasoactive intestinal peptide) - molecular markers for

inhibitory interneuron subtypes – following sensitization to METH across the PRL and the OFC. To achieve this, behavioral sensitization and qPCR was employed using in-house designed PCR primers to compare the relative mRNA expression of molecular markers between METH-sensitized and controls in the PFC subregions.

The expression of mRNAs encoding for the neuropeptides somatostatin, vasoactive intestinal peptide and cholecystokinin were increased in the PRL, indicating increased demand from somatostatin-martinotti, vasoactive intestinal peptide-multipolar and cholecystokinin-basket cells following sensitization to METH. An additional finding of Chapter 5 was increased mRNA expression for calcium binding proteins, calbindin and calretinin, in the PRL following sensitization to METH, which likely reflects increased bursting and irregular spiking of bouquet interneurons. Specifically within the OFC, expression of vasoactive intestinal peptide and cholecystokinin mRNA was increased in GABA neurons that target other interneurons (multipolar cells) or the perisomatic region of pyramidal cells (basket cells) (Chapter 5; Figure 5). Also, increased mRNA expression of calbindin and parvalbumin may suggest increased bursting and fast-spiking firing of double bouquet, basket and chandelier cells in the OFC following sensitization to METH. The finding of increased parvalbumin expression in the OFC was significant, as fast-spiking interneurons are hypothesized to mediate the neuronal oscillations requisite for cognitive function. As such, the findings from Chapter 5 provide evidence that that METH sensitization may lead to changes in the OFC that could have profound consequence on behavioral phenotypes mediated by the PFC.

6.2.5 Do Inhibitory Changes following Sensitization Correlate to a Specific Cellular Subtype across the Prelimbic and Orbitofrontal Cortices?

As an extension of the fourth study of this thesis (Chapter 5), together with the fact

that the findings from Chapter 4 and Chapter 5 derived from the same cohort of METH-sensitized rats, the last analysis investigated whether the mRNA expression of GABAergic changes identified in Chapter 4 correlated with interneuronal mRNA expression. It was assumed that if certain GABA changes co-localized within specific cell types, then these changes should correlate with the expression of interneuronal markers.

Altered GABA neurotransmission within parvalbumin-containing neurons, including basket and chandelier cells, was indicated by the correlation of expression deficits in several gene products, including GAD₆₇ and GAT₁. The mRNA expressions of GAD₆₇, GAD₆₅ and GAT₁ also correlated with the expression of cholecystokinin, suggesting that these deficits are also occurring within basket cells that target the initial axon segment of the pyramidal neuron. GABA_Aβ2 mRNA expression was also correlated with the expression of while VGAT correlated with the expression of calretinin following sensitization to METH in the PRL. The expression of GABA_Aα1 and GABA_Aα5 both differentially correlated with the expression of somatostatin in the OFC following sensitization to METH, suggesting they both may be implicated in the phasic and tonic inhibition of martinotti cells, however, no other correlations between GABA_A receptor mRNA expression and interneuronal markers were identified in the OFC. Therefore, altered GABA neurotransmission mediated by ionotropic and metabotropic receptors in the OFC may be localized to glutamatergic pyramidal cells.

6.3 Theoretical and Clinical Implications

The major findings of this thesis demonstrate that markers of inhibitory neurotransmission, either at the cellular or synaptic level, appear to be impaired across both global and localized regions of the PFC, with several of these transcripts potentially localized to specific inhibitory interneurons. These findings have important theoretical and clinical implications regarding the role of GABAergic neurotransmission in the PFC in sensitization

to psychostimulants, the biological underpinnings that subserve METH psychosis and with respect to whether inhibitory changes to the PFC are conserved across psychoses.

6.3.1 Inhibitory Regulation following Sensitization to Psychostimulants – the Role of the Prefrontal Cortex in Sensitization Circuitry

The studies conducted throughout this thesis demonstrate that inhibitory neurons and GABAergic circuitry in the PFC undergo adaptive changes in response to METH sensitization. Therefore, this thesis provides further support that the PFC is implicated in the circuitry of sensitization to psychostimulants (Pierce & Kalivas, 1997; Steketee & Kalivas, 2011) and that the underlying neurobiology of sensitization extends beyond the dopaminergic and glutamatergic systems, both of which have been studied in considerable detail across the literature. Furthermore, as these changes were identified at the point of the expression of behavioral sensitization, they may indicate that inhibitory changes could subserve the maintenance, rather than the development, of sensitized neural networks.

The expression of sensitization derives from increased dopamine and glutamate transmission in the nucleus accumbens (NAc) (Pierce & Kalivas, 1997; Steketee & Kalivas, 2011; Cornish & Kalivas, 2001). Additionally, persistent changes to the ventral tegmental area (VTA) include increased glutamatergic and reduced GABAergic transmission, both of which stimulate the firing of dopamine neurons and the dopamine efflux in the nucleus accumbens that modulate increased locomotor behavior (Garris, Ciolkowski, Pastore, & Wightman, 1994; Johnson, Seutin, & North, 1992; Lacey, Mercuri, & North, 1988; RPierce & Kalivas, 1997; Suaud-Chagny, Chergui, Chouvet, & Gonon, 1992). Sensitization is also dependent on additional nuclei throughout the motivational circuit that work in concert to maintain increased sensitivity to psychostimulants. For example, the mesolimbic pathway can be activated by glutamatergic projections from the mPFC to the VTA (Li et al., 1999) while

activity of the VTA can be mediated by afferent projections from the laterodorsal tegmentum (LDT) (Nelson, Wetter, Milovanovic, & Wolf, 2007; Omelchenko & Sesack, 2005), basolateral amygdala (Lintas et al., 2012) and hippocampus (Lodge & Grace, 2008). Therefore, several important brain structures are implicated in the circuitry of sensitization.

The data outlined in Chapter 3 suggests that METH sensitization is concomitant with multiple presynaptic and postsynaptic changes to the GABAergic system in the PFC, while the results of Chapters 4 extend these findings by describing that presynaptic GABAergic changes are localized to the PRL and changes to GABA receptors are predominantly central to the OFC. In light of the differential GABAergic changes following METH sensitization across the PRL and OFC, these findings also suggest that analyzing the PFC as one homogeneous region may be too broad an anatomical dimension to accurately encapsulate the different roles of localized inhibitory circuits in sensitization to psychostimulants. Consequently, these experiments suggest that both the OFC and the PRL differentially regulate the circuitry that subserves the expression of sensitization.

6.3.1.1 Inhibitory Regulation of the Prelimbic Cortex in Sensitization

Several lines of evidence suggest that the PRL is excitable during the expression of sensitization. For example, lidocaine-induced blockade of the mPFC blocks locomotor sensitization to amphetamine (Degoulet et al., 2009) while sensitization to amphetamine is associated with increased burst firing of neurons within the same region (Aguilar-Rivera et al. (2015). These findings are also supported by the fact that dopamine D₂ and group II metabotropic glutamate receptor function is reduced in the mPFC (Beyer & Steketee, 2002; Bowers et al., 2004), resulting in increased excitability of projection neurons. Indeed, extracellular glutamate is increased in the mPFC following amphetamine and METH exposure (Del Arco et al., 1998; Stephans & Yamamoto, 1995) suggesting that glutamatergic

efferents of the PRL are involved in the expression of sensitization. Therefore, the finding that GABA_T was elevated in the PRL (Chapter 4) provides molecular evidence that a hyperglutamatergic environment may be maintained by increased GABAergic metabolism. As glutamatergic pyramidal neurons provide major excitatory afferents to the mesolimbic pathway, the increased excitation of the PRL following sensitization can be interpreted as increased excitatory drive from the PFC to the pathways heavily implicated in sensitization. Indeed, Fanous et al. (2011) found that fos expression was increased in PRL neurons that project to the VTA following sensitization to amphetamine. As glutamate and GABA_B receptors in the VTA promote and inhibit psychostimulant-induced motor activity (Johnson & North, 1992; Kalivas, Duffy, & Eberhardt, 1990), respectively, the increased sensitivity of glutamatergic efferents from the PRL may promote elevated locomotor activity elicited by METH challenge. As such, the findings described here may promote increased excitation of sensitization circuitry that maintains the sensitivity to relapse.

While the glutamatergic innervation of the PFC to the VTA has been well established, this does not diminish the possibility that elevated PRL activity could be strengthening the connection between the PFC and other important nuclei. Indeed, Ahn et al. (2013) found altered oscillatory patterns between the PFC and the hippocampus following repeated amphetamine injections, raising the possibility that increased excitation of the PRL could mediate altered activation of the hippocampus at the point of the expression of sensitization to amphetamine. Furthermore, using a retrograde label with dual fos immunoreactivity, Morshedi and Meredith (2008) found that fos expression was specifically upregulated in PRL neurons that project to the lateral hypothalamus and not the NAc or basolateral amygdala following amphetamine challenge. Collectively, these findings suggest that excited neurons of the PRL may activate multiple pathways in addition to the VTA connection, rendering the ability to delineate the efferents regulated by the GABA changes described here increasingly

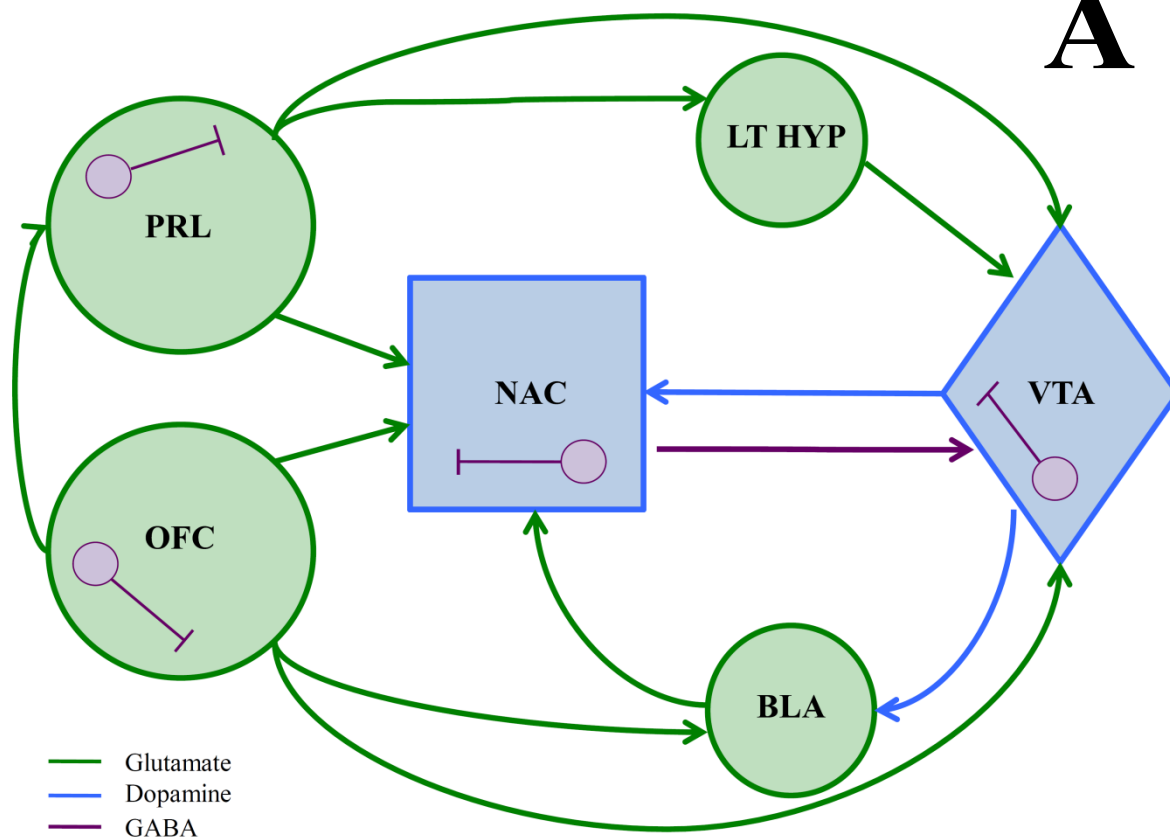
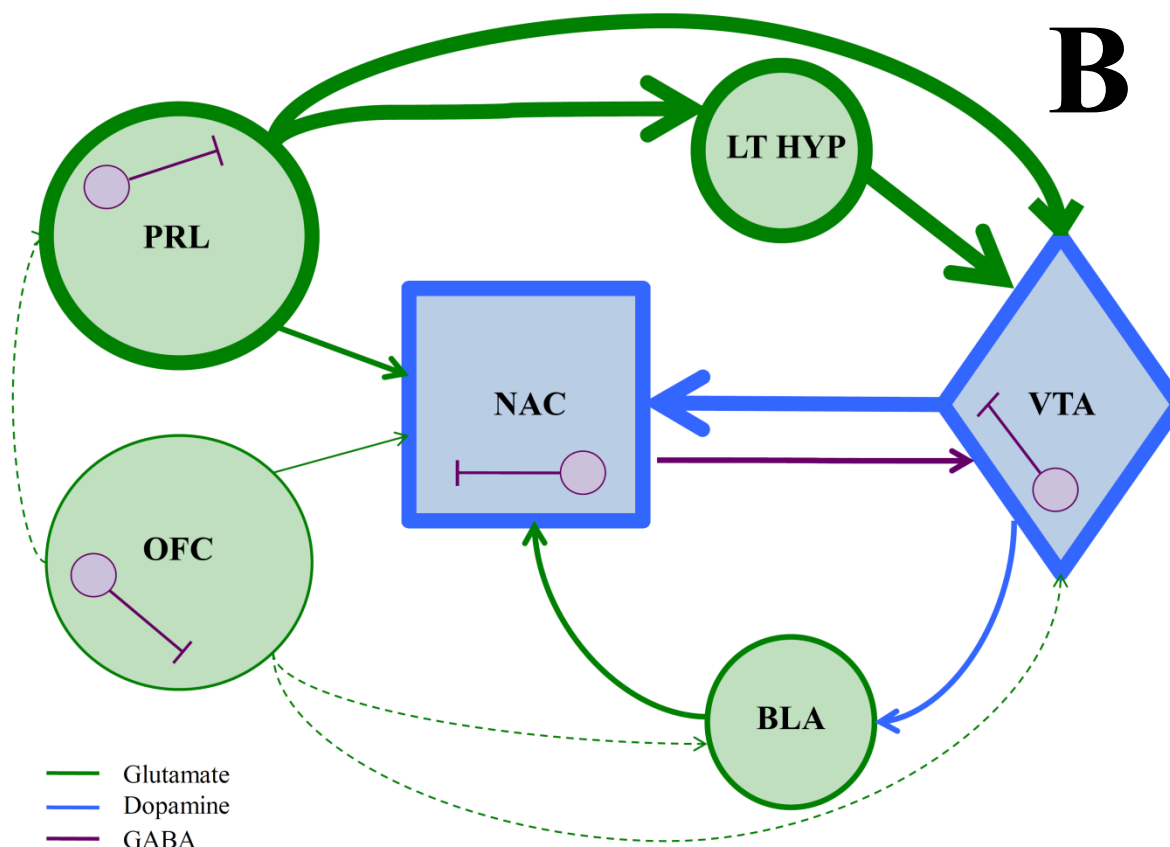
A**B**

Figure 1. A simplified representation of the circuitry subserving the expression of methamphetamine-induced behavioral sensitization under both normal (A) and sensitized conditions (B). As shown in Figure A, the nucleus accumbens (NAc) is the output to motor circuits and also regulates the expression of psychosis. The NAc is modulated by dopamine (blue) projections from the ventral tegmental area (VTA) and glutamatergic (green) projections from the prelimbic cortex (PRL), orbitofrontal cortex (OFC) and basolateral amygdala (BLA). The VTA, lateral hypothalamus (LT HYP) and BLA also receive glutamatergic afferents from the PRL and OFC. The OFC, PRL, VTA and NAc are also regulated by local inhibitory neurons (interneurons; purple). While other regions of the circuit express inhibitory neurons, the main regions of interest in the study have been those of the PFC with reference to the mesolimbic pathway. Following sensitization (Figure 1B), the hyperexcitable PRL maintains excitatory drive with projections that specifically target the LT HYP and VTA. This elevated excitation maintains the sensitivity of the VTA-NAc pathway (mesolimbic) to system challenge. Glutamatergic projections from the OFC are underactive, which also maintain the sensitivity of the mesolimbic system to locomotor response and psychotic relapse following challenge. It is hypothesized that this sensitivity could be mediated by reduced excitation on local interneurons at efferent targets such as the VTA. Bolded lines represent overactive areas or pathways while stippled lines represent underactive regions of the circuit.

complex (Figure 1).

The findings of this thesis indicate that the PRL is associated with increased production, release and reuptake of GABA following sensitization to METH (Chapter 4), potentially within cholecystokinin and parvalbumin-containing interneurons (Chapter 5). While these findings can be interpreted as an attempt to compensate for increased excitation of this region, it is also possible that these changes could promote elevated activity of some glutamatergic outputs while the increase in GABAergic production could be inhibiting others. It will be important for future studies to determine whether the changes describe here are specifically modulating efferents implicated in the expression of sensitization, and how these modulate sensitized behavioral output.

6.3.1.2 The Role of GABA in the Orbitofrontal Cortex in Sensitization Circuitry

Several GABA receptor transcripts were upregulated in the OFC following sensitization to METH (Chapter 4), and with the exception of the relationship between GABA_Aα1 and α5 and somatostatin interneuronal mRNA expression, there were few correlations between the GABAergic changes and the expression of interneuronal makers (Chapter 5). Consequently, these findings raise the possibility that GABA receptor changes following METH sensitization may be predominantly localized on pyramidal glutamatergic cells in the OFC. The results from this thesis, therefore, suggest a decrease in excitatory output of the OFC secondary to elevated inhibition of glutamatergic circuits, and may provide evidence that the GABAergic system promotes reduced excitatory drive to distal brain regions during sensitization. There is a paucity of research on the role of the OFC in mediating sensitized behavior and its relationship amongst the associated circuitry of sensitization. As such, while these findings are the first to implicate the GABAergic system of the OFC in the neurobiology of METH sensitization, the specific glutamatergic pathways regulated by

increased GABA_A receptor inhibition are currently unknown.

Prior studies have shown that the OFC projects to nuclei of the motive circuit, including the ventral tegmental area (Johnson, Rosvold, & Mishkin, 1968), striatum (Ferry, Ongur, An, & Price, 2000), the caudate nucleus (Haber, Kim, Maily, & Calzavara, 2006), the subthalamic nucleus (STN) and additional regions of the PFC, including the cingulate cortex and the medial PFC (Price, 2007). Consistent with the findings described above for the PRL, the OFC also has strong connections with the amygdala (Barbas, 2007) and the lateral hypothalamus (Rolls, Burton, & Mora, 1976). Therefore, the efferents of the OFC suggest that this region is in a position to modulate sensitized circuitry and motor behavior. Indeed, studies have shown increased locomotor activity following lesions to the OFC (de Bruin, van Oyen, & Van de Poll, 1983; Bryan Kolb, 1974; Kolb, 1984), suggesting that the OFC has an inhibitory role on motor output. These findings therefore suggest that the increased inhibition of the OFC during the expression of sensitization to METH could also mediate elevated activity of the mesolimbic pathway and subsequent locomotor activity (Figure 1). It is likely that the OFC could influence locomotor output through an intermediary brain structure rather than directly inhibiting glutamatergic afferents to the mesolimbic system, or alternatively, the OFC could mediate locomotor responses by providing reduced excitation to GABAergic interneurons in the VTA, which would have the overall effect of neuronal excitation (Figure 1). Overall, in light of the changes described throughout this thesis, the OFC may be a feature of the sensitized circuitry that should be thoroughly explored in future studies, particularly with respect to how inhibitory changes in the OFC affect the functioning of downstream areas that directly innervate the mesolimbic pathway.

6.3.2 The Relevance of Inhibitory Alterations to Methamphetamine Psychosis

Consistent with the idea that METH challenge can induce an elevated behavioral

response in animals previously exposed to a chronic regime of psychostimulants, individuals with chronic METH psychosis can also experience the emergence of psychosis from a single low-dose re-exposure to METH (Sato, 1992; Yui et al., 2003; Yui et al., 1997; Yui, Ishiguro, Goto, Ikemoto, & Kamata, 1999). Furthermore, psychosis can be elicited by additional triggers, including alternative drugs of abuse or stress (Sato, 1992; Yui et al., 2000), suggesting that sensitized neural networks may be involved in mediating a persistent vulnerability to psychotic relapse following METH use. Indeed, in light of the clear role of sensitization in METH psychosis, locomotor sensitization is often regarded as an animal model of stimulant-induced psychosis (Robinson & Becker, 1986; Ujike & Sato, 2004). Therefore, several of the findings described throughout this thesis could inform the biological underpinnings of METH psychosis.

Several lines of evidence suggest that the PFC could regulate subcortical dopamine neurotransmission in psychosis. For example, neuroimaging has shown that PFC activity correlates with increased striatal dopamine levels in individuals with schizophrenia (Meyer-Lindenberg et al., 2002) and those at high risk of psychosis (Fusar-Poli et al., 2010; Fusar-Poli et al., 2011). Additionally, amphetamine-induced behavioral changes in humans is accompanied by a decrease in dopamine D₂ receptor binding in the striatum following re-exposure to amphetamine, which is indicative of increased dopaminergic activity in the mesolimbic pathways (Boileau et al., 2006). Furthermore, previous studies have shown that the expression of the dopamine transporter (DAT) in the striatum of METH users with psychosis correlates with the severity of psychotic symptoms (Sekine et al., 2001), with these relationships similarly reported in the OFC and DLPFC (Sekine et al., 2003). Collectively, these findings suggest that mesoaccumbens dopamine transmission is heavily involved in the expression of psychoses.

A main implication of the findings of this thesis is that altered inhibitory control of

subregions and efferent projections of the PFC could lead to dopamine dysregulation of the mesolimbic system. Therefore, while these findings suggest that GABAergic changes may maintain sensitized neural circuits, these same networks could similarly mediate an elevated vulnerability to psychotic relapse. Indeed, changes to GABAergic transcripts in the PRL could maintain glutamatergic outflow to the VTA and lateral hypothalamus that lead to elevated dopamine responsiveness in the nucleus accumbens upon re-exposure to METH, while elevated GABA_A receptor expression on glutamatergic projections in the OFC could maintain an predisposition to psychosis through decreased excitatory drive to additional sensitization nuclei (Figure 1).

While GABAergic changes are traditionally interpreted in the context of cognition, research has shown that GABA is also implicated in amphetamine-type stimulant psychosis. For example, GABA deficits induced by the benzodiazepine receptor inhibitor, iomanzenil, predisposes healthy subjects to the psychotic effects of amphetamine at a dose that does not produce psychosis in healthy controls (Ahn et al., 2015). However, a major limitation of this study is the systemic approach used, and therefore the brain regions that could be mediating this sensitivity are not known. Here, as an extension to these findings, we suggest that the sensitivity to the psychotomimetic effects of amphetamine may derive from altered inhibitory control of the PFC to additional sensitized circuitry. Taken together, GABAergic dysfunction may be an important mediator in sensitization to psychostimulants, and place the inhibitory environment of the PFC in a position to contribute to the development and maintenance of psychotic-related behavior.

6.3.3 Do Schizophrenia and METH Psychosis have Conserved Inhibitory Pathology?

A growing body of literature has suggested that METH psychosis and schizophrenia are characterized by comparable positive, negative and cognitive symptoms, raising the

possibility that METH psychosis and schizophrenia represent the same disorder. As the results described above provide face and construct validity to the sensitized paradigm as a model of METH psychosis, an additional aim of this thesis was to determine whether the GABAergic changes observed reflect the inhibitory alterations reported in schizophrenia.

The findings throughout the experimental chapters indicate that METH sensitization is associated with: down-regulated expression of multiple GABAergic proteins in the global PFC (Chapter 2); upregulated expression of various mRNAs encoding for GABAergic genes in the global PFC (Chapter 3); increased mRNA expression for various presynaptic and postsynaptic GABAergic genes across the PRL and the OFC (Chapter 4), respectively; and, elevated interneuronal mRNA expression across the PRL and OFC of the PFC (Chapter 5). While several similarities were found across these studies, particularly in the *type* of inhibitory changes observed across psychoses, the majority of these findings are diametrically opposed to the directional changes routinely identified in the PFC of schizophrenic brains (Table 1). For example, GAD₆₇ and GAT₁, together with various interneuron markers parvalbumin, somatostatin, calbindin and cholecystokinin, are reliably reduced across dorsolateral PFC in schizophrenia (Table 1), while the same genes were upregulated across the studies presented in this thesis. Additionally, we failed to identify changes to GABAergic mRNA expression that are reliably found in the PFC of schizophrenia, such as the decreased expression of the $\gamma 2$ subunit of the GABA_A receptor (Table 1), while several of the findings in these analyses, such as the increased mRNA expression of GAD₆₅ and calretinin in the PRL following sensitization to METH (Chapter 4 & 5; Table 1), are unaltered in schizophrenia. In light of the divergent expression of inhibitory markers in the PFC, the findings of this thesis suggest that METH psychosis – at least with respect to the METH sensitized model – is characterized by a distinct inhibitory pathology of the PFC compared to schizophrenia.

Table 1. Changes to GABAergic network in the PFC across the PFC and METH sensitization: enzymes, transporters, synaptic GABA_A receptors, extrasynaptic GABA_A receptors, metabotropic GABA_B receptors, calcium binding proteins and neuropeptides.

Subunit	Schizophrenia	References	METH Sensitization	Chapter
GABA Enzymes				
GAD ₆₇	<p>↓ Protein & mRNA expression in DLPFC & OFC</p> <p>↓ expression correlated with GAT₁</p> <p>↓ expression in PVALB neurons</p>	Akbarian et al., 1995; Blum & Mann, 2002; Curley et al., 2011; Gonzalez-Burgos et al., 2010; Guidotti et al., 2000; Hashimoto et al., 2008; Joshi et al., 2012; Straub et al., 2007; Volk et al., 2000	<p>↓ Protein global PFC ↔ mRNA global PFC ↔ mRNA OFC</p> <p>↑ mRNA PRL; positively correlated with GAD₆₅, GAT₁ & GAT₃; Negatively correlated with GABA_T</p> <p>↑ mRNA PRL positively correlated with PVALB and CCK expression</p>	<p>2</p> <p>3</p> <p>4</p> <p>4 & 5</p> <p>5</p>
GAD ₆₅	↔ unaltered expression	Hashimoto et al., 2008; Huang et al., 2007; Volk et al., 2012	<p>↔ mRNA global PFC ↔ OFC</p> <p>↑ PRL; positively correlated with GAD₆₇, GAT₁, GAT₃; negatively correlated with GABA_T</p> <p>↑ PRL positively correlated with CCK</p>	<p>3</p> <p>4</p> <p>4 & 5</p> <p>5</p>
GABA _T	↔ unaltered expression	Gluck, Thomas, David & Haroutunian, 2002	<p>↔ OFC</p> <p>↑ PRL; positively correlated with VGAT; negatively correlated with GAD₆₇, GAD₆₅, VGAT, GAT₁ and GABA_{B1}</p> <p>↑ PRL positively correlated with NPY</p>	<p>4</p> <p>4 & 5</p> <p>5</p>
GABA Transporters				
GAT ₁	<p>↓ protein and mRNA in PFC</p> <p>↓ expression correlated with GAD₆₇</p> <p>↓ expression in PVALB neurons</p>	Gonzalez-Burgos et al., 2009; Hoftman et al., 2015; Ohnuma et al., 1999; Schleimer et al., 2004; Gonzales-Burgos et al., 2010; Hashimoto et al., 2003; Curley et al., 2011	<p>↑ mRNA global PFC ↔ OFC</p> <p>↑ PRL; positively correlated with GAD₆₇, GAD₆₅ and GABA_{B1}; negatively correlated with GABA_T.</p> <p>↑ PRL correlated PVALB & CCK</p>	<p>3</p> <p>4</p> <p>4 & 5</p> <p>5</p>

GAT ₃	↓ in DLPFC	Schleimer et al., 2004	↑ mRNA global PFC ↔ OFC ↑ PRL; positively correlated with GAD ₆₇ , GAD ₆₅ , GABA _A β2	3 4 4 & 5
VGAT	↓ mRNA expression in PFC	Hoftman et al., 2015	↔ OFC ↑ PRL; positively correlated with GABA _T ; negatively correlated with GABA _B 1 ↑ PRL positively correlated with CRET	4 4 & 5 5
Synaptic GABA_A R				
GABA _A α ₁	↑ mRNA and protein expression in PFC ↓ mRNA expression in layers 3 and 4	Increased: Impagnatiello et al., 1998; Ishikawa et al., 2004; Ohnuma et al., 1999 Decreased: Akbarian et al., 1995; Glausier & Lewis, 2011; Beneyto et al., 2010	↔ mRNA global PFC ↔ PRL ↑ OFC; positively correlated with GABA _A α ₃ ; negatively correlated with GABA _A α ₅ ↑ OFC positively correlated with SST	3 4 4 & 5 5
GABA _A α ₂	↑ mRNA and protein expression in PFC	Volk et al., 2002; Beneyto et al., 2010	↔ mRNA global PFC ↔ PRL ↔ OFC	3 4 4
GABA _A α ₃	↔ unaltered mRNA expression	Beneyto et al., 2010	↑ mRNA global PFC ↔ PRL ↑ OFC; positively correlated with GABA _A α ₁	3 4 4 & 5
GABA _A β ₁	↔ unaltered mRNA expression	Beneyto et al., 2010	↑ mRNA global PFC ↑ PRL ↔ OFC	3 4 4
GABA _A β ₂	↑ protein expression ↓ mRNA expression in layers 3 and 4	Akbarian et al., 1995; Glausier & Lewis, 2011; Lewis et al., 2012	↔ mRNA global PFC ↑ PRL; positively correlated with GAT ₃ and GABA _B 1 ↑ OFC ↑ PRL positively correlated with SST	3 4 & 5 4 & 5 5

GABA _A γ 2	↓ Protein & mRNA expression in DLPFC	Akbarian et al., 1995; Huntsman et al., 1998	↔ mRNA global PFC ↑ PRL ↔ OFC	3 4 4
Extrasynaptic GABA_A Receptors				
GABA _A α 5	↑ mRNA expression ↓ mRNA expression	Inc: Impagnatiello et al., 1998 Decr: Beneyto et al., 2010	↔ mRNA global PFC ↔ PRL ↑ OFC; negatively correlated with GABA _A α ₁ ↑ OFC negatively correlated with SST and CALB	3 4 4 & 5 5
GABA _A δ	↓ mRNA expression in DLPFC	Maldonado-Aviles et al., 2009	↔ mRNA global PFC ↔ PRL ↔ OFC	3 4 4
GABA_B receptors				
GABA _B 1	↓ Protein expression on pyramidal cells	Ishikawa et al., 2005	↑ mRNA global PFC ↔ OFC ↑ PRL; positively correlated with GAT ₁ , and GABA _A β ₂ ; negatively correlated with VGAT & GABA _T	3 4 4 & 5
GABA _B 2	↓ Protein expression on pyramidal cells	Ishikawa et al., 2005	↔ mRNA global PFC ↑ PRL ↑ OFC	3 4 4
Calcium Binding Proteins				
Parvalbumin	↓ mRNA and protein expression in PFC ↓ GAD ₆₇ and GAT ₁ mRNA expression in PVALB neurons Positively correlated with GAD ₆₇ and GAT ₁	Gonzalez-Burgos et al., 2010; Hashimoto et al., 2003; Glausier et al., 2013; Curley et al., 2011	↓ Protein global PFC ↔ PRL ↑ OFC	2 5 5

Calretinin	↔ Unaltered expression	Lewis et al., 2005; Fung et al., 2014	↑ PRL ↔ OFC	5 5
Calbindin	↑ Protein-labeled neurons in BA 9 and 46 ↓ Protein in BA 9 ↑ mRNA in DLPFC	Daviss & Lewis, 1995 Beasley et al., 2002 Fung et al., 2014	↑ PRL ↑ OFC	5 5
Neuropeptides				
Somatostatin	↓ mRNA in DLPFC ↓ mRNA in OFC	Fung et al., 2014; Hashimoto et al., 2008	↑ PRL ↔ OFC	5 5
Cholecystokinin	↓ mRNA in PFC ↔ unaltered mRNA in DLPFC and OFC	Hashimoto et al., 2008 Fung et al., 2014	↑ PRL ↑ OFC	5 5
Neuropeptide Y	↓ mRNA in DLPFC ↔ unaltered mRNA in OFC	Hashimoto et al., 2008 Fung et al., 2014	↔ PRL ↔ OFC	5 5
Vasoactive Intestinal peptide	↓ mRNA in DLPFC ↔ unaltered mRNA in OFC	Fung et al., 2014	↑ PRL ↑ OFC	5 5
Other				
Gephyrin			↑ Protein global PFC	2
Neuroigin2	Mutations found in schizophrenia	Sun et al., 2011	↓ Protein global PFC	2

The results of this thesis provide evidence that chronic METH use does not cause symptoms of schizophrenia by inducing schizophrenia pathology, at least with regard to the GABAergic network in the PFC. Therefore, in light of the divergence in biology between these conditions, it is possible that chronic METH psychosis and schizophrenia represent distinct and separate disorders and raises the possibility that chronic METH psychosis should be treated as such. Current diagnostic protocol dictates that any persistent psychosis that extends beyond the acute effects of the drug should be diagnosed as a primary psychotic disorder (American Psychiatric Association, 2013). However, careful consideration should be placed on the diagnostic entity of METH psychosis as a primary psychotic disorder in light of differential neurobiology in the PFC. Indeed, diagnosing those with chronic METH psychosis as schizophrenic may be unhelpful and ineffective, particularly given that the long-term outcomes and rehabilitation prospects may be markedly different for these individuals. It will be important for future studies to examine the long-term changes associated with chronic METH psychosis, as the trajectory of biology of the disorder could be distinct from schizophrenia and could therefore inform targeted treatment therapies for this population. The need for greater understanding of the factors causing and subserving chronic METH psychosis is particularly important given the increasing crystallized METH use on a global scale, which will no doubt be associated with increased rate of METH-induced psychosis.

6.4 Considerations and Future Directions

6.4.1 Alternative Interpretations to Differential Inhibitory Profiles

There are several alternative interpretations regarding the finding that METH psychosis has a distinct GABAergic profile of the PFC compared to schizophrenia. An important consideration of these findings is the progression of GABAergic changes throughout sensitization. As we have only examined inhibitory expression at one time point,

the findings described here only have direct relevance to the underlying circuitry that maintains sensitivity to psychotic relapse (i.e. the expression of sensitization). As many of the findings in schizophrenia derive from post-mortem analyses on brain tissue, GABAergic changes in the PFC of schizophrenia may not reflect sensitized neural networks and the inhibitory status of the PFC at the point of psychosis, but rather, the status of inhibitory neurotransmission when the sensitivity to psychosis may have declined or retracted. Thus, it is possible that the GABAergic system may be differentially regulated throughout periods of heightened psychosis sensitivity in the sensitized paradigm. Support for this idea derives from the fact that many of the changes in the PFC following sensitization to METH were qualitatively similar to the inhibitory profile of the PFC in schizophrenia, yet they differed in the direction of change. As studies of schizophrenia have shown no association between GAD₆₇ mRNA expression and length of illness and age (Curley et al., 2011), it is possible that the time point chosen for the analysis of GABAergic genes in the current study may not appropriately reflect the status of GABAergic dysfunction in the PFC of schizophrenia patients. Therefore, it will be important for future research to examine the time course changes of GABAergic mRNA throughout the initiation, expression and long-term withdrawal of METH sensitization to determine whether these findings converge with the GABAergic deficits reported in schizophrenia.

The results of this thesis may provide evidence that schizophrenia and METH sensitization are characterized by distinct inhibitory pathologies, however it is also possible that the increase in various GABAergic mRNAs may be the result of inhibitory homeostasis of the PFC. That is, the inhibitory profile we have identified in the current set of experiments may reflect the restoration of GABAergic deficits induced by repeated METH exposure that restore and mediate altered excitatory and inhibitory neurotransmission. This raises the possibility that GABAergic genes and proteins are not unidirectional and may be in a constant

state of change throughout the initiation and maintenance of behavioral sensitization. If so, the fact that the GABA deficits in schizophrenia appear to be stable throughout the progression of the disorder (Lewis et al., 2005) may suggest that certain biological factors prevent the resolution of GABA dysfunction in the PFC of schizophrenia. Further support for this argument derives from the fact that cognitive dysfunction in schizophrenia, which is believed to be mediated by GABAergic changes, are also stable throughout the progression of the illness (Bozikas & Andreou, 2011; Lewandowski et al., 2011), while there is some evidence of cognitive recovery and resolution of pathology within the PFC following chronic psychostimulant exposure (Chang et al., 2002). While this interpretation is purely speculative, it is possible that the expression patterns described in this thesis are a reflection of the adaptive PFC attempting to mediate neuronal homeostasis. Therefore, examining the biological factors that suppress this homeostasis in schizophrenia may lead to effective treatment strategies across psychoses.

6.4.2 Treatment Protocol

Sensitization to repeated METH administration was used as the behavioral paradigm for several reasons. Firstly, research has reported sensitized behavior across both METH psychosis (Akiyama et al., 2011; Sato et al., 1983) and schizophrenia (Ohmori et al., 1999; Olivares et al., 2013), with locomotor sensitization to psychostimulants proposed as an animal model of stimulant-induced psychosis (Featherstone et al., 2007; Ujike, 2002). Secondly, the development and maintenance of behavioral change as a result of chronic METH administration indicates that neuronal adaptations must be involved. Therefore, this model was used as a tool for studying how neurons in the PFC adapt in response to sensitization and how these changes could mediate vulnerability to psychotic relapse in psychoses. Sensitization is a robust behavioral phenomenon, and the paradigm described throughout

these chapters was similar to other previously published methods (Bartoletti et al., 2005; Morshedi & Meredith, 2007; Pierce et al., 1998), which adds further validation to the findings of the behavioral analyses. Furthermore, we found no evidence of elevated responding to saline challenge on acclimation, suggesting that the behavioral change to METH challenge can be attributed to the sensitized effects of METH and not secondary to conditioned responding to the locomotor cages or to the injection procedure.

An important characteristic of the current analysis was the withdrawal period employed, whereby rats were assessed for sensitized responding by a challenge dose of METH 14 days following cessation of the chronic METH regime. Several previous analyses have been hampered by sensitization protocols whereby challenge is administered following two to three days of withdrawal. This presents a significant issue in delineating conclusions from molecular changes as it is uncertain whether the results are due to sensitization or the direct effects of withdrawal. As such, it has been proposed that studies should leave at least one-week withdrawal following chronic psychostimulant exposure for full manifestation of behavioral sensitization to challenge drug administration (Pierce & Kalivas, 1997). Furthermore, as sensitization was used as a model of METH psychosis, the abstinence period was an important consideration in light of the fact that diagnostic guidelines for psychotic disorders only become ambiguous when the psychosis can no longer be attributed to the acute effects of the drug or withdrawal (American Psychiatric Association, 2013). Therefore, while these findings at this time point may not reflect the alterations typically observed in schizophrenia, they represent an important reference point for the expression of sensitization and the role of GABAergic dysfunction in chronic METH psychosis.

An important consideration in the interpretation of the molecular changes in this thesis is the effect of the METH challenge on GABAergic protein and mRNA expression. Throughout the experimental chapters, rats were either allocated to a chronic METH or saline

regime, and following 14 days of withdrawal, both groups were challenged with acute METH and euthanized one hour later for molecular analyses. Therefore, it is possible that the results described throughout this thesis may reflect the effect of sensitization and acute METH compared to acute METH. However, several factors indicate that the METH challenge did not confound these results. Firstly, the genes examined throughout these experiments are not considered early response transcripts in the production of protein. As phosphorylated protein expression can be detected at approximately 30 minutes while peak expression of early response mRNA, such as *c-fos* and *zif*, is typically observed between 30 and 90 minutes following stress (Bertaina & Destrade, 1995; Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Xiu et al., 2014), it is unlikely that METH challenge would alter mRNA expression within this time frame. Indeed, previous studies have shown that mRNA expression for *GAD₆₅* is increased at 3 hours while *GAD₆₇* and *GABA_Aα1* mRNA was increased only at 72 hours following acute administration of the D1/D5 agonist SKF-81297 (Yamamoto & Soghomonian, 2008). In support for these findings, proteomic analysis at 24 hours following acute METH administration showed no changes to the GABAergic network in the PFC (Kobeissy et al., 2008) while protein examination of the PRL following METH sensitization - using a 2 x 2 design - revealed no changes to the GABAergic system at 1 hour following challenge (unpublished data from our laboratory). Similarly, we have also found that GABAergic protein expression is unchanged in the ventral hippocampus at 1 hour following METH and saline challenge in METH-sensitized rats (Sauer et al., in prep). Overall, we found no evidence to suggest that alternations to the GABAergic system would be altered within one hour following METH challenge. It was therefore concluded that, in the benefit of reduction of animals and experimental groups, the effect of the acute METH challenge did not need to be controlled for in these experiments. Thus, the findings described throughout this thesis likely represent the state of the sensitized system at the time-point of the expression of METH

sensitization. However, for the purpose of clarity, it would be beneficial for future research to examine the time-course changes of GABAergic mRNA expression following METH challenge in METH sensitized and saline-treated controls. Not only would this provide insight into the potential confounding effects of the METH challenge in these studies, but it would also determine whether METH sensitization alters the time course of inhibitory mRNA and protein production.

6.4.3 Proteomic Analysis

The proteomic analysis used in Chapter 2 has been used extensively to determine the effect of environmental and pharmacological manipulations on plants and neural tissue, including published research from our own laboratory (Francis, Mirzaei, Pardey, Haynes, & Cornish, 2013; Mirzaei, Pascovici, Atwell, & Haynes, 2012; Mirzaei et al., 2011; Neilson et al., 2013; Neilson et al., 2011; Wearne et al., 2014). However, there are some limitations to the method that should be taken into consideration when interpreting the proteomic results presented in this thesis.

Consistent with previous studies, particularly those that have analyzed brain tissue in the rat (Francis et al., 2013), three animals from each treatment group were randomly selected for proteomic analysis with mass spectrometry. Consequently, this low sample size may shed doubt that there is sufficient power to reliably detect differences between treatment conditions. However, we detected altered protein expression in 8% of the total proteins identified in the PFC. Furthermore, given that variance negatively affects significance (Cohen, 1992), it is noteworthy that there was minimal variability between the numbers of peptides counted in each nano LC-MS/MS run between biological replicates and across conditions. This therefore increased the probability of finding a true difference across these samples and adds argument that the proteomic research in Chapter 2 was not underpowered.

While increasing sample size is always going to increase the likelihood that differences will be detected, this also increases the risk of making a Type 1 error. In keeping with this idea, there was a relative standard deviation of approximately 3% across treatment groups in Chapter 2. This suggests that the number of identified peptides was highly consistent and given our previous experience has shown that protein expression can be reliably detected and confirmed by additional molecular approaches when the calculated %RSD is less than 10% (Lee et al., 2011; Pascovici et al., 2012), the low variability between replicates confirmed that validation through the use of additional measures, such as western blot, was not required.

In the proteomic study presented in Chapter 2, 1317 proteins were reproducibly identified in the PFC across the samples analyzed. However, this does not represent all the proteins that can be identified in the PFC. A potential explanation for this discrepancy is that low abundance and membrane proteins can be difficult to identify in the presence of more abundant nuclear, cytosolic or cytoskeletal proteins. This may therefore explain why protein expression for inhibitory GABA_A receptors was not identified in the PFC in Chapter 2. Furthermore, proteins can be expressed in more than one organelle in the cell, which presents the possibility that differential expression in different organelles could compensate and cancel each other to prevent differences from being detected. Therefore, this suggests that there may have been Type 2 errors in the differences detected in Chapter 2. This also raises the issue that if a particular protein is expressed in more than one cellular location, then employing a proteomic approach will make it impossible to state where this difference may be occurring. In order to overcome these limitations, fractionating the sample to isolate different organelles can improve sensitivity of identifying significant differences in low abundance proteins (Boisvert, Lam, Lamont, & Lamond, 2010; Cox & Emili, 2006).

Lastly, consideration needs to be made in the interpretation of the proteomic results in Chapter 2 with regard to the comparisons made with schizophrenia. In this study, we

examined whether the changes to the proteome of the PFC in the METH sensitized model was comparable to changes identified in the PFC of schizophrenic brains. To achieve this, we used the output from the Ingenuity Pathway Analysis (IPA) report and conducted literature searches for the individual proteins differentially expressed in the PFC. While this was advantageous in that the literature searches significantly expanded the breath of schizophrenia-related pathology identified by the IPA report, this also increased the chance of making Type 2 errors in this analysis. That is, this approach is limited to the current status of the literature, and therefore salient proteins could have been overlooked solely on the basis that they had not been previously explored in the schizophrenia field. In order to control for this limitation, this analysis could have been strengthened by the inclusion of a schizophrenia control group, whereby the PFC of METH sensitized-rats were compared to the PFC of schizophrenic brains and saline-treated controls. However, this approach is also hindered by the availability and accessibility of these tissue samples.

6.4.4 Quantitative Polymerase Chain Reaction

Similar to the proteomic analysis used in Chapter 2, the analysis of gene expression in Chapters 3 to 5 was conducted using a valid technique (quantitative polymerase chain reaction, qPCR) that has also been used extensively by our own research group. There are many strengths to the PCR approach used in this thesis. Firstly, qPCR is considered the gold standard molecular technique in the analysis of gene expression in homogenate tissue (VanGuilder, Vrana, & Freeman, 2008). Secondly, the decision to design PCR primers in-house was advantageous as all templates were made under similar parameters, and ensured specificity of these gene sequences through gel electrophoresis, DNA sequencing and by running a BLAST search against the rat genome. Additionally, all samples used throughout these experiments were reverse transcribed simultaneously and the housekeeping gene was

analyzed on each PCR plate. This enabled analyses to be conducted without the differences between plates and the efficiency between analyses (i.e. PCR runs) affecting the results. To this end, we found that GAPDH protein (Chapter 2) and mRNA expression (Chapters 3 to 5) was unaltered by METH sensitization across the global and localized regions in the PFC. This was an important finding, as changes to the expression of housekeeping gene could result in changes that are not the result of METH sensitization but due to normalization process.

The homogenate approach used throughout the qPCR analyses could be considered both a strength and weakness. Firstly, in light of the tissue-based approaches used in Chapters 2 & 3, it was appropriate to examine subregions of the PFC as a progression towards more cellular based analyses. Secondly, the aim of these experiments was to determine the relative mRNA expression of multiple genes of the GABAergic networks, including GABA receptors. Previous findings have shown that postsynaptic GABA_A receptors can be difficult to identify through imaging methods when the brains are prepared through aldehyde perfusion (Fritschy & Panzanelli, 2014). In order to control for these limitations, gene analysis of unfixed tissue through PCR allowed for quantification of GABA receptor transcripts without the need to search for alternative perfusion and imaging techniques. However, there are some limitations to this approach. While the manual dissection method used in these studies can lead to some lack of specificity in the brain regions analyzed, which could have affected the results, care was taken in this approach and the clear differentiation of results across the subregions of the PFC indicates that these regions likely reflect distinct anatomical nuclei. Additionally, qPCR does not allow for examination of the spatial distribution amongst particular nuclei. This is an important consideration in light of the findings of Chapter 5 where we identified multiple changes to interneuronal mRNA expression following sensitization to METH.

The choice to analyze inhibitory cell markers through PCR was done for two reasons. Firstly, by using qPCR we were able to use the same tissue samples used to examine the

mRNA expression of GABAergic transcripts described in Chapter 4. This is advantageous as it meant that fewer behavioral experiments had to be conducted and animal numbers could be reduced, complying with the ethical guidelines for the animal testing in the sciences (Balls & Fentem, 2008; Russell, Burch, & Hume, 1959). Furthermore, it also allowed for the evaluation of the interneuron markers under the same conditions as the GABAergic mRNA expression in Chapter 4, meaning that mRNA expression could be directly compared in the same animals. However, in light of the expression changes presented throughout this thesis, it will be important for future studies to examine the cellular distribution of these findings throughout the PFC, particularly given that previous studies that have shown lamina specific changes to parvalbumin following sensitization (Morshedi & Meredith, 2007). This could be achieved through detailed immunohistochemical or in situ hybridization analysis to visualize the identified changes and to co-localize interneuronal and GABAergic mRNA.

It should be noted that the majority of these interneuronal subtypes identified in Chapter 5 are unable to be differentiated solely on the basis of their molecular profile. That is, no calcium binding protein or single neuropeptide correlates with the electrophysiological properties or anatomical expression of a specific type of interneuron. Indeed, an interneuron can express a mixture of up to five neuropeptides and calcium binding proteins (Markram et al., 2004), thereby making the identification of interneurons irrevocably complex. While calretinin, calbindin and parvalbumin tend to correspond to three broad interneuron classes, there is some overlap in the expression of calbindin and calretinin, together with calbindin and parvalbumin (Kawaguchi & Kubota, 1997). Furthermore, even though combinations of neuropeptides map exclusively to certain interneuronal subtypes, there is co-localization of somatostatin – neuropeptide Y (Kawaguchi & Kubota, 1997), vasoactive intestinal peptide-cholecystokinin (Kubota & Kawaguchi, 1997), vasoactive intestinal peptide - calretinin (Porter et al., 1998) and calretinin - cholecystokinin (Kubota & Kawaguchi, 1997), and

calbindin is expressed with almost all neuropeptides and calcium binding proteins (Markram et al., 2004), meaning that molecular markers should be used with caution. Therefore, these findings suggest that the conclusions drawn in Chapter 5 may represent the expression of several interneurons within the same cell. Nevertheless, molecular markers are still useful indicators of distinct anatomical interneuronal subtypes and are routinely used across both molecular and disease-focused analyses.

Another potential limitation of Chapter 4 and 5 is that these conclusions were drawn solely on the basis of mRNA and not protein expression. However, similar to mRNA, protein expression is inherent with limitations in the interpretation of results. As noted above, protein expression as determined through homogenate approaches, such as western blot, includes the collective expression of multiple proteins throughout distinct organelles and cell types. Furthermore, while mRNA expression consists of transcripts localized to cell bodies within the PFC, protein expression represents the protein made within the soma of these cells together with protein derived from the synaptic terminals of afferent projections (Carr & Sesack, 2000; Gritti et al., 1997). Therefore, changes to protein expression make it difficult to localize changes to the particular brain region of interest or to the particularly source of change within the cell. This is particularly important for the conclusions drawn regarding the interneuronal markers in Chapter 5, as these changes can be localized to these particular brain regions. Therefore, while it cannot be assumed that mRNA expression would accurately reflect protein expression, only cells that contain mRNA can express protein for that gene, meaning that mRNA expression examined throughout this thesis is still insightful into the regulation of protein for these genes in the PFC.

Lastly, many of the changes found across our mRNA analyses of this thesis could be considered “small” in magnitude. However, an important consideration to this assumption is the point at which a change in expression becomes functionally and physiologically relevant.

This is a difficult question, and depending on the gene, protein and biological process, the size of a change to be considered relevant varies. Indeed, relatively small changes in expression for some products can be disastrous to function while large changes in others can be accommodated for through homeostasis variance before there are negative consequences. For example, slight variations in blood pH can be lethal while blood glucose levels are dynamic and can double in concentration after meals. As such, for many processes, a ‘goldilocks’ zone is typically present whereby a particular product can vary without any ramification on function, and evidence of these same principles in the context of the expression of genes and proteins has begun to accrue. For example, over and under expression of some proteins can have different implications on function while the maintenance of protein expression within a definitive range has been found for some brain proteins (Ash, Vanderweyde, Youmans, Apicco, & Wolozin, 2014; Grayson, Seeley, & Sandoval, 2013; Johnson & Giulivi, 2005; Marty, Dallaporta, & Thorens, 2007). Furthermore, genetic deletion of some genes is lethal while other knockout models fail to demonstrate any observable changes to their phenotypes (McMahon et al., 1996; Schluter et al., 1999). Furthermore, studies have shown copy-number variations can occur in the general population without any change to biological function (Vogler et al., 2010). Therefore, determining the normal range of expression for proteins in the brain is an important area of research, particularly concerning threshold expression limits at which changes could have functional relevance.

The findings throughout the experiments described in this thesis are, for the most part, descriptive in nature, rendering the ability to draw conclusions regarding the functional significance of these changes difficult. Furthermore, it has yet to be determined whether the differential changes in expression of GABAergic mRNAs found throughout this thesis have changed sufficiently to have biological consequences. However, it has been suggested that even modest decreases in mRNA expression of GAD₆₇ in schizophrenia can have critical and

functional consequences (Hashimoto al., 2008) while genetic knock-out of the GAD₆₇ gene is lethal (Buddhala, Hsu, & Wu, 2009). Furthermore, as an optimal level of GABAergic function is critical for the appropriate execution of different PFC-mediated behaviors, such as executive functioning, these findings suggest that the molecular alterations identified throughout this thesis may have significant consequences on cells, regions, circuitry and behavior. In support of this, the best validation for these findings will be to carry out causal experiments that can determine whether changes in GABAergic expression in these regions of the brain are sufficient to drive behavioral or biological changes. For example, manipulation of GABA receptors in the OFC could assist in determining whether these changes do alter sensitization and its underlying neural circuitry.

6.5 Concluding Remarks

In conclusion, the research conducted throughout this thesis demonstrates that METH sensitization results in multiple changes to GABAergic proteins, genes and interneuronal markers across global and localized subregions of the PFC. These effects appear to occur in a regionally-dependent manner and our preliminary analyses suggests that these GABAergic changes may also be occurring within particular subsets of inhibitory cells across the PFC. These findings, therefore, provide evidence that GABAergic neurotransmission plays an adaptive role once the PFC has been sensitized to the effects of METH. They may also explain how altered inhibitory control of efferent networks from the PFC may maintain sensitized circuitry and a persistent vulnerability to psychotic relapse in chronic METH psychosis. However, a number of unexpected findings were revealed with respect to the GABAergic changes in primary psychotic disorders, as the inhibitory changes observed across the PFC were quantitatively, and to some degree qualitatively, distinct from those typically identified in the PFC of schizophrenia. As such, this thesis provides potentially new important evidence that schizophrenia and METH psychosis, at least with respect to the GABAergic system, may be associated with distinct neuropathology of the PFC and may represent distinct disorders. Future investigation into the cellular changes identified in these experiments is warranted while behavioral and cellular manipulations into the mediating effects of GABA could add further insight into whether GABAergic changes are compensatory to additional biological changes, or whether they reflect primary mediators of sensitized behavior.

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Appendix A

Methamphetamine-Induced Sensitization Is Associated with Alterations to the Proteome of the Prefrontal Cortex: Implications for the Maintenance of Psychotic Disorders

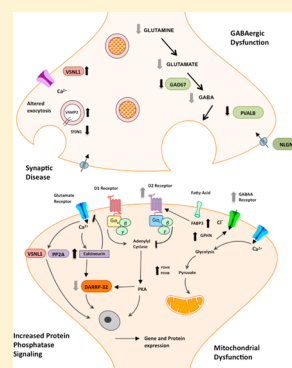
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S Supporting Information

ABSTRACT: Repeat administration of psychostimulants, such as methamphetamine, produces a progressive increase in locomotor activity (behavioral sensitization) in rodents that is believed to represent the underlying neurochemical changes driving psychoses. Alterations to the prefrontal cortex (PFC) are suggested to mediate the etiology and maintenance of these behavioral changes. As such, the aim of the current study was to investigate changes to protein expression in the PFC in male rats sensitized to methamphetamine using quantitative label-free shotgun proteomics. A methamphetamine challenge resulted in a significant sensitized locomotor response in methamphetamine pretreated animals compared to saline controls. Proteomic analysis revealed 96 proteins that were differentially expressed in the PFC of methamphetamine treated rats, with 20% of these being previously implicated in the neurobiology of schizophrenia in the PFC. We identified multiple biological functions in the PFC that appear to be commonly altered across methamphetamine-induced sensitization and schizophrenia, and these include synaptic regulation, protein phosphatase signaling, mitochondrial function, and alterations to the inhibitory GABAergic network. These changes could inform how alterations to the PFC could underlie the cognitive and behavioral dysfunction commonly seen across psychoses and places such biological changes as potential mediators in the maintenance of psychosis vulnerability.

KEYWORDS: Proteomics, psychosis, prefrontal cortex, methamphetamine, protein expression, schizophrenia, nanoflow LC–MS/MS, GABA, mitochondrial function, synaptic proteins



1. INTRODUCTION

Psychotic disorders, such as schizophrenia, represent a class of heterogeneous, chronic, and complex illnesses that is deleterious for quality of life and traditionally associated with poor treatment outcomes.^{1–3} While seminal work in the psychiatric field placed the pathogenesis of psychotic disorders in the context of dysfunctional monoamine and neurotransmitter systems in the brain, with particular emphasis on dopamine⁴ and glutamate,⁵ treatment regimes that aim to rectify the catecholamine changes in the central nervous system are unable to ameliorate all psychotic symptomatology, such as affective states and cognitive dysfunction.^{6,7} As such, research has consequently attempted to unravel the molecular mechanisms underlying psychotic disorders with the expectation of finding downstream mediators that could represent therapeutic targets that normalize all aspects of psychotic pathology. Even though research describing these biological markers has accrued, knowledge concerning the etiology of a psychotic state, or the biological underpinning that maintains this vulnerability, is still relatively unknown.

Methamphetamine (METH) is a potent psychostimulant that has a high prevalence of psychotic symptoms among both recreational⁸ and chronic users,^{9–11} with METH contributing

to the development of psychosis even after controlling for additional drug use.¹² While most psychotic symptoms are transient and resolve once the drug is ceased, anecdotal and observational research has suggested that METH-induced psychosis may develop into a more persistent psychotic syndrome that is indistinguishable from schizophrenia,^{13–16} with METH users more likely to have a schizophrenia diagnosis than controls.^{17,18} The fact that chronic METH use can result in the development of a chronic schizophrenia-like psychosis suggests that METH may induce certain brain changes that are consistent with schizophrenia pathology or that chronic METH use and schizophrenia share a common biological vulnerability.

Researchers have placed the neurobiological vulnerability to psychosis in the context of behavioral sensitization.^{19,20} Behavioral sensitization refers to the unique phenomenon whereby repeat exposure to a stimulus results in a progressively increased behavioral response to the stimulus following a period of abstinence.²¹ Indeed, METH users can experience a

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relapsed psychotic state from a single low-dose re-exposure to METH or alternative psychostimulants after decades of abstinence,^{22,23} whereas schizophrenia patients can relapse to a more severe psychosis once their medication is discontinued²⁴ or after they experience a significant stressor.²⁵ Interestingly, patients with schizophrenia can experience a psychotic relapse following exposure to METH at a dose that does not cause psychosis in healthy controls.²⁶ These findings suggest that both schizophrenia and METH-induced psychosis share common neuronal mechanisms that initiate and maintain a persistent vulnerability to psychosis, with sensitization as a key mediating factor that links these conditions to each other. Importantly, sensitization to psychostimulants can be reliably and effectively induced in experimental animals, with locomotor sensitization (hyperlocomotion) regarded as an animal model of human stimulant-induced psychosis.^{19,27} As such, to further understand the molecular markers that characterize sensitization will help to understand not only the etiology of drug-induced psychosis but also the pathogenesis of schizophrenia in humans.

The prefrontal cortex (PFC) is critically involved in mediating higher-order cognitive processes relating to behavioral and cognitive control.^{28,29} It is therefore not surprising that a large body of neuroimaging,³⁰ neuropsychological,³¹ and post-mortem studies³² have implicated significant PFC dysfunction in the pathogenesis of schizophrenia, with executive impairment regarded as an inherent characteristic of the disorder.⁶ Similarly, chronic METH use is associated with executive dysfunction and damage to the PFC,³³ with METH-induced psychosis associated with cognitive dysfunction that is indistinguishable from schizophrenia.³⁴ Furthermore, METH sensitization is associated with PFC-mediated cognitive dysfunction, such as deficits to attention, prepulse, and latent inhibition,²⁷ suggesting common executive dysfunction across psychotic syndromes. While numerous transmitter systems within the PFC are proposed to mediate the etiology and maintenance of behavioral sensitization,^{21,35} understanding of the underlying biological mechanisms that serve as common substrates across METH sensitization, METH-induced psychosis, and schizophrenia within the PFC is ongoing.

Proteomics provides a high-throughput method of evaluating the differential expression of multiple proteins, and therefore functional output, of a biological system or diseased state. While previous research has examined the effect of METH at the mRNA and protein levels for multiple researcher-selected targets, the proteome of the PFC has been examined only at 24 h after acute METH administration exposure³⁶ or after 8 days of METH exposure in adolescent rats.³⁷ As such, no research has examined the effect of behavioral sensitization to chronic METH exposure on the PFC proteome in adult rats, nor have any studies placed their results in the context of psychotic disorders.

The aim of the current study was to investigate differential changes in protein expression in the PFC following behavioral sensitization to chronic METH exposure using quantitative label-free shotgun proteomics. Using this information, we provide an analysis of the proteins identified as being differentially expressed with respect to previous schizophrenia and psychoses literature. By identifying common molecular pathways that mediate the PFC-driven symptomatology in these disorders, the molecular pathogenesis and maintenance of psychosis following METH and in schizophrenia can be better understood.

2. MATERIALS AND METHODS

2.1. Animals

Twelve experimentally naïve male Sprague–Dawley rats (Animal Resource Centre, WA, Australia), weighing an average of 261 ± 6 g at the start of testing, were used. Animals were housed in groups of four in plastic high-top cages [(64 cm (L) \times 40 cm (W) \times 20 cm (H))] that were kept in a humidity- and temperature-controlled room (21 ± 2 °C, 60% humidity) and maintained on a 12 h light/12 h dark cycle (lights on at 0600 h). All experimentation was carried out during the light period. The rats were given ad libitum access to food and water in their home cages for the duration of the experiment. Rats were acclimated to their new surroundings for 1 week and then handled daily for an additional week prior to drug treatment and behavioral testing. All experimental procedures were approved by the Macquarie University Animal Ethics Committee (reference no. ARA 2010/045) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2004).

2.2. Methamphetamine-Induced Behavioral Sensitization

2.2.1. Drug Schedule. On day 1, all rats received an injection of saline (0.9%, 1 mL/kg, i.p.) and were placed into the measurement apparatus to record locomotor activity. Rats were then allocated to treatment groups (METH $n = 6$ or saline $n = 6$) based on baseline locomotor activity such that there was no significant difference between groups prior to the commencement of the drug schedule ($p = 0.67$). Rats in the METH group were treated with once daily 1.0 mg/kg of METH (i.p.) on days 2 and 8, while the same rats received once daily injections of 5.0 mg/kg METH (i.p.) on days 3–7. These doses were selected to represent low to moderate administration, whereby exposure would be unlikely to cause neurotoxicity while simultaneously inducing locomotor sensitization, and are consistent with previous methods.^{38,39} Rats in the control group received daily injections of 1.0 mL/kg saline (i.p.) from days 2–8. On days 9–22, all rats were given a 14 day withdrawal period in their home cages. On day 23, all rats were injected with 1.0 mL/kg saline (i.p.) to test for conditioned baseline responding. The next day, all rats were assessed for methamphetamine-induced sensitization via a challenge dose of 1 mg/kg METH (i.p.).

2.2.2. Behavioral Measures. In order to quantify the behavioral effects of the METH administration schedule and to confirm the development of behavioral sensitization, locomotor activity was recorded on days 1, 2, and 8 of the drug regime and on days 15 and 16 of drug withdrawal, respectively. Sixteen standard chambers [25 cm (L) \times 31 cm (W) \times 50 cm (H)] consisting of aluminum tops and side panels, together with plexi-glass front and back panels with a metal rod floor (16 rods, 6 mm diameter, 15 mm apart), were used. Each chamber was equipped with four infrared photobeam detectors (Quantum PIR motion sensor, part no. 890-087-2, NESS Security Products, Australia) positioned on the front and back panels approximately 50 mm apart and 30 mm above the floor. Locomotor activity was quantified as the number of photobeam interruptions and recorded via a computer equipped with Med-IV PC software (Med Associates, St. Albans, VT, USA). Rats were placed in the test chamber 15 min prior to drug injection to reduce novelty-induced increases in activity before locomotor activity was recorded (60 min). Each chamber was cleaned with ethanol solution (70%) between trials.

2.2.3. Drugs. Methamphetamine hydrochloride (METH) was purchased from the Australian Government Analytical Laboratories (Pymble, NSW) and was dissolved in 0.9% saline. Intraperitoneal injections (i.p.) were made at a volume of 1 mL/kg with control rats treated with saline (0.9%).

2.3. Statistical Analysis for Methamphetamine-Induced Sensitization

All results are reported as means \pm SEM. To determine locomotor sensitization, mean locomotor activity in response to METH challenge was compared between saline and METH pretreated rats using a two-tailed independent *t*-test. Additionally, a paired *t*-test was used to compare mean baseline locomotor activity on day 1 for METH pretreated rats to the mean locomotor activity in response to METH challenge. Statistical analyses were performed using SPSS, version 17, and the significance level was set at $p < 0.05$.

2.4. Brain Dissection and Proteomic Analyses

2.4.1. Sacrifice. One hour following the METH challenge, rats were euthanized via rapid decapitation, and their brains were removed, rinsed, and cooled in wet ice/water mixture. The PFC was dissected out on dry ice and stored at -80°C until analysis. The dissection method has previously been described in detail.⁴⁰

2.4.2. Protein Extraction and Fractionation by SDS-PAGE. PFC samples were homogenized in buffer (0.32 mM sucrose, 2 mM EDTA, 1% SDS) with a dounce homogenizer. The solution was centrifuged at 14 000 rpm for 15 min at 4°C , the supernatant was removed, and the pellet was stored at -20°C until further analysis. Fifty microliter aliquots of protein were combined with 20 μL of a 5 \times SDS sample buffer containing 200 mM DTT and then separated using a Bio-Rad 10% Tris-HCl SDS-PAGE gel. Coomassie Brilliant Blue G-250 (Bio-Rad) was used to stain the gel overnight. A solution of 50% H_2O , 40% methanol, and 10% acetic acid and was used to destain the gel for 2 h prior to in-gel digestion.

2.4.3. Trypsin In-Gel Digestion. Using a scalpel, each of the 6 gel lanes was cut into 16 equal pieces, with each piece further divided into 4 equal pieces before being transferred to a 96-well plate. Gel pieces were briefly washed with 100 mM NH_4HCO_3 and then 3 times with 200 μL of ACN (50%)/100 mM NH_4HCO_3 (50%), each for 10 min. Fractions were then dehydrated with 100% ACN for 5 min, air-dried, and then reduced using 50 μL of 10 mM DTT/ NH_4HCO_3 (50 mM) at 37°C for 1 h. Samples were cooled at room temperature in the dark before being alkylated with 50 μL of 50 mM iodoacetamide/ NH_4HCO_3 (50%) for 45 min and then washed with 100 mM NH_4HCO_3 for 5 min and washed twice with 200 μL of ACN (50%)/100 mM NH_4HCO_3 (50%), each for 10 min. Gel pieces were then dehydrated with 100% ACN and air-dried. Finally, samples were placed on ice and digested with 20 μL of trypsin (12.5 ng/mL 50 mM NH_4HCO_3) for 30 min before being covered and left to digest overnight at 37°C .

2.4.4. Peptide Extraction. Remaining solutions from trypsin digestion were transferred to individual Eppendorf tubes, and 50 μL of ACN (50%)/formic acid (2%) was added before being incubated for 30 min. This was repeated twice to give a final extraction volume of approximately 90 μL for each of the 16 fractions. Extracts were then dried using a vacuum centrifuge and reconstituted to 10 μL with 2% formic acid.

2.4.5. Nanoflow LC-MS/MS. Nanoflow liquid chromatography/tandem mass spectrometry with a LTQ-XL linear ion top mass spectrometer (Thermo, San Jose, CA) was used as

previously described.^{41,42} Briefly, reversed-phase columns were packed in-house using 100A, 500m Zorbax C18 resin (Agilent Technologies, CA, USA) to approximately 7 cm (100 mm i.d.) in a fused silica capillary with an integrated electrospray tip. The tip was prepared using a Sutter Instruments P-2000 laser puller and had a diameter of approximately 10–15 μm . A 1.8 kV electrospray voltage was applied upstream of the C18 column via a liquid junction. Using a surveyor autosampler, each sample was injected onto the column followed by a wash of buffer A (5% v/v ACN, 0.1% v/v formic acid) for 10 min at 1 mL/min. Samples were then eluted from the column using buffer B (95% v/v ACN, 0.1% v/v formic acid) at 500 nL/min at 0–50% for 58 min followed by 50–90% for 5 min and were directed into the mass spectrometer's nanospray ionization source. Spectra over the range of m/z 400–1500 were scanned, and automated peak recognition, dynamic exclusion (repeat count 1, repeat duration 30 s, list size 500, exclusion duration 90 s, exclusion by mass with 1.5 Da tolerance), and MS of the top six most intense precursor ions at 35% normalization collision energy were performed using Xcalibur software (version 2.06, Thermo). The samples were injected in rows of 8 from the 96-well plate, with the three replicates of control tissue analyzed before the three replicates from the METH condition. Standards were run before and after data acquisition to ensure continued optimum system performance.

2.4.6. Database Search for Protein/Peptide Identification. Raw data files were converted to mzXML format and analyzed using the global proteome machine software (GPM, version 2.1.1) and the X!Tandem algorithm by searching tandem mass spectra against the NCBI *Rattus norvegicus* reference sequence database (94 699 proteins, April 2013). To evaluate the false discovery rate (FDR), additional searching against a reversed sequence database was used. For each replicate, the 16 fractions were individually and sequentially searched, and the output was merged into a single file using GPM software. The merged data contained only protein identifications with $\log(e)$ values < -1 . A 0.4 Da fragment mass error was used for peptide identification. Variable modifications were set for oxidation of methionine, while fixed modifications were set for carbamidomethylation of cysteine. For X!Tandem searches, the mass tolerance for fragment ions was 0.4 Da, and the tolerance for parent ions was +3 Da and -0.5 Da. The enzyme specificity was set to trypsin.

2.4.7. Data Processing and Quantitation. GPM output files were combined for each condition using the Scrappy program.⁴³ Proteins were retained for quantification if they were present across all three replicates with a total spectral count greater than or equal to 6. Normalized spectral abundance factors (NSAF) were calculated for each protein as previously described.⁴³ When summarizing protein abundance for experimental conditions, the mean NSAF value across triplicates was used.

2.5. Statistical Analysis of Differentially Expressed Proteins

Only proteins (i.e., spectral count > 6) present in all three replicates for at least one experimental condition were included in the data set. The protein false discovery rate was calculated using the reverse database as decoy (i.e., $\text{FDR} = \text{no. reverse proteins identified} / \text{total proteins}$) in addition to the peptide false discovery rate ($\text{peptide FDR} = \text{no. reverse peptide identification} / \text{total peptides}$). In order to determine whether proteins were differentially expressed between experimental conditions, independent *t*-tests were performed on the log-

transformed NSAF values for individual proteins. The significance level was set at $p < 0.05$ for all comparisons. Differentially expressed proteins were then further differentiated based on their protein–protein relationships, signaling pathways, and cellular and biological functions using Ingenuity Pathway Analysis (IPA) software. The data was searched against the Ingenuity Pathways Knowledge Base (IPKB), a continuously updated knowledge base of known proteins from peer-reviewed scientific publications.

3. RESULTS AND DISCUSSION

3.1. Sensitization to Methamphetamine

Methamphetamine challenge resulted in a significant increased locomotor response in METH pretreated rats compared to saline controls, $p < 0.05$ (Figure 1A). METH pretreated rats also showed a significant increased locomotor response on challenge when compared to their locomotor response on day 1 of METH exposure, $p < 0.01$ (Figure 1A). Furthermore, as shown in Figure 1B, time-course analysis of the locomotor response to METH challenge revealed that METH pretreated rats were significantly more active than saline pretreated controls from 15 to 35 min following challenge administration, $p < 0.001$. Collectively, these results suggest that repeated METH administration induced locomotor sensitization in response to METH challenge and are consistent with a large body of literature that has shown increased hyperlocomotion following repeated psychostimulant administration.^{21,44} Importantly, given that locomotor sensitization is regarded as an animal model of human stimulant-induced psychosis,^{19,27} with sensitized behavior observed in both METH psychosis^{22,23} and schizophrenia,^{24,25} the clear sensitized response to METH would suggest that any protein changes detected could represent biological factors that may contribute to the vulnerability to psychosis.

3.2. Proteomic Analysis

Table 1 represents a summary of proteins and peptides identified across replicates in our experimental conditions. A total of 1317 nonredundant proteins were identified across both control and METH-sensitized conditions, with 1312 protein reproducibly identified in the control condition and 1314 proteins identified across the METH-sensitized group (Table 1). False discovery rates (FDRs) were calculated after combining replicate data and were consistently low at the peptide level (FDR = 0.00768%; Table 1) and protein level (FDR = 0.0759%; Table 1), indicating that the data was of adequate stringency and further filtering was not necessary. There was also minimal variability between the numbers of peptides counted in each nano LC–MS/MS run between biological replicates and across conditions, with a relative standard deviation of approximately 3% across treatment groups. This suggests that the number of identified peptides was highly consistent and, given that our previous experience has shown that protein expression can be reliably detected and confirmed by additional molecular approaches when the calculated %RSD is less than 10%,^{45,46} the low variability between replicates confirmed that validation through the use of additional analytical measures was not required.

3.3. Proteins Related to METH Sensitization and Schizophrenia

Given that the replicates were highly reproducible, with little variability between samples (<3% RSD), a threshold cutoff of

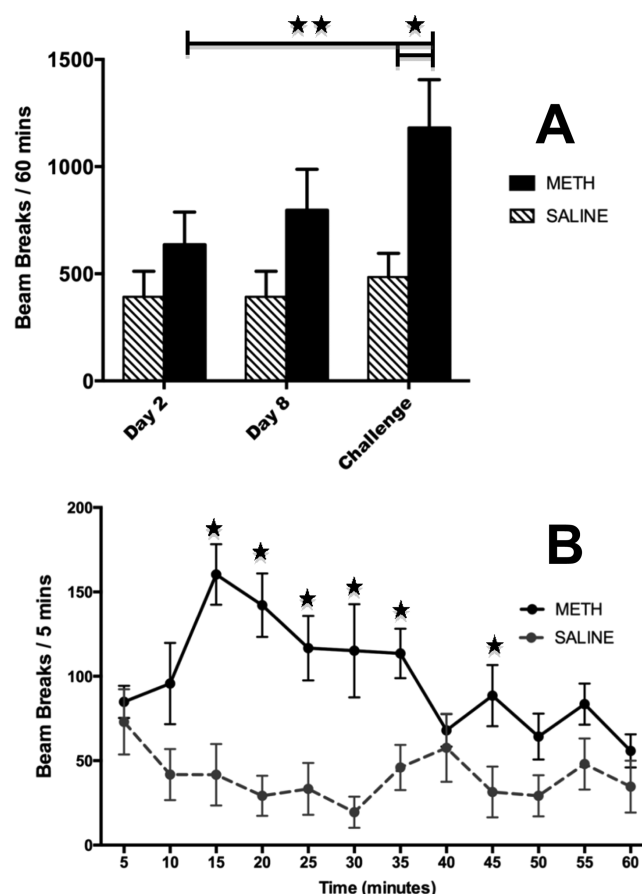


Figure 1. Locomotor sensitization to repeated METH administration. Rats were assigned to undergo either repeated METH (1 mg/kg intraperitoneal (i.p.) days 1 and 7; 5 mg/kg i.p. days 2–6, $n = 6$) or saline (1 mg/kg i.p., $n = 6$) treatment for 7 days. Following 14 days of withdrawal, both METH and saline rats were challenged with an acute METH (1 mg/kg, i.p.) injection. (A) Total beam breaks across days 2 and 8 and challenge. Rats showed a progressively increased locomotor response to repeated METH administration, with a METH challenge resulting in a significant sensitized locomotor response in METH pretreated animals when compared to saline controls (*). METH-treated rats also showed a significant increase in locomotor activity between day 2 and challenge (**). (B) Time course of locomotor activity for METH and saline pretreated rats over 60 min post challenge administration. Data are represented as mean + SEM beam break in each 5 min period. METH-treated rats displayed significantly higher locomotor activity 15–35 and 45 min after challenge injection (*). Solid lines represent METH, and the dashed lines represent the saline condition.

1.3-fold change was applied to the differentially expressed proteins. METH-treated rats showed a significant down-regulation of 32 proteins (Table 2) in the PFC, while 64 proteins were significantly upregulated (Table 3) compared to saline controls. To discuss and interpret the biological significance of each of these changes individually is beyond the scope of this article, although each variation in protein expression could represent a significant area of potential investigation. To overcome this, differentially expressed proteins were further characterized based on their biological functions and cellular processes through the use of IPA (Table 4). The top biological functions in order of significance were cellular assembly and organization, cell-to-cell signaling and interaction, cellular function and maintenance, small molecule

Table 1. Summary of Peptide and Protein Analysis for Both the Control and Methamphetamine Groups

	total	control			methamphetamine			control	methamphetamine	FDR (%)
		1	2	3	1	2	3	average (\pm RSD%)	average (\pm RSD%)	
proteins ^a	1317	2322	2344	2397	2324	2329	2339	1312 ^c	1314 ^c	0.0759
peptides ^b	143 185	24 406	25 200	23 867	22 189	23 555	23 968	24 491 (\pm 1.58%)	23 237 (\pm 2.31%)	0.00768

^aLow-stringency proteins. ^bLow-stringency peptides. ^cHigh-stringency reproducibly identified proteins.

Table 2. Downregulated Proteins (Fold Change > 1.3) in the Prefrontal Cortex Following Behavioral Sensitization to Chronic Methamphetamine Exposure Presented by Fold Change

Ensembl no.	symbol	Entrez gene name	fold	p-value
ENSRNOG00000030628	EIF4A1	eukaryotic translation initiation factor 4A1	−10.242	0.0020
ENSRNOG00000000007	GAD1	glutamate decarboxylase 1 (brain, 67 kDa)	−8.283	0.0000
ENSRNOG000000026705	DGKI	diacylglycerol kinase, iota	−5.615	0.0250
ENSRNOG000000008744	COPS2	COP9 signalosome subunit 2	−5.346	0.0180
ENSRNOG000000000841	DDX39B	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B	−4.396	0.0470
ENSRNOG00000002989	NMT1	N-myristoyltransferase 1	−4.191	0.0190
ENSRNOG00000009495	SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue (avian)	−3.781	0.0180
ENSRNOG00000019298	DCTN4	dynactin 4 (p62)	−2.987	0.0290
ENSRNOG00000014984	DMWD	dystrophin myotonia, WD repeat containing	−2.985	0.0270
ENSRNOG00000017852	NARS	asparaginyl-tRNA synthetase	−2.787	0.0130
ENSRNOG00000023529	RPL5	ribosomal protein L5	−2.236	0.0210
ENSRNOG00000004806	STRN	striatin, calmodulin binding protein	−2.175	0.0290
ENSRNOG00000015430	NLGN2	neuroligin 2	−2.109	0.0040
ENSRNOG00000010042	WDFY3	WD repeat and FYVE domain containing 3	−1.939	0.0030
ENSRNOG00000014718	ACSL3	acyl-CoA synthetase long-chain family member 3	−1.88	0.0090
ENSRNOG00000018795	RPL18A	ribosomal protein L18a	−1.78	0.0100
ENSRNOG00000023373	SEC24B	SEC24 family, member B (<i>S. cerevisiae</i>)	−1.763	0.0380
ENSRNOG00000002339	MARK1	MAP/microtubule affinity-regulating kinase 1	−1.708	0.0450
ENSRNOG00000018326	PGLS	6-phosphogluconolactonase	−1.659	0.0420
ENSRNOT00000007554	VAPB	VAMP (vesicle-associated membrane protein)-associated protein B and C	−1.644	0.0240
ENSRNOG00000007518	NCKAP1	NCK-associated protein 1	−1.628	0.0400
ENSRNOG00000003782	ACOT9	acyl-CoA thioesterase 9	−1.605	0.0390
ENSRNOG00000046502	LONP1	lon peptidase 1, mitochondrial	−1.558	0.0000
ENSRNOG00000026930	NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9, 39 kDa	−1.556	0.0060
ENSRNOG000000009155	NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30 kDa	−1.538	0.0410
ENSRNOG000000006471	PVALB	parvalbumin	−1.52	0.0090
ENSRNOG00000005836	FAM49A	family with sequence similarity 49, member A	−1.499	0.0220
ENSRNOG00000008996	DPYSL5	dihydropyrimidinase-like 5	−1.43	0.0440
ENSRNOG00000030371	MT-CO2	cytochrome c oxidase subunit II	−1.419	0.0050
ENSRNOG00000008961	MAPRE3	microtubule-associated protein, RP/EB family, member 3	−1.401	0.0100
ENSRNOG00000011142	CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	−1.366	0.0040
ENSRNOG00000017428	MAP1B	microtubule-associated protein 1B	−1.335	0.0310

biochemistry, and cell morphology. There were 235 canonical pathways linked to METH sensitization in the PFC, with the top representing mitochondrial dysfunction. The proteins associated with these functions are displayed in Table 5.

In addition, neurological and psychological disorders were the top disease and disorders identified through the IPA analysis. As such, we show here that METH sensitization shares many of the similar molecular changes in the PFC as those reported in schizophrenia. Specifically, 20% of differentially expressed proteins identified in the current study have previously been implicated in schizophrenia pathology (Figure 2).^{47–58} Using ontology classification and categorization of biological functions and pathway membership of the differentially expressed proteins from the IPA analysis, we identified biological and functional categories that appear to be commonly altered across both METH sensitization and schizophrenia, including mitochondrial function, synaptic

proteins, protein phosphatase signaling, and alteration to the inhibitory GABAergic network.

3.4. Top Canonical Pathway: Mitochondrial Dysfunction

A total of 8 proteins related to mitochondrial function and energy expenditure were differentially expressed in the PFC of METH-sensitized rats, with mitochondrial dysfunction isolated as the top canonical pathway altered in our experimental paradigm (Table 5). Specifically, proteins involved in oxidative phosphorylation were differentially expressed, with decreases in the expression of two NADH dehydrogenase subunit proteins (NDUFA9, NDUFS3), while two subunits were upregulated (NDUFA6, NDUFS8). Specifically, NDUFS3 is important in the assembly and enzymatic activity of complex I,⁵⁹ suggesting that a downregulation of this enzyme likely contributes to the overall reduced activity of the complex and possibly oxidative phosphorylation. We also found cytochrome oxidase II (MT-CO2) was downregulated and cytochrome oxidase VI (COX6C) was upregulated in the PFC of rats sensitized to

Table 3. Upregulated Proteins (Fold Change > 1.3) in the Prefrontal Cortex Following Behavioral Sensitization to Chronic Methamphetamine Exposure Presented by Fold Change

Ensembl no.	symbol	Entrez gene name	fold	p-value
ENSRNOG00000015182	PPP2CB	protein phosphatase 2, catalytic subunit, beta isozyme	32.57	0.0000
ENSRNOG00000009760	PALM	paralemmin	7.774	0.0122
ENSRNOG00000018700	MOBP	myelin-associated oligodendrocyte basic protein	6.392	0.0001
ENSRNOG00000008203	SYNPR	synaptoporin	5.059	0.0035
ENSRNOG000000025715	DYNLRB1	dynein, light chain, roadblock-type 1	5.043	0.0000
ENSRNOG000000025539	VPS13A	vacuolar protein sorting 13 homologue A (<i>S. cerevisiae</i>)	4.327	0.0452
ENSRNOG00000019740	HDGFRP3	hepatoma-derived growth factor, related protein 3	4.289	0.0161
ENSRNOG00000014109	PSMD13	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	4.289	0.0161
ENSRNOG00000015320	ATP5G2	ATP synthase, H ⁺ transporting, mitochondrial Fo complex, C2 (subunit 9)	3.875	0.0157
ENSRNOG00000028366	GPHN	gephyrin	3.786	0.0209
ENSRNOT00000006542	SEC14L2	SEC14-like 2 (<i>S. cerevisiae</i>)	3.437	0.0230
ENSRNOG00000012724	C1orf123	chromosome 1 open reading frame 123	3.428	0.0260
ENSRNOG00000048862	MRPS36	mitochondrial ribosomal protein S36	3.382	0.0265
ENSRNOG00000027408	PPID	peptidylprolyl isomerase D	3.344	0.0110
ENSRNOG00000014635	CLTA	clathrin, light chain A	3.295	0.0480
ENSRNOG00000010807	COX6C	cytochrome c oxidase subunit VIc	3.054	0.0250
ENSRNOG00000036835	COPZ1	coatamer protein complex, subunit zeta 1	3.027	0.0270
ENSRNOG00000018556	TOMM40	translocase of outer mitochondrial membrane 40 homologue (yeast)	2.998	0.0460
ENSRNOT00000012915	TST	thiosulfate sulfurtransferase (rhodanese)	2.84	0.0070
ENSRNOG00000018457	PPP2R4	protein phosphatase 2A activator, regulatory subunit 4	2.795	0.0350
ENSRNOG00000013300	ATPIF1	ATPase inhibitory factor 1	2.739	0.0450
ENSRNOG00000049075	FABP5	fatty acid binding protein 5, epidermal	2.633	0.0170
ENSRNOG00000043210	PPP3R1	protein phosphatase 3, regulatory subunit B, alpha	2.382	0.0020
ENSRNOG00000003975	PFN1	profilin 1	2.341	0.0410
ENSRNOG00000012084	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	2.328	0.0430
ENSRNOG000000027149	RPL10	ribosomal protein L10	2.298	0.0230
ENSRNOG00000045928	MYL6	myosin, light chain 6, alkali, smooth muscle and nonmuscle	2.284	0.0270
ENSRNOG000000004146	CORO7	coronin 7	2.125	0.0210
ENSRNOG00000007134	STRAP	serine/threonine kinase receptor associated protein	2.112	0.0160
ENSRNOG00000016580	RPS23	ribosomal protein S23	2.094	0.0110
ENSRNOG00000046705	Snx3	sorting nexin 3	2.085	0.0150
ENSRNOG00000020715	DDB1	damage-specific DNA binding protein 1, 127 kDa	2.073	0.0110
ENSRNOG00000002440	RALB	v-ral simian leukemia viral oncogene homologue B	1.983	0.0030
ENSRNOG00000010434	DYNC1LI1	dynein, cytoplasmic 1, light intermediate chain 1	1.971	0.0490
ENSRNOG00000002642	PTGES3	prostaglandin E synthase 3 (cytosolic)	1.969	0.0400
ENSRNOG00000005924	DSTN	destrin (actin depolymerizing factor)	1.909	0.0460
ENSRNOG00000002693	NME1	NME/NM23 nucleoside diphosphate kinase 1	1.887	0.0210
ENSRNOG00000003990	GRB2	growth factor receptor-bound protein 2	1.876	0.0490
ENSRNOG000000019189	ACAT2	acetyl-CoA acetyltransferase 2	1.84	0.0360
ENSRNOG00000017446	NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23 kDa	1.803	0.0440
ENSRNOG00000007806	ARF5	ADP-ribosylation factor 5	1.759	0.0210
ENSRNOG00000011550	KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	1.744	0.0500
ENSRNOG00000012879	FABP3	fatty acid binding protein 3, muscle and heart	1.71	0.0120
ENSRNOG00000011857	MTPN	myotrophin	1.701	0.0230
ENSRNOG000000027006	HNRNPA3	heterogeneous nuclear ribonucleoprotein A3	1.696	0.0110
ENSRNOG00000016257	COTL1	coactosin-like 1 (<i>Dictyostelium</i>)	1.693	0.0300
ENSRNOG00000004494	LTA4H	leukotriene A4 hydrolase	1.685	0.0360
ENSRNOG00000016507	SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16 kDa	1.682	0.0040
ENSRNOG00000047247	PTPRS	protein tyrosine phosphatase, receptor type, S	1.674	0.0210
ENSRNOG000000014868	HSPE1	heat shock 10 kDa protein 1 (chaperonin 10)	1.644	0.0390
ENSRNOG00000001559	MTX2	metaxin 2	1.633	0.0180
ENSRNOG00000016251	DNAJA2	DnaJ (Hsp40) homologue, subfamily A, member 2	1.545	0.0060
ENSRNOG00000000840	ATP6 V1G2	ATPase, H ⁺ transporting, lysosomal 13 kDa, V1 subunit G2	1.505	0.0240
ENSRNOG00000005345	VSNL1	visinin-like 1	1.499	0.0010
ENSRNOG00000006947	PDHX	pyruvate dehydrogenase complex, component X	1.487	0.0330
ENSRNOG00000018680	RPL17	ribosomal protein L17	1.487	0.0310
ENSRNOG00000007895	PDHB	pyruvate dehydrogenase (lipoamide) beta	1.483	0.0150
ENSRNOG00000000805	GJA1	gap junction protein, alpha 1, 43 kDa	1.481	0.0340
ENSRNOG00000012999	PHB2	prohibitin 2	1.478	0.0170
ENSRNOG00000012594	SUGT1	SGT1, suppressor of G2 allele of SKP1 (<i>S. cerevisiae</i>)	1.412	0.0000

Table 3. continued

Ensembl no.	symbol	Entrez gene name	fold	p-value
ENSRNOG00000008569	NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14 kDa	1.405	0.0430
ENSRNOG00000003365	CADM3	cell adhesion molecule 3	1.396	0.0430
ENSRNOG00000047374	GNAS	GNAS complex locus	1.367	0.0080
ENSRNOG00000018282	GDA	guanine deaminase	1.334	0.0140

Table 4. Ingenuity Pathway Analysis of the PFC Following Methamphetamine Sensitization

top networks					
associated network functions					score
Network	cardiovascular disease, genetic disorder, metabolic disease				53
	organismal survival, cellular assembly and organization, cellular function and maintenance				39
	cellular compromise, cell death and survival, nervous system development and function				37
	neurological disease, cell death and survival, nervous system development and function				30
	cardiac arteriopathy, cardiovascular disease, gene expression				30
top disease and disorders			top tox lists		
	p-value	no.		p-value	ratio
neurological disease	3.88×10^{-6} to 4.17×10^{-2}	36	mitochondrial dysfunction	5.06×10^{-6}	0.051
psychological disorders	3.88×10^{-6} to 4.56×10^{-2}	29	decreased permeability of mitochondria	4.17×10^{-2}	0.143
skeletal and muscular disorders	2.18×10^{-5} to 4.75×10^{-2}	24	xenobiotic metabolism signaling	6.31×10^{-2}	0.014
hereditary disorder	3.95×10^{-5} to 4.17×10^{-2}	31	hypoxia-inducible factor signaling	6.74×10^{-2}	0.029
inflammatory response	9.35×10^{-5} to 4.11×10^{-2}	6	aryl hydrocarbon receptor signaling	7.35×10^{-2}	0.019
molecular and cellular functions			top canonical pathways		
	p-value	no.		p-value	ratio
cellular assembly and organization	4.03×10^{-5} to 4.69×10^{-2}	32	mitochondrial dysfunction	4.38×10^{-6}	0.042
cell-to-cell interaction	9.35×10^{-5} to 4.75×10^{-2}	16	EIF2 signaling	1.29×10^{-4}	0.035
cellular function and maintenance	9.35×10^{-5} to 4.43×10^{-2}	34	telomerase signaling	1.29×10^{-4}	0.048
small molecule biochemistry	1.96×10^{-4} to 4.75×10^{-2}	30	remodeling of epithelial junctions	1.29×10^{-4}	0.059
cell morphology	4.81×10^{-4} to 4.43×10^{-2}	26	P13K/AKT signaling	1.29×10^{-4}	0.035

Table 5. Proteins Associated with the Top Molecular and Cellular Functions Altered in the PFC Following Methamphetamine Sensitization

proteins associated with top molecular and cellular functions				
function	p-value	upregulated proteins	downregulated proteins	
cellular assembly and organization	4.03×10^{-5} to 4.69×10^{-2}	PPID, NME1, GJA1, PFN1, MYL6, RAB3A, GRB2, RALB, SNX3, PALM, GPHN, VAMP2, TST, DYNC1L1, GNAS, DSTN, SNRPD1, GFAP, HDGFRP3	MYO6, SRC, STRN, NLGN2, MAP1B, MARK1, DPYSL5, TUBA1A, DDX39B, SYNJ1, LONP1, MAPRE3, NCKAP1	
cell-to-cell signaling and interaction	9.35×10^{-5} to 4.75×10^{-2}	GJA1, NME1, RAB3A, GRB2, RALB, PALM, GPHN, KCNAB2, VAMP2, PPP3R1, GFAP, FABP3, VSNL1	MYO6, SRC, SYNJ1, NLGN2	
cellular function and maintenance	9.35×10^{-5} to 4.43×10^{-2}	NME1, GJA1, PFN1, RAB3A, GRB2, RALB, PALM, VAMP2, DYNC1L1, HSP90AB1, PPP3R1, GNAS, DSTN, HSPE1, GFAP, HDGFRP3, NAPB	MYO6, PVALB, SRC, SYNJ1, NLGN2, MAP1B, MARK1, DPYSL5, LONP1, MAPRE3, NCKAP1	
small molecule biochemistry	1.96×10^{-4} to 4.75×10^{-2}	LTA4H, NME1, GJA1, ACAT2, COTL1, VAMP2, TST, ATP1F1, FABP5, HSP90AB1, PPP2R4, HSPE1, SEC14L2, FABP3, ATP6 V1G2, PDHB	MYO6, SRC, PVALB, ACSL3, CYB5B, PGLS, MAP1B, ACOT9, SYNJ1, GAD1, SLC6A1, OGDH, LONP1	
cell morphology	4.81×10^{-4} to 4.43×10^{-2}	PPID, GJA1, NDUFS8, NDUFA6, PPP3R1, DSTN, RALB, TST	SRC, STRN, TUBA1A, MAP1B, DPYSL5, NCKAP1	

METH, suggesting such changes could have salient consequences on the energy production of the mitochondrial cellular environment within the PFC, particularly given that complex IV (COX) synthesizes over 80% of the ATP required for cellular processes.⁶⁰

Accumulating morphological,⁶¹ imaging,^{62,63} and genetic^{64,65} evidence has suggested a critical and prominent role of mitochondrial dysfunction in the pathogenesis and/or progression of schizophrenia, with alterations of subunits within ATPases and within complexes I–IV of the electron transport chain (ETC) differentially expressed in the PFC of

schizophrenia patients.^{64,66} Interestingly, mitochondrial diseases are frequently comorbid with psychotic symptoms⁶⁷ and can be misdiagnosed as schizophrenia,⁶⁸ while mitochondrial conditions such as myopathy and lactic acidosis have been observed in patients with schizophrenia.⁶⁹ Given that the etiological cause for these disorders are mutations of mitochondrial DNA and mRNA coding for mitochondrial proteins, these observations increase the probability that psychotic symptoms are either the cause of or are secondary to mitochondrial dysfunction. As such, transgenic mice that have mitochondrial DNA deletions display dysfunctional PFC-

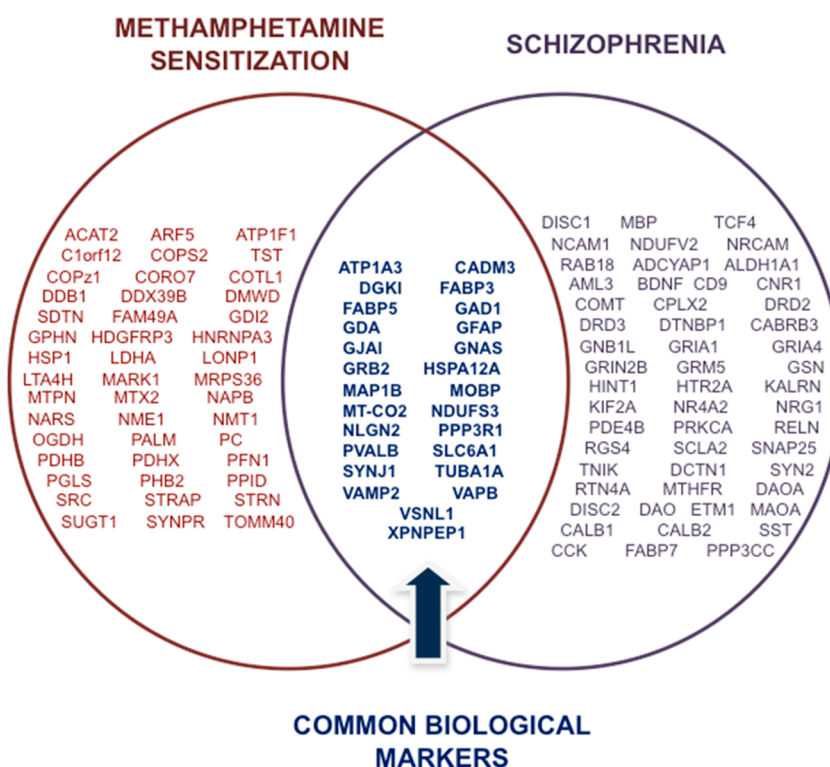


Figure 2. Venn diagram of the overlap between proteins involved in the neurobiology of methamphetamine sensitization and schizophrenia. The left represents proteins that were differentially expressed in the current study that had not been previously implicated in the neurobiology of schizophrenia and therefore reflect the effects of METH. The right represents proteins that have previously been implicated in the neurobiology of schizophrenia and are regarded as markers of dysfunction in the disorder (not measured in the current study).⁵⁸ Proteins that are altered in both disorders are shown in the overlap and are in bold.

mediated behavior that is also altered in schizophrenia, such as startle response.⁷⁰ Thus, the changes in oxidative phosphorylation and energy production detected here may underlie certain behavioral changes associated with sensitization to METH and schizophrenia, such as psychotic symptoms or cognition (Figure 3).

3.5. Molecular and Cellular Functions

3.5.1. Cell-to-Cell Signaling and Interaction: Synaptic Proteins. A total of 15 proteins were differentially expressed in the PFC following sensitization to METH that could be involved in vesicle trafficking, synaptogenesis, gap junctions, and neurotransmitter release, with many of these proteins being previously implicated in the pathophysiology of schizophrenia. Specifically, gap junction protein 1, 43 kDa (GJA1; connexin43) was upregulated in the PFC of METH-sensitized rats. GJA1 mediates gap junction communication between glial cells and neurons and safeguards against propagation of neuronal inactivation by reuptake of glutamate and potassium.⁷¹ Interestingly, knockout mice for the GJA1 gene display increased locomotor activity and exploratory behavior, suggesting an anxiolytic effect of GJA1 deletion.⁷² It has also been hypothesized that changes to the function of gap junctions between astrocytes and neurons could contribute to the cognitive dysfunction reported in schizophrenia.⁷³

Furthermore, 3 proteins (VSNL1, FABP3, and GPHN) were upregulated in the PFC of METH-sensitized rats that are involved in the regulation of synaptic receptors. VSNL1 is a neuronal calcium sensor protein that binds to synaptic receptors in a calcium-dependent manner,⁷⁴ leading to increased signaling, internalization, and/or surface expression

of acetylcholine and glutamate receptors. Furthermore, FABP3 has been shown to regulate dopamine D2 receptors,⁷⁵ while GPHN is specifically involved in the scaffolding and clustering of GABA receptors at postsynaptic sites.⁷⁶ These findings likely suggest an increased density of postsynaptic receptor types, potentially as a compensatory mechanism for decreased neurotransmitter levels at afferent sites. VSNL1 has previously been shown to be upregulated in the PFC of schizophrenia⁷⁷ and is linked to both functional and morphological deficits in the disorder,⁷⁸ particularly in pyramidal cells.⁷⁹ Single-nucleotide polymorphisms in VSNL1 have also been linked to schizophrenia cognitive impairments.⁸⁰ Furthermore, increased expression of dopamine D2⁸¹ and GABA receptors is reported in the PFC of schizophrenia, which could be linked to an upregulation of FABP3 and GPHN, respectively. Overall, these findings suggest that changes to pre- and postsynaptic proteins may be a potential contributor to the pathogenesis and maintenance of these psychotic conditions (Figure 3).

3.5.2. Cellular Function and Maintenance: Protein Phosphatase Signaling. Three specific subunits (PPP2CB, PPP2R4, and PPP3R1) of serine/threonine phosphoprotein phosphatases (PPPs) were upregulated in the PFC of rats sensitized to METH. PPPs mediate intracellular signaling and dephosphorylation⁸² and have been implicated in a range of synaptic functions,⁸³ with PPP3 (calcineurin) being a key regulator of axonal guidance, endocytosis, exocytosis, and signal transduction.^{84,85} Importantly, calcineurin regulates intracellular calcium levels, making it a mediator of NMDA receptor function and long-term depression (LTD). As such, an upregulation of calcineurin is negatively correlated with cognitive performance,⁸⁶ while forebrain-specific PP2(B1)-

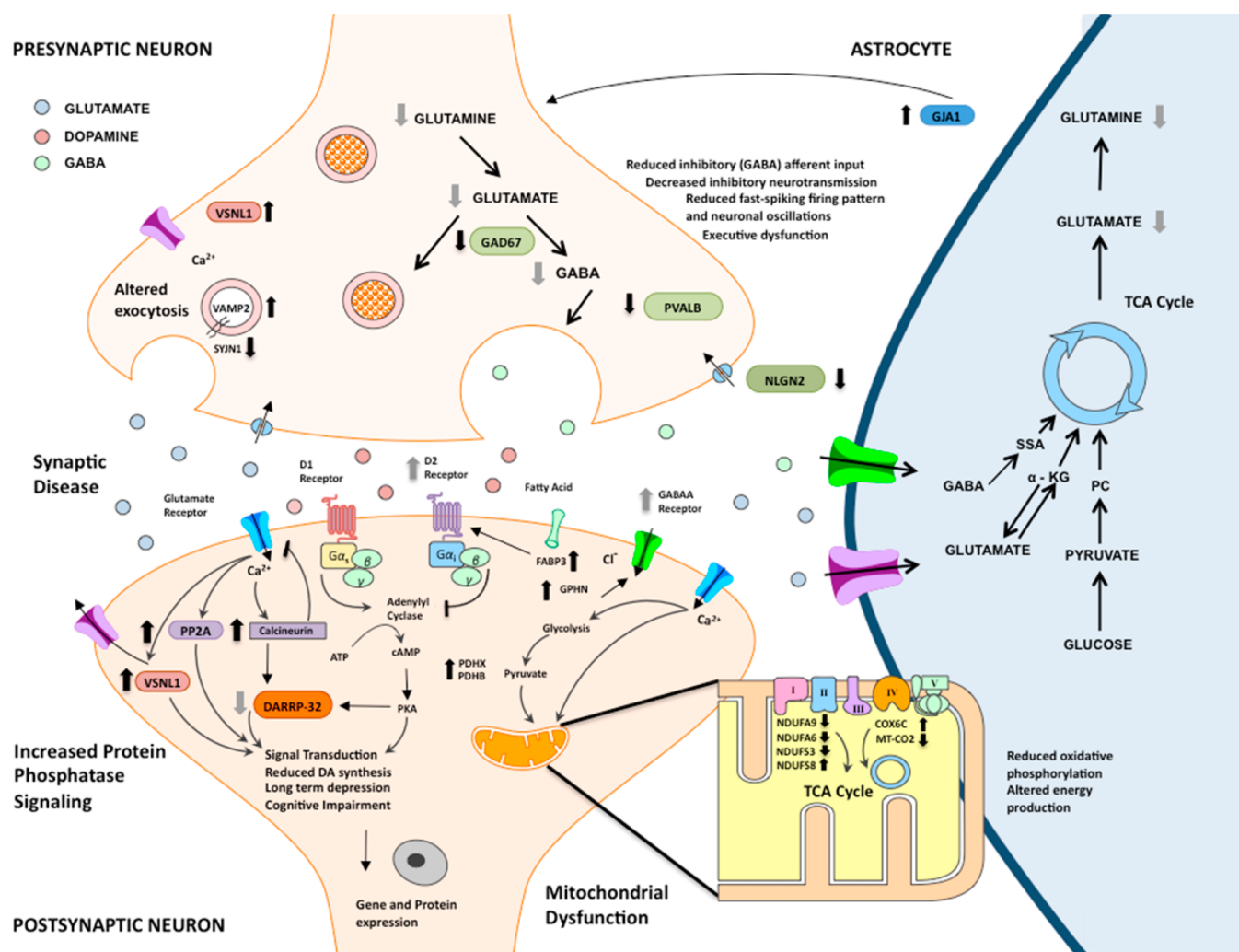


Figure 3. Depiction of the proposed common neurobiological mechanisms underlying the vulnerability to psychosis in the prefrontal cortex (PFC). Black arrows adjacent to protein names represent proteins that were differentially expressed in the current study, with the direction of the arrow representing whether the protein was down or upregulated following METH sensitization. Gray arrows represent hypothesized changes based on previous research on METH sensitization and schizophrenia. GPHN and FABP3, which were increased in the current study, regulate the expression of GABAA receptors and dopamine D2 receptors, respectively, with D2 receptors negatively involved in the regulation of cAMP and corresponding intracellular downstream regulators such as PKA and DARPP-32. The NMDA and AMPA glutamate receptors allow influx of calcium into the cell, with altered calcium levels induced by METH treatment leading to increased expression of VSNL1, PP2A, and PPP3R1 (calcineurin), which also negatively regulate the expression of DARPP-32. Collectively, alterations to these proteins are thought to lead to changes in signal transduction, dopamine synthesis, long-term depression, and cognitive dysfunction. The disturbed intracellular calcium levels also affect energy pathways via modulation of pyruvate metabolism and oxidative phosphorylation, ultimately leading to changes in TCA activity and energy output. The decreased expression of GAD67 results in reduced production of GABA and decreased inhibitory neurotransmission within the PFC. This most likely occurs in parvalbumin (PVALB) expressing interneurons, which, together with neuroligin 2 (NLGN2), regulates interneuronal firing patterns and the oscillations requisite for cognitive tasks. As such, the downregulation of PVALB and NLGN2 is hypothesized to result in reduced oscillatory power and executive dysfunction. Drawing designed and created by adapting various slides purchased from Motifolio (<http://motifolio.com/>).

knockout mice exhibit decreased working memory, latent and prepulse inhibition.^{87,88} Furthermore, regulatory subunits of the PPP2A complex are negatively implicated in the phosphorylation of tyrosine hydroxylase, the rate-limiting precursor in dopamine synthesis,⁸⁹ while elevated PPPs regulate the dephosphorylation of DARPP-32,⁹⁰ particularly following activation of dopamine D2 or NMDA receptors. As such, changes in both dopaminergic and glutamatergic signaling within the mesolimbic system are regarded as being critically involved in the development of locomotor sensitization,^{91,92} which, according to the current results, may be secondary to elevated PPPs.

Importantly, calcineurin is increased in the PFC of schizophrenia,⁹³ and there is a significant association between single-nucleotide polymorphisms of calcineurin γ catalytic subunit (PPP3CC) and the disorder.⁹⁴ Furthermore, the PPP3CC gene is significantly associated with poor performance on PFC-mediated cognitive tasks such as the Wisconsin Card Sorting Test.⁹⁵ Schizophrenia is also characterized by reduced dopamine and overactive D2 receptors⁸¹ in the PFC, which may be the result of or secondary to elevated PP2A subunits, particularly given that DARPP-32 is also downregulated in the PFC in schizophrenia.⁹⁶ Overall, the PPP pathway may provide a biological mechanism that mediates the changes between monoamine systems and the phosphorylation status of

downstream mediators not only in schizophrenia but also in METH sensitization, leading to long-term depression and cognitive dysfunction (Figure 3).

3.5.3. Small Molecule Biochemistry: Inhibitory GABAergic Network. Given that GABA is synthesized from the metabolism of glutamate, together with the changes to the glutamine-glutamate network described above, it serves that several alterations to the GABAergic inhibitory network were identified following sensitization to METH in the PFC. Indeed, glutamate dehydrogenase 1 (GAD67), which is involved in the synthesis of GABA from glutamate, was significantly downregulated in METH-sensitized rats. Given that over 90% of GABA production is derived from GAD67 activity⁹⁷ and that GAD67 and the concentration of GABA in interneurons are activity-dependent,⁹⁸ the reduced GAD67 expression likely reflects decreased GABA within the PFC, and, indeed, GABA is reduced in the PFC following METH sensitization.⁹⁹ Alterations to GABAergic neurotransmission are the most consistent findings in the PFC of schizophrenic brains,¹⁰⁰ with the mRNA and protein expression of GAD67^{101,102} consistently reported as downregulated.

Neurologin2 (NLGN2), a cell adhesion molecule that is specifically found at inhibitory synapses,¹⁰³ was downregulated in the PFC of METH-sensitized rats. Alterations to NLGN2 expression affects GABAergic synaptogenesis¹⁰⁴ and the pool of vesicular reserve in frontal cortex synapses,¹⁰⁵ suggesting a key role for NLGN2 in synaptic signaling at inhibitory junctions in the PFC. Importantly, overexpression or knockout of NLGN2 increases¹⁰⁵ and decreases¹⁰⁶ GABAergic neurotransmission, respectively, and mutations of NLGN2 have previously been found in schizophrenia.¹⁰⁷ Deletion of NLGN2 has also been found to selectively decrease GABAergic synaptic transmission within fast-spiking cells,¹⁰⁸ suggesting that changes to NLGN2 expression following sensitization to METH may mediate reduced activity of GABAergic circuits within the PFC specifically in fast-spiking interneurons.

Indeed, parvalbumin (PVALB), a calcium-binding protein that is associated with a fast-spiking firing pattern,¹⁰⁹ was downregulated in the PFC of METH-sensitized rats. Not only is the expression of PVALB downregulated in the PFC of schizophrenia^{101,110} but also the downregulation of GAD67 is most prominent in PVALB-expressing interneurons.^{101,111} PVALB-expressing GABAergic neurons regulate the temporal organization of cortical networks through control of pyramidal cells and neuronal oscillations.^{112,113} Interestingly, fast-spiking PVALB-GABA cells are believed to mediate oscillations and neural synchrony during cognitive tasks,¹¹⁴ particularly those mediated by the PFC,¹¹⁵ with schizophrenia characterized by significant alterations to PFC oscillatory patterns.¹¹⁶ These findings have ultimately led to the hypothesis that dysfunctional inhibitory control of the PFC could underlie the executive deficits inherent to schizophrenia.¹¹⁷ As such, METH sensitization is also associated with global changes to the inhibitory GABAergic network within the PFC, with changes possibly localized to PVALB-containing fast-spiking cells. In light of the significant overlap in both the executive dysfunction and changes to the GABAergic system between METH sensitization and schizophrenia, alterations to the GABAergic network may present a common biological substrate that could underpin the executive dysfunction seen across psychoses (Figure 3).

4. CONCLUDING REMARKS

This is the first shotgun proteomics study to examine the differential protein expression of the PFC of adult rats sensitized to METH. We found multiple proteins that were differentially expressed following sensitization, which collectively form an integrated signaling network whose dysfunction shares many of the same biological changes commonly observed in schizophrenia. While original research on METH sensitization placed particular salience on the dopaminergic network, much like schizophrenia, the current results suggest an increasingly clear role for mitochondrial proteins, synaptic proteins, protein phosphatases, and inhibitory GABAergic proteins in the neurobiology of both schizophrenia and METH sensitization within the PFC. Specifically, alterations to the protein phosphatase or GABAergic networks may subserve common clinical symptoms between METH sensitization and schizophrenia, particularly executive dysfunction. As such, this research should serve as an important step for further investigation with particular proteins of interest to further understand how these changes could mediate clinical symptoms as well as to inform the development of new therapeutic strategies.

■ ASSOCIATED CONTENT

📄 Supporting Information

Table S1: The complete set of 1317 proteins identified reproducibly from all samples in this study, including numbers of peptides assigned to each protein in each biological replicate experiment. Also includes normalized spectral abundance factors for individual replicates, average normalized spectral abundance factors for each condition, Student's *t*-test *p*-values and FDR adjusted *p*-values, and expression ratios calculated from averaged normalized spectral abundance factors. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACN, acetonitrile; ARA, Animal Research Authority; AGAL, Australian Government Analytical Laboratories; ATP, adenosine triphosphate; D2, dopamine 2 receptor; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ETC, electron transport chain; FDR, false discovery rate; GABA, gamma-aminobutyric acid; GPM, global proteome machine; HCL, hydrochloric acid; I.P., intraperitoneal injections; IPA, Ingenuity Pathway Analysis; LC-MS, liquid chromatography with mass spectrometry; METH, methamphetamine; mRNA, messenger RNA; NADH, nicotinamide adenine dinucleotide; NH_4HCO_3 , ammonium bicarbonate; NSAF, normalized spectral abundance factor; RSD, relative standard deviation; PAGE, polyacrylamide gel electrophoresis; PFC, prefrontal cortex; PPP, phosphoprotein phosphatase; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TCA, tricarboxylic acid

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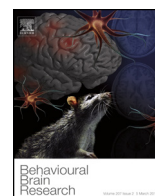
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Appendix B



Research report

GABAergic mRNA expression is upregulated in the prefrontal cortex of rats sensitized to methamphetamine



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HIGHLIGHTS

- METH sensitization altered pres and postsynaptic GABA transcripts in the PFC.
- GAT₁ and GAT₃ mRNA were upregulated in PFC following METH sensitization.
- GABA_Aα3, GABA_Aβ1 and GABA_B1 subunit mRNAs were upregulated following sensitization.

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ABSTRACT

Inhibitory gamma-aminobutyric acid (GABA)-mediated neurotransmission plays an important role in the regulation of the prefrontal cortex (PFC), with increasing evidence suggesting that dysfunctional GABAergic processing of the PFC may underlie certain deficits reported across psychotic disorders. Methamphetamine (METH) is a psychostimulant that induces chronic psychosis in a subset of users, with repeat administration producing a progressively increased vulnerability to psychotic relapse following subsequent drug administration (sensitization). The aim here was to investigate changes to GABAergic mRNA expression in the PFC of rats sensitized to METH using quantitative polymerase chain reaction (qPCR). Male Sprague–Dawley rats ($n = 12$) underwent repeated methamphetamine (intraperitoneal (i.p.) or saline injections for 7 days. Following 14 days of withdrawal, rats were challenged with acute methamphetamine (1 mg/kg i.p.) and RNA was isolated from the PFC to compare the relative mRNA expression of a range of GABA enzymes, transporters and receptors subunits. METH challenge resulted in a significant sensitized behavioral (locomotor) response in METH pre-treated animals compared with saline pre-treated controls. The mRNAs of transporters (GAT₁ and GAT₃), ionotropic GABA_A receptor subunits (α3 and β1), together with the metabotropic GABA_B1 receptor, were upregulated in the PFC of sensitized rats compared with saline controls. These findings indicate that GABAergic mRNA expression is significantly altered at the pre and postsynaptic level following sensitization to METH, with sensitization resulting in the transcriptional upregulation of several inhibitory genes. These changes likely have significant consequences on GABA-mediated neurotransmission in the PFC and may underlie certain symptoms conserved across psychotic disorders, such as executive dysfunction.

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1. Introduction

Methamphetamine (METH) is a potent psychostimulant that can induce psychosis among recreational and chronic users [1–3]. However, while METH psychosis is typically transient, some users

may develop a persistent psychotic syndrome that is indistinguishable to schizophrenia, with positive, negative, and cognitive deficits that persist after long periods of abstinence from drug use [4–7]. Previous research has interpreted the significant similarities between chronic METH psychosis and schizophrenia in the context of behavioral sensitization [8,9], a phenomenon whereby repeated exposure to a stimulus results in a progressively increased sensitivity to that stimulus following a period of abstinence [10]. Indeed, both chronic METH psychosis and schizophrenia are characterized by psychotic relapse even during abstinence from METH use or

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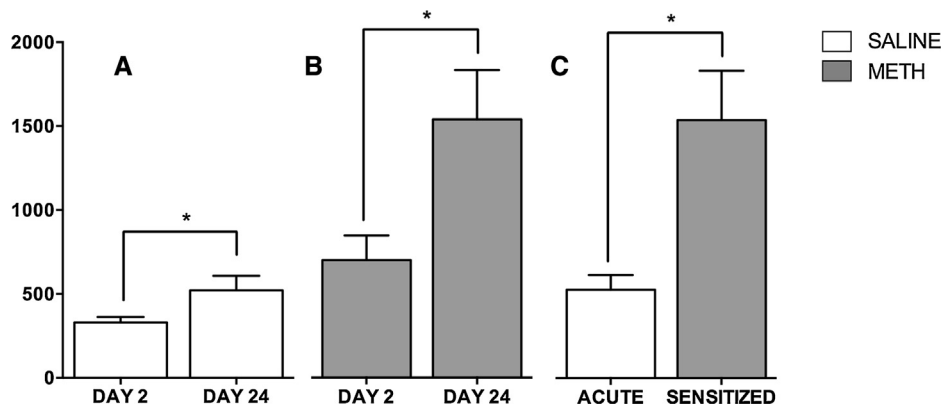


Fig. 1. Locomotor sensitization to repeated METH administration. Rats were assigned to undergo either repeated METH (1 mg/kg intraperitoneal (i.p.) days 2 and 8; 5 mg/kg i.p. days 3–7, $n=6$) or saline (1 mg/kg i.p. $n=6$) treatment for 7 days. Following 14 days of withdrawal, both METH and saline rats were challenged with an acute METH (1 mg/kg, i.p.) injection. (A) shows that saline pre-treated rats showed increased locomotor response to acute METH administration on Day 24. (B) shows that METH-treated rats displayed increased locomotor response upon challenge administration of METH compared to their first day of METH exposure on Day 2. Together, METH-treated rats displayed significantly more locomotor activity compared to saline pre-treated controls in response to METH challenge and therefore demonstrated sensitization to METH, as shown in (C). * $p<0.05$.

if neuroleptic medication is discontinued [11–13], while patients with schizophrenia can experience a psychotic relapse following exposure to METH at a dose that does not induce psychosis in healthy controls [14]. These findings suggest that chronic METH psychosis and schizophrenia may be the result of overlapping neurobiological factors that mediate the expression of similar phenotypes together with a persistent sensitivity to psychotic relapse. As such, understanding the molecular mechanisms that mediate METH sensitization could help understand the neurobiology of schizophrenia.

Normal brain function is dependent on the delicate balance of excitatory and inhibitory neurotransmission predominantly mediated by glutamatergic and gamma-aminobutyric acid (GABA) signaling, respectively. GABA-mediated processes facilitate inhibitory control of both inhibitory interneurons and excitatory pyramidal cells, thus providing both direct and indirect mechanisms of altering the activity of neural networks [15]. Furthermore, the GABAergic system contributes to the production of synchronized network oscillations during cognitive tasks [16,17], particularly those mediated by the prefrontal cortex (PFC), a brain region associated with higher-order cognitive and behavioral processes [17–19]. GABAergic disturbances are some of the most consistent findings in post-mortem analyses of the PFC in schizophrenic brains, with reduced protein and mRNA expressions of glutamate

decarboxylase 1 (GAD₆₇) and GABA transporter 1 (GAT₁) routinely found in the PFC in schizophrenia [20]. Given that executive dysfunction is considered a core feature of the disorder [21], dysfunctional GABAergic signaling of the PFC has been proposed to underlie the altered gamma oscillatory patterns and executive deficits inherent to schizophrenia [21–23].

Previous studies have shown that METH administration changes GABAergic function. Specifically, METH sensitization increases GAD₆₇ and GABA_Aα2 protein expression in the caudate nucleus and decreases the same protein in the nucleus accumbens core and shell [24]. Furthermore, METH sensitization has been shown to decrease the concentration of GABA in the striatum [25] and PFC [26]. METH sensitization is also associated with PFC-mediated cognitive dysfunction [27], with gamma oscillation disturbances reported in both chronic METH users [28] and METH sensitized animals [29]. These data suggest that GABAergic dysfunction may be a common biological substrate underlying the PFC-mediated behavioral and cognitive dysfunction that appears to be conserved across psychotic disorders. While PFC GABAergic disturbances in schizophrenia have been well documented, GABAergic dysfunction in the PFC following METH sensitization has yet to be described.

Sensitization to psychostimulants can be reliably induced in experimental animals, with METH sensitized locomotor activity regarded as an animal model of the neurobiological changes

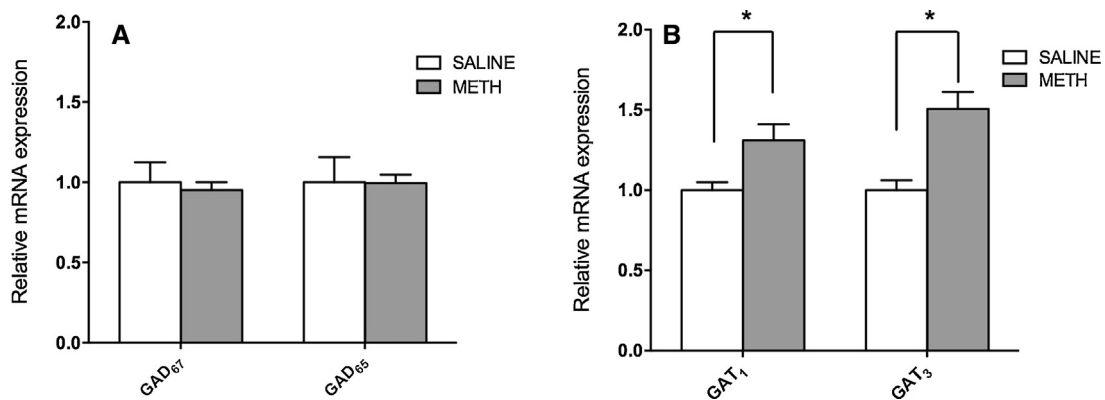


Fig. 2. GABA enzymes and transporter mRNA expression after saline and METH sensitization ($n=6$ per group) as determined using quantitative RT-PCR. Each mRNA is expressed as the mean \pm SEM fold change relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method. (A) shows no significant difference in the expression of GAD₆₅ and GAD₆₇ between METH sensitization and saline controls. (B) shows that GAT₁ and GAT₃ were both significantly upregulated following sensitization to METH. *Indicates GABA mRNA was significantly different ($p<0.05$) when compared with saline controls. All error bars were produced with consideration of the exponential nature of qPCR using the $2^{-\Delta\Delta Ct}$ method.

associated with METH-induced psychosis [8,27]. We have previously found that protein expression for GAD₆₇ and gephyrin – a scaffolding protein involved in the clustering of GABA_A and glycine receptors at postsynaptic sites [30] – were down-regulated and upregulated in the PFC following METH sensitization, respectively [31]. As an extension of these findings, the primary aim of the current study was to investigate changes to gene expression of GABA related proteins (including enzymes, transporters, and receptor subunits) in the PFC of rats sensitized to METH, with particular focus on mRNA that was both expressed within the PFC and that had previously been implicated in the pathophysiology of schizophrenia. Based on our previous findings, it was hypothesized that GAD₆₇ mRNA would be downregulated while GABA_A receptor mRNA would be upregulated in the PFC of METH sensitized rats compared with controls.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Macquarie University Animal Ethics Committee (reference number ARA 2010/045) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 8th Edition, 2013). Twelve experimentally naïve male Sprague-Dawley rats (Animal Resource Center, WA, Australia), weighing an average of 261 ± 5 g at the start of testing, were used. Animals were housed in groups of four in plastic high top cages [(64 cm (L) \times 40 cm (W) \times 20 cm (H))] that were kept in a humidity- and temperature-controlled room ($21 \pm 2^\circ\text{C}$, 60% humidity) and maintained on a 12 h light: 12 h dark cycle (lights on at 0600 h). All experimentation was carried out during the light period. The rats were given *ad libitum* access to food and water in their home cages for the duration of the experiment.

2.2. Methamphetamine-induced behavioral sensitization

Methamphetamine-induced sensitization was performed as previously described (Wearne et al., 2014). Briefly, on Day 1, all rats received a saline injection (0.9%, 1 mg/kg, i.p.) and were allocated to treatment groups such that there was no significant difference in baseline locomotor activity between METH and saline-treated rats prior to drug administration ($p = 0.997$). Rats were then assigned to undergo repeated METH (once daily 1 mg/kg intraperitoneal (i.p.) on days 2 and 8; 5 mg/kg i.p. days 3–7) or saline (1 mg/kg i.p.) injections for 7 days, consistent with previously published methods [32,33]. Following a 14-day withdrawal period (Days 9–22), rats were injected with 1 mg/kg saline (i.p.) to test for conditioned baseline locomotor responding (Day 23) before being assessed for locomotor sensitization via a challenge dose of 1 mg/kg METH (i.p.) on Day 24. Locomotor activity was recorded on Days 1, 2, 8, 23 and 24 using standard Med Associates chambers equipped with 4 infrared photobeam detectors 30 mm above the floor on the front and back panels 50 mm apart. Locomotor activity was quantified as the number of photobeam interruptions recorded via a computer equipped with Med-IV PC software (Med Associates, St. Albans, VT, USA). Rats were placed in the test chamber 15 min prior to drug injection to reduce novelty-induced increases in activity before locomotor activity was recorded for 1 h following treatment. Immediately following the challenge locomotor session (60 min post-injection), rats were euthanized via rapid decapitation and the PFC was dissected out on dry ice as previously described [31,34].

2.2.1. Drugs

Methamphetamine hydrochloride was purchased from the Australian Government Analytical Laboratories (Pymble, NSW) and

was dissolved in 0.9% saline. Intraperitoneal injections (i.p.) were delivered at a volume of 1 ml/kg.

2.3. Statistical analysis for methamphetamine-induced sensitization

Locomotor results are reported as mean \pm SEM. Mean locomotor activity on Day 1 and Day 23 were compared between saline and METH treated rats using an independent *t*-test. Mean locomotor activity following METH challenge was compared between saline and METH treated rats using an independent *t*-test. A paired *t*-test was used to compare mean baseline locomotor activity on Day 2 for saline and METH pretreated rats to the mean locomotor activity in response to METH challenge. Results are reported as mean \pm SEM. Analyses were performed using SPSS version 21 and the significance level was set at $p < 0.05$.

2.4. Quantitative polymerase chain reaction (qPCR)

2.4.1. Gene choice, primer design and validation

As indicated above, the focus of the current study was on mRNA of GABA related proteins that were both expressed within the PFC and that had previously been implicated in the pathophysiology of schizophrenia. Thus, the expression of GABA enzymes, GAD₆₇ and GAD₆₅ [15,35], GABA transporters, GAT₁ and GAT₃, [36], together with metabotropic GABA_B receptors, GABA_{B1} and GABA_{B2} [37], were examined in the current study. While GABA_A receptors are composed from a pool of 19 distinct subunits, combinations of the $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 2$, $\beta 3$ and $\gamma 2$ subunits represent over 80% of benzodiazepine sensitive GABA_A receptors in the adult brain [38]. However, given that the $\beta 3$ subunit is predominantly found in the striatum (Pirker et al., 2000) and that $\beta 1$ and δ have also been implicated in schizophrenia [39–41], the expression of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 2$, $\gamma 2$ and δ subunits were examined in the current study.

Table 1 summarizes the primer sequences, GeneBank accession numbers, PCR product sizes and efficiencies for each gene. Primers were designed using NCBI Nucleotide Primer Design software with parameters: 18–22 nucleotides length, 70–150 base pair product size, 55–60 $^\circ\text{C}$ melting point and 50% GC content. Primers did not distinguish between splice variants. PCR products were sequenced at the Macquarie University DNA Analysis Facility and specificity was verified through the use of a BLAST search against the rat genome. A four-point standard curve was measured for each primer set and the primer efficiencies were calculated using MxPro software (Stratagene). Similar primer efficiencies are necessary to compare genes and all were shown to have $r^2 > 0.975$ (Table 1).

2.4.2. Tissue extraction, RNA isolation and reverse transcription

RNA was extracted from PFC tissue using the SV total RNA isolation system according to the manufacturer's protocol (Promega SV Total RNA Isolation Kit, Promega, Madison, Wisconsin, USA). The concentration and integrity of total RNA was measured using a Nanodrop 5000 spectrophotometer (Beckman-Coulter DU-800; Fullerton, California, USA) before 500 μg of RNA was reverse transcribed with oligo-dT primers according to the manufacturer's protocol (Promega Improm II kit). All samples were reversed transcribed simultaneously.

2.4.3. Quantitative real time PCR

Template cDNA from each sample was used for quantitative real-time PCR using the DNA-binding dye SYBR Green (Stratagene Brilliant II Mastermix). Each reaction consisted of 12.5 μl of Mastermix (with 2.5 mM/l MgCl_2), 1.0 μl forward primer (300 nmol), 1.0 μl reverse primer (300 nmol), 1 μl (500 ng) cDNA and 9.5 μl nuclease-free PCR-grade water were combined in a 25 μl reaction. Forty-five cycles of real time PCR was carried out on a Stratagene

Table 1

Summary of primers, GeneBank accession numbers, forward and reverse sequences and PCR-product sizes for the 14 GABA related genes analyzed in the current study. The relative expressions of GABAergic genes were normalized to the housekeeping gene, GAPDH. Genes were chosen based on abundance in the PFC and whether they had been previously implicated in the neurobiology of schizophrenia [15,35–38,40,41].

Primer	Sequence GeneBank ID	Forward (5'–3')	Reverse (5'–3')	Product size	Efficiency
GAD ₆₇	NM.017007.1	ACAAATGCCTGGAGCTGGCTGAAT	TTGTGTGCTCAGGCTCACCATTGA	93	0.995
GAD ₆₅	NM.012563.1	TCTCAAAGGTGGCGCCAGTGATTA	TTGGTGAGTTGCTGCAGGGTTTGA	131	0.995
GAT ₁	NM.02437.1	GCGCAACATGCACCAATGACA	AGACCACCTTTCCAGTCCATCCAA	140	0.993
GAT ₃	NM.024372.2	TGGGCATGAGTGGAACACAGAGAA	AGGTTCCCGATGTGTTCAATGCCA	159	0.985
GABA _{α1}	NM.183326.2	TGTCITTTGGAGTGACGACCGTTCT	ACACGAAGGCATAGCACACTGCAA	125	0.977
GABA _{α2}	NM.001135779.4	TCCAGGATGACGGAACATTGCTGT	TTCCGGCTTGACTGTAAGCCTCAT	53	0.975
GABA _{α3}	NM.017069.2	TCTGGATGGCTATGACAACCGACT	ACTTCAGTCACTGCATCTCCAAGC	57	0.995
GABA _{α5}	NM.017295.1	AACATCAGCACCAGCACAGGTGAA	TGACTGTATGATGCAGGGAAGGT	112	0.976
GABA _{β1}	NM.012956.1	TTGTGTTCTGTTCTGGCTCTAC	GGGCATCAACCTGGACTTTGTTC	150	0.982
GABA _{β2}	NM.012957.2	GCTGTCTGTCTCAGAGTGCAAT	CAGAAACCATATCGATGCTGGCGA	166	0.998
GABA _{γ2}	NM.183327.1	TCGCCAAATACATGGAGCACTGGA	TTTGGCTAGTGAAGCCTGGGTAGA	112	0.978
GABA _δ	NM.017289.1	ATGGGCCAGAGCAATGAATGA	TTCTGAGATGTGGTCAATGCTGGC	182	0.990
GABA _{B1}	NM.031028.3	ACCTGAAGCGTCAAGATGCTCGAA	AGTTGTGAGCATACCACCGATGA	141	0.998
GABA _{B2}	NM.031802.1	AATGATCCTGCACCAGCGTCAA	AACATGCTCTCTCGAAGGCACAA	119	0.983
GAPDH	NM.017008.4	TGAAGTTCGGTGTGAACGGATTG	AGCCTTGACTGTGCCGTTGAACCT	176	0.999

MX3000P real time PCR machine (Agilent Biosciences) with an initial 10 min denaturing step at 95 °C. Each cycle consisted of 30 s at 95 °C (denaturation), 60 s at 60 °C (annealing) followed by 60 s at 72 °C (extension). Fluorescent data was acquired at the end of each extension cycle at 85 °C for 10 s to eliminate primer dimer artifact. Following the 45 cycles, a melt curve was performed to determine amplification of all primer sets and to ensure that a single PCR product was formed in the reaction.

2.4.4. Normalization and data analysis for real time PCR

Measurements from each sample were performed in duplicate and 'no template control' samples did not produce any product. Cycle threshold (Ct) values were obtained for each target using the MxPro software and normalized via a housekeeping gene, GAPDH. GAPDH protein was unchanged in the PFC following METH sensitization (Wearne et al., 2014) and initial analyses revealed GAPDH mRNA was also unaltered in the current study ($p=0.68$). Expression levels were calculated relative to controls using the $2^{-\Delta\Delta Ct}$ method [42]. For statistical analysis, outliers (± 2 SD from the mean) were removed from the control and METH sensitized conditions (1% of values). All genes were normally distributed except for 3 genes in the saline group (GAT₁, $\alpha 2$, $\alpha 5$) and two genes in the METH sensitized condition ($\gamma 2$ and δ), although further analysis revealed that the violations against normality did not alter the interpretation of the results. Independent t -tests were consequently used to determine significant changes in gene expression between METH-sensitized rats and saline controls. All analyses were performed using SPSS version 21 and the significance level was set at $p < 0.05$.

3. Results

3.1. Sensitization to methamphetamine

There was no difference in the locomotor activity of METH- and saline-treated rats in response to saline challenge on Day 1 ($p=0.997$) and on Day 23 ($p=0.40$). Locomotor activity was increased on Day 24 for drug naïve rats compared with their locomotor activity on Day 2, suggesting that acute METH increased locomotor activity. (Fig. 1A). METH pre-treated rats also showed a significantly increased locomotor response on challenge day when compared with their locomotor response on Day 2 of METH exposure, $p < 0.01$ (Fig. 1B). However, METH challenge resulted in a significant increased locomotor response in METH pre-treated rats compared with saline controls, $p < 0.05$ (Fig. 1C).

3.2. Relative expression of GABAergic mRNA in the PFC following METH sensitization

3.2.1. GABA related enzymes

There was no significant difference in the expression of GAD₆₇ ($p=0.68$) and GAD₆₅ ($p=0.97$) mRNA in the PFC between saline and METH sensitized rats (Fig. 2A).

3.2.2. GABA transporters

GAT₁ (1.31-fold, $p < 0.01$) and GAT₃ (1.50-fold, $p < 0.0005$) were significantly upregulated in METH-treated rats compared with saline-treated controls (Fig. 2B).

3.2.3. GABA_A receptors

Ionotropic GABA_A receptor subunits $\alpha 3$ (1.34-fold, $p < 0.0001$) and $\beta 1$ (1.26-fold, $p < 0.005$) were significantly upregulated in METH-treated rats compared with saline controls. There was no significant difference in the expression of subunits $\alpha 1$ ($p=0.24$), $\alpha 2$ ($p=0.41$), $\alpha 5$ ($p=0.07$), $\beta 2$ ($p=0.08$), $\gamma 2$ ($p=0.47$) and δ ($p=0.18$) in the PFC between METH-treated rats and saline-treated controls (Fig. 3A and B).

3.2.4. GABA_B receptors

GABA_{B1} (1.40-fold, $p < 0.0001$) mRNA was significantly upregulated in METH-treated rats compared with saline controls (Fig. 3C). There was no significant difference in the expression of GABA_{B2} mRNA between METH-treated rats and saline-treated controls ($p=0.16$).

4. Discussion

The main findings of the current study were that a METH challenge produced a significant sensitized locomotor response in METH pre-treated rats compared to saline controls and that the mRNA expression of GAT₁, GAT₃, GABA_A $\alpha 3$, GABA_A $\beta 1$ and GABA_{B1} were significantly upregulated in the PFC of rats sensitized to METH. The behavioral findings confirm the expression of METH-induced behavioral sensitization and are consistent with a large body of literature that has shown increased locomotor activity following a chronic psychostimulant administration and withdrawal regime [10,31,43]. Furthermore, the clear lack of behavioral activation following saline challenge on Day 23 confirms that the rats were neither conditioned to the locomotor cages nor to the injection, suggesting that the change in behavior can be attributed to the sensitizing effects of METH.

The current findings suggest that METH sensitization is associated with multiple presynaptic and postsynaptic changes to the

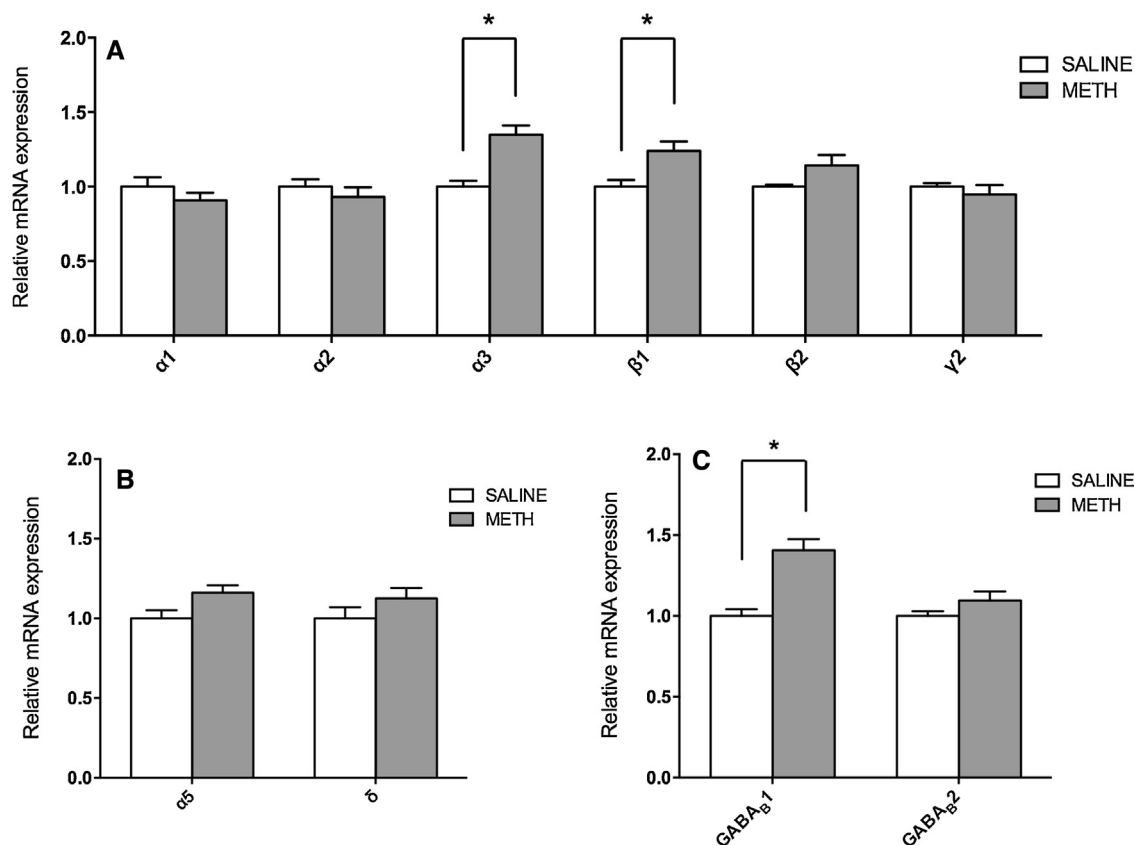


Fig. 3. GABA receptor subunit mRNA expression after saline and METH sensitization ($n = 6$ per group) as determined using quantitative RT-PCR. (A) shows that two GABA_A receptor subunits, $\alpha 3$ and $\beta 1$, were upregulated following sensitization to METH. (B) depicts that the expression of extrasynaptic GABA_A subunits were unchanged following METH. (C) shows that the expression of metabotropic GABAB receptors, GABA_B, was upregulated following METH sensitization. *Indicates GABA mRNA was significantly different ($p < 0.05$) when compared with saline controls. All error bars were produced with consideration of the exponential nature of qPCR using the $2^{-\Delta\Delta Ct}$ method.

GABAergic system in the PFC. The fact that multiple GABAergic genes were unaltered following METH sensitization indicates that changes to the GABAergic system are subtype specific, and therefore reflect particular changes to GABAergic neurotransmission and inhibitory regulation secondary to the functional requirements of the cellular environment and/or PFC tissue. As such, these findings confirm that the GABAergic network plays an adaptive role in the PFC following METH sensitization. As sensitization is typically described in the context of dopamine and glutamate systems [10,44], the current findings extend this body of literature by providing evidence of alterations to the PFC inhibitory GABAergic network following METH sensitization.

Two GABA_A receptor subunits, $\alpha 3$ and $\beta 1$, were upregulated in the PFC of METH sensitized rats, partially supporting the hypothesis that GABA_A receptor mRNA expression would be increased in the PFC following METH sensitization. The increased expression of these subunits may correspond with increased clustering of GABA_A receptors, as we have previously found an upregulation of gephyrin protein in the PFC following METH sensitization [31]. While the functional significance of these specific subunit changes is not clear, different combinations of receptor subtypes likely reflect altered postsynaptic inhibitory signaling, particularly given that GABA_A receptors containing the $\alpha 3$ and $\beta 1$ subunits mediate postsynaptic phasic inhibition and the hyperpolarization of efferent projections [45]. Furthermore, in light of the fact that METH sensitization is associated with a reduced level of GABA in the PFC [26], these receptor changes may represent a compensatory mechanism that attempts to restore and/or maintain GABAergic mediated inhibition of efferent projections. Importantly, locomotor sensitization is augmented following microinjection of GABA_A

receptor antagonist, dicentrine, into the PFC [46], whereas total knock-out of the $\alpha 3$ subunit gene results in elevated locomotor activity and sensorimotor gating deficits but does not appear to alter amphetamine-induced locomotor activity [47]. These findings suggest that while the subunits expressed in GABA_A receptors are important in mediating dopaminergic neurotransmission, cognitive function and locomotor activity, GABA_A receptor activation in the PFC may be specifically involved in mediating sensitization to amphetamine type psychostimulants.

The finding that GAT₁ and GAT₃ mRNA expression was upregulated in the PFC following METH sensitization indicates increased reuptake of GABA at inhibitory synapses [48] and may explain the postsynaptic GABA_A receptor changes in the PFC described above. There are several possible reasons for increased mRNA expression of GAT₁ and GAT₃ in the PFC following METH sensitization. First, GAT₁ and GAT₃ are believed to regulate the tonic inhibition of pyramidal neurons [49], suggesting that their increased expression may modify inhibitory modulation of pyramidal cells in the PFC following METH sensitization. Additionally, given that GAT₁ is predominantly localized to presynaptic terminals and astrocytes [48,50], while GAT₃ is exclusively found in astrocytes [48], the increased expression of GABA transporters may relate to increased reuptake of GABA to the glutamate/glutamine cycle in astrocytes, where GABA is metabolized into succinate [51]. Indeed, succinic acid semialdehyde, an intermediate in the catabolism of GABA, is increased following METH sensitization in the PFC [26], adding further support that accelerated removal and metabolism may be responsible for reduced GABA in the PFC. An additional explanation may be that increased GAT₁ and GAT₃ mRNA expression may be compensatory to decreased protein expression. In support for

this hypothesis, we have previously found that GAT₁ protein levels were down-regulated in the PFC following sensitization to METH [31], although, the magnitude of the fold-change (–1.23-fold) failed to reach the threshold cutoff.

Based on our previous work [31], we expected to find reduced mRNA expression for GAD₆₇ in the PFC, however we found no differential expression of GAD₆₇ mRNA following METH sensitization. While previous studies have found reduced mRNA and protein expression of GAD₆₇ following sensitization to METH [24,25], these findings have been localized to the striatum, which may be differentially affected following METH sensitization. As such, it is possible that GAD₆₇ protein may be down-regulated in our previous work due to either post-translational modifications or increased GAD₆₇ protein metabolism (Wearne et al., 2014). Alternatively, GAD₆₇ protein expression represents the protein found in soma, axons and terminals within the PFC while GAD₆₇ mRNA expression likely consists mainly of transcripts localized to GABAergic cell bodies [52,53], suggesting that reduced GAD₆₇ protein following METH sensitization may be due to reduced afferent inhibitory drive to the PFC. In keeping with this idea, this potential decrease in inhibitory input due to lack of GAD₆₇ protein from distant sources could explain the upregulated expression of GAT₁ mRNA, as elevated reuptake of GABA to the presynaptic neuron may counteract disinhibited afferent projections by facilitating GABA exocytosis from recycled GABA stores.

While locomotor sensitization is regarded as an animal model of human psychostimulant-induced psychosis [8,27], many of the transcriptional changes identified in the current study do not reflect the expression typically observed in schizophrenia. For example, compensatory changes to GABA_A receptors have been associated with upregulated expression of GABA_Aα2 mRNA [15] while the expression of GABA_A3 is unchanged in the PFC of schizophrenia [54,55]. Additionally, analyses consistently indicate that the mRNA expressions for GAD₆₇ and GAT₁ are down-regulated in the PFC in schizophrenia [56,57]. As such, while both METH sensitization and schizophrenia are characterized by changes to the GABAergic network, there appears to be both qualitative and quantitative differences in the expression of specific GABAergic genes between the two conditions. While the differences detected may be due to species-specific effects, an alternative explanation may be due to the global approach used in the current study. That is, the PFC is a heterogeneous structure, with schizophrenia research showing differential inhibitory changes across multiple areas of the PFC, including the dorsolateral prefrontal cortex [36] and orbitofrontal cortex [58], while amphetamine sensitization is blocked by ibotenic acid lesion of the medial prefrontal cortex [59] but not the total PFC [60]. Thus, the regional expression patterns of GABAergic mRNA may have escaped detection with the global approach used in the current study. Consequently, localized analysis of the PFC will be needed to determine the expression of GABAergic genes with respect to METH sensitization.

5. Conclusions

GABAergic mRNA expression is significantly altered at the pre and postsynaptic level in the PFC of rats sensitized to METH, with sensitization resulting in the transcriptional upregulation of several GABAergic markers. Even though GABAergic alterations could underlie certain aspects of psychosis symptomatology, it is unclear whether the changes identified here represent primary changes that mediate vulnerability to psychosis (i.e., sensitization) or secondary compensatory changes due to an unknown primary etiological cause (e.g., dopamine or glutamate dysregulation). While the results of the current study clearly implicate GABAergic dysfunction of the PFC in METH sensitization – suggesting that

GABAergic disturbances may be common across psychotic syndromes – the underlying mechanisms mediating these inhibitory disturbances may not be consistent across conditions. As such, future research should determine the underlying nature of these changes in relation to regional and cellular expression profiles together with psychotic and cognitive phenotypes.

Author contributions

T.W. and J.C. designed the study; T.W. and J.F. performed the behavioral experiments; T.W. and L.P. carried out the PCR experiments; T.W. analyzed the data; T.W. wrote the initial version of the manuscript with subsequent contribution from J.C. and A.K. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix C

ANIMAL RESEARCH AUTHORITY

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Macquarie University NSW 2109
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Associate Investigators:

A/Prof Ann Goodchild 0410 601 302
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In case of emergency, please contact:

Animal Welfare Officer Dr Sally Smith: 9850 7758 / 0439 497 383
Central Animal House Manager Christine Sutter: 9850 7780 / 0428 861 163
or the Principal Investigator / Associate Investigator named above

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: The role of the prefrontal cortex in cardiovascular regulation: effects of repeated methamphetamine administration

Type of animal research and aims of project:

Research (pharmacological/cardiovascular) – This project aims to determine how repeated METH administration changes the role the medial prefrontal cortex (MPFC) plays in influencing the tonic and reflex regulation of the cardiovascular and respiratory systems, and to link these with associated changes in phosphorylation states and proteomics of related chemicals in the MPFC.

Surgical Procedures category: 6 (Minor Physiological Challenge)

All procedures must be performed in accordance with the AEC approved protocol.

Numbers approved:

Species	Strain	Age/Sex/Weight	Year 1	Year 2	Year 3	Total	Supplier/Source
Rat	Sprague-Dawley (SD)	3 months / 250g	104	64		168	ARC Perth

Location of research:

Location	Full street address
Australian School of Advanced Medicine	Level 1, Clinic Building, 2 Technology Place, Macquarie University NSW 2109
Central Animal House Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109

Amendments approved by the AEC since initial approval: N/A

Approval is subject to compliance with the following conditions: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

This authority remains in force from **1 November 2010 to 31 October 2011**, unless suspended, cancelled or surrendered, **and will only be renewed upon receipt of a PROGRESS REPORT before the end of this period.**



Prof Michael Gillings
Chair, Animal Ethics Committee

Date: 27 October 2010

Appendix D

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2012/047

Date of Expiry: 31 October 2013

Full Approval Duration: 01 November 2012 to 31 October 2014 (24 Months)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).

Principal Investigator:

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Associate Investigators:

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Jane Franklin 0432 219 402

In case of emergency, please contact:

the Principal Investigator / Associate Investigator named above

Or Manager, CAF: 9850 7780 / 0428 861 163 and Animal Welfare Officer: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: Inhibitory regulation of the prefrontal cortex in methamphetamine-induced sensitization in the rat: the role of the GABAergic system

Purpose: 4 - Research: Human or Animal Biology

Aims: To determine the effect of chronic METH in altering inhibitory control in localized areas of the prefrontal cortex

Surgical Procedures category: 3 - Minor Conscious Intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
Rat	Sprague Dawley	3 months/Male/250g	112	ARC Perth
		TOTAL	112	

Location of research:

Location	Full street address
Central Animal House Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109
ASAM	Building F10A, Technology Place, Macquarie University NSW 2109

Amendments approved by the AEC since initial approval: N/A

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.



Prof Michael Gillings (Chair, Animal Ethics Committee)

Approval Date: 18 October 2012

