The Contribution of System Xc-to Prefrontal Cortical Mediated Behaviors Associated with Schizophrenia

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THE CONTRIBUTION OF SYSTEM X_C^- TO PREFRONTAL CORTICAL MEDIATED BEHAVIORS ASSOCIATED WITH SCHIZOPHRENIA

by

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ASTRACT

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Schizophrenia is a debilitating disorder involving impaired cognition, disorganized thinking, and auditory hallucinations that has a tremendous unmet medical need, potentially because current antipsychotics insufficiently target the pathophysiology of the disease. The neural basis of schizophrenia appears to involve abnormal activity within the dorsolateral prefrontal cortex (PFC) in which efferent neurons exhibit disorganized firing patterns. Synchronization of cortical activity is regulated by complex inter-neuronal connections, which is compromised in schizophrenia. Schizophrenic's exhibit reduced PFC volume that may reflect reduced arborization leading to diminished inter-neuronal connectivity. An unexplored explanation is that reduced volume reflects changes in astrocytes, cells positioned to control synchronized firing at up to 100K synapses. Glutamate release, including from system x_c^- which exchanges one intracellular glutamate for an extracellular cystine, is emerging as a component of astrocytic neuronal regulation and is altered in the PFC of schizophrenics. To determine the importance of system x_c^- dysregulation, we examined the impact of increased or decreased activity to glutamate levels in the PFC and PFC mediated behaviors that are used to model schizophrenia.

Decreased system x_c^- activity, achieved using the inhibitor sulfasalazine (SSZ), produced a phenotype that mirrored aspects of schizophrenia and reduced extracellular glutamate levels in the PFC. Specifically, SSZ produced deficits in sensorimotor gating, cognition, and anxiety – all of which involve the PFC and are altered in schizophrenia. Similar to schizophrenia, system x_c^- dysregulation was detected, and appears to be central to the cognitive deficits in the methylazoxymethanol acetate (MAM) neurodevelopmental model of schizophrenia.

Increased system x_c^- activity, achieved using the cysteine prodrug N-acetylcysteine, reversed multiple behavioral deficits present in preclinical models of schizophrenia. Namely, N-acetylcysteine reversed sensorimotor gating deficits produced by phencyclidine, an acute model of schizophrenia, and reversal learning deficits in the MAM model. Notably, chronic N-acetylcysteine attenuated behavioral deficits and normalized aspects of system x_c^- activity in MAM-treated rats.

Collectively, these data position system x_c^- as a key regulator of behavioral output from the prefrontal cortex and indicate that system x_c^- dysregulation in schizophrenia may be an important component of the pathology of the disease. In addition, increased system x_c^- may represent an effective therapeutic endpoint.

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ABBREVIATIONS

AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ApN, aminopeptidase N

ARE, antioxidant response element

CC, cystine

CGI, clinical global impression

CNS, central nervous system

CPG, (*S*)-4-carboxyphenylglycine

DNA, deoxyribonucleic acid

D_2 receptor, dopamine type 2 receptor

EAAT1 and EAAT2, excitatory amino acid transporters 1 and 2

EEG, electroencephalography

EPSPs, excitatory post synaptic potentials

fMRI, functional magnetic resonance imaging

GABA, γ-aminobutyric acid

$GABA_A$ and $GABA_B$ receptor, γ-aminobutyric acid receptor type A or B

GAD_{65} and GAD_{67}, glutamic acid decarboxylase 65 and 67 kilodalton

GAT1 , GABA transporter 1

GCLM or GCLC, glutamate-cysteine ligase modifier or catalytic subunit

GGT, γ-glutamyl transpeptidase

GI, gastrointestinal system

GSH, glutathione

GSS, glutathione synthetase
HAT, heteromeric amino acid transporter

IPSPs, inhibitory post synaptic potentials

MAM, methylazoxymethanol acetate

mGluR, metabotropic glutamate receptor (1-8)

MK801, dizocilpine

mPFC, medial prefrontal cortex

mRNA, messenger ribonucleic acid

Mrp1, multidrug resistance protein 1

NAAG, N-acetyl aspartyl glutamate

NAC, N-acetylcysteine

NMDA receptor, *N*-methyl-D-aspartate receptor

Nrf2, nuclear factor erythroid-2-related factor

PANSS, positive and negative symptoms scale

PCP, phencyclidine

PND, post-natal day

PPI, prepulse inhibition

PV, parvalbumin

SSZ, sulfasalazine

System x_c^-, cystine-glutamate exchange or cystine-glutamate antiporter

TBOA, *threo*-β-Benzyloxyaspartic acid

I. INTRODUCTION:

SCHIZOPHRENIA AND SYSTEM X_C^-

This dissertation will characterize the involvement of system x_c^- or the cystine-glutamate antiporter in schizophrenia. Specifically, acute and neurodevelopmental models of schizophrenia will be utilized to examine whether cystine-glutamate exchange is altered in these models. In addition, the ability of direct manipulation of system x_c^- to produce behavioral deficits similar to those observed in schizophrenia will be examined.

OVERVIEW OF SCHIZOPHRENIA:

Schizophrenia is a debilitating lifelong neuropsychiatric disorder affecting up to 1% of the world's population (Lewis and Lieberman, 2000; McGrath et al., 2008). It is characterized by a range of symptoms including positive (hallucinations, paranoia, delusions), negative (anhedonia, blunted affect, social deficits) and cognitive deficits (executive function, behavioral flexibility, memory, attention, learning) (Andreasen, 1995; Elvevag and Goldberg, 2000; Blanchard and Cohen, 2006). Cognitive deficits, particularly executive functioning which includes directed attention and planning working memory (Smith and Jonides, 1999; Eisenberg and Berman, 2010), are the best predictors of functional disability of the disorder and are poorly treated with current antipsychotic medication (Elvevag and Goldberg, 2000; Liddle, 2000; Kurtz et al., 2005; Lewis and Moghaddam, 2006). Further, schizophrenics have persistent cognitive impairments below psychiatrically healthy groups (Heinrichs and Zakzanis, 1998). The typical diagnosis occurs in late teens to early twenties with the onset of positive symptoms, however, negative symptoms such as social withdrawal and cognitive deficits typically occur throughout neurodevelopment (Andreasen, 1995; Chua and Murray,

1996). The estimated lifetime cost of each schizophrenic in the United States can exceed over US$2 million dollars (Blomqvist et al., 2006) and therefore discovering treatments that target cognitive deficits will not only improve quality of life for the patient, but also alleviate some financial burden on society.

The etiology of schizophrenia remains largely unknown. The genetic contribution is supported by twin studies where a monozygotic twin has a greater chance of developing schizophrenia than a dizygotic twin if the other is affected (Sullivan et al., 2003; Gottesman and Wolfgram, 1991; Kendler, 2001). However, 40% of schizophrenics have no familial history of schizophrenia (Gottesman and Erlenmeyer-Kimling, 2001). Further, there is no single causative gene directly linked to the illness and likely results from multiple risk genes acting additively to predispose an individual to schizophrenia (Weinberger et al., 2001; Lewis and Levitt, 2002; Harrison and Weinberger, 2005). Current research suggests that there are multiple contributing factors including genetic predisposition and environmental stressors leading to abnormal neurodevelopment (Weinberger, 1987; Chua and Murray, 1996; Lewis and Levitt, 2002; Lang et al., 2007; Brown, 2011). Pre and perinatal stressors that have been linked to increased risk of developing schizophrenia include maternal malnutrition, infections, stress and obstetrical complications (Lewis and Levitt, 2002; Lante et al., 2007; Malaspina et al., 2008; Brown, 2011; Brown and Patterson, 2011). Likely, schizophrenia results from a combination of genetics and environmental stressors leading to abnormal neurodevelopment and the wide range of symptoms associated with this disorder.

STRUCTURAL ABNORMALITIES:

Dorsolateral prefrontal cortex: Abnormal dorsolateral prefrontal cortical activity has been found in schizophrenics in the absence of neurodegeneration or lesions (Bunney and Bunney, 2000; Callicott et al., 2003; Eisenberg and Berman, 2010). The rodent analog to human dorsolateral prefrontal cortex is the medial prefrontal cortex (Uylings et al., 2003). Pyramidal neurons comprise about 75% of total neurons in this region, are the main source of glutamate and the main targets of the majority of glutamate containing axons; the remaining 25% are interneurons (Lewis, 2004). Schizophrenics have decreased dorsolateral prefrontal cortex volume (Andreasen et al., 1994; Nopoulos et al., 1995; Cannon et al., 2002; Giuliani et al., 2005; Fornito et al., 2009) in the absence of a change in neuronal number (Akbarian et al., 1995b; Thune et al., 2001). However, there is evidence of increased pyramidal neuron density (Selemon et al., 1995, 1998) and decreased pyramidal neuron soma size with the greatest reductions found in deep layer 3 (Rajkowska et al., 1998; Glantz and Lewis, 1997; Pierri et al., 2001; Sweet et al., 2003; Sweet et al., 2004). Reduced soma size correlates to measures of the dendritic tree (Hayes and Lewis, 1996; Jacobs et al., 1997) and axonal arbor (Gilbert and Kelly, 1975; Lund et al., 1975) indicating reductions in those structures as well. In support, decreased synapsin, a marker for axon terminals (Glantz and Lewis, 1997) has been found in schizophrenics suggesting a decrease in the number of terminals. Further, a decrease in neuropil (axon terminals, dendritic spines, glial processes that occupy space between neurons) has also been suggested (Selemon and Goldman-Rakic, 1999). All of these

findings suggest abnormal connectivity within the dorsolateral prefrontal cortex of schizophrenics.

Abnormal dorsolateral prefrontal cortex activity has been shown using functional magnetic resonance imaging (fMRI) which measures changes in blood flow although there is some controversy. Studies have found both hyper and hypofrontality in schizophrenics (for review see Eisenberg and Berman, 2010); increased activity is thought to result from inefficient prefrontal processing (Callicott et al., 2003; Manoach et al., 1999; Potkin et al., 2009). To explain these controversial results, authors suggest an inverted U-shaped load-response curve where as task demand increases, there is elevated prefrontal cortical activity which peaks out at physiological capacity, after which, activity falls (Fletcher et al., 1998). Further, the curve appears to be shifted to the left in schizophrenics (Perlstein et al., 2003; Jansma et al., 2004) so lower tasks will result in hyperactivation (inefficient signaling with similar performance to controls) and hypoactivation occurs during more difficult tasks (when schizophrenic's performance is likely to be worse than controls) compared to controls (Callicott et al., 2003; Manoach, 2003).

Hyper and hypofrontality is less relevant when considering the contribution of synchronized pyramidal cell activity creating oscillations in the prefrontal cortex and hippocampus (Buzsaki and Draguhn, 2004) which have been shown to be necessary for cognitive processing (Howard et al., 2003; Haenschel et al., 2009; Lewis et al., 2012). Different frequencies of oscillation magnitude have been identified including slow oscillations in delta (0.5-3 Hz) theta (3-8 Hz) and beta (8-30 Hz) ranges to fast oscillations in gamma (30-90 Hz) and ultrafast (90-200 Hz) ranges (Buzsaki and

Draguhn, 2004; Gonzalez-Burgos and Lewis, 2008). Greater oscillation magnitude occurs with a greater amount of regular and synchronized pyramidal neuron activity. One neuron regularly firing generates rhythmic postsynaptic membrane potential in all target cells. Greater neuronal synchrony amplifies the postsynaptic membrane potential generating synchronization in a greater population of target cells leading to network oscillations (Gonzalez-Burgos and Lewis, 2008). Fast oscillation gamma bands have been linked to cognitive processing (Lisman and Idiart, 1995; Lisman, 1999; Howard et al., 2003; Tallon-Baudry et al., 2004; Sejnowski and Paulsen, 2006). Parvalbumin (PV) containing, basket cell GABAergic interneuron activity is necessary and sufficient for high frequency gamma oscillations based on axonal positioning onto pyramidal neuronal soma, innervation of numerous pyramidal neurons and the high degree of interneuronal connectivity (Buzsaki and Draguhn, 2004; Gonzalez-Burgos and Lewis, 2008). Schizophrenics have irregular prefrontal gamma range oscillations which may contribute to symptoms of the illness (Spencer et al., 2003; Cho et al., 2006; Uhlhaas et al., 2006; Haenschel et al., 2009; Carlino et al., 2012). Taken together, evidence supports abnormal dorsolateral prefrontal cortical function underlying symptoms of schizophrenia.

Additional structural abnormalities: Additional structures have been implicated in schizophrenia including the hippocampus, mediodorsal thalamus, striatum and anterior cingulate cortex. Specifically in the hippocampus reports of reduced hippocampal volume (Nelson et al., 1998; Wright et al., 2000; Honea et al., 2005; Weiss et al., 2005) and pyramidal cell size (Benes et al., 1991; Arnold et al., 1995; Zaidel et al., 1997) but see (Highley et al., 2003) who reported no changes. Additionally, reduced dendritic spine density (Rosoklija et al., 2000), synaptic protein levels (Browning et al., 1993;

Young et al., 1998; Sawada et al., 2005) and alterations in ionotropic glutamatergic receptors (Harrison et al., 1991; Gao et al., 2000) are also observed. Further, it has been suggested that early abnormal hippocampal activity and connectivity to the dorsolateral prefrontal cortex results in blunted maturation of the dorsolateral prefrontal cortex resulting in aberrant connectivity back to the hippocampus (Lipska et al., 2002). Also, aberrant hippocampal-prefrontal interactions have been observed following executive tasks specifically working memory (Meyer-Lindenberg et al., 2005). Further investigations of cortical-subcortical interactions and the precise involvement of each structure are necessary to understand abnormal activity and the contributions of each structure to the pathophysiology of schizophrenia.

PATHOPHYSIOLOGY OF SCHIZOPHRENIA: NEUROTRANSMITTERS

Along with structural abnormalities observed in schizophrenia, many neurotransmitter systems have also been implicated in the pathophysiology of the disorder. The dopamine hypothesis was the first to emerge since dopamine agonists including amphetamine induce a schizophrenia-like psychosis and the first antipsychotics acted as antagonists at dopamine type 2 receptors; however, these medications treat positive systems with few having any effect on negative or cognitive deficits (Coyle, 2006; Carlsson, 2006). More recently abnormal glutamate and GABA neurotransmission have emerged as popular theories underlying schizophrenia that have gained support and will be discussed in greater depth. This section will give a brief overview of abnormal activity of each of these neurotransmitter systems that is observed in schizophrenia. Of

note are the contributions of other neurotransmitters implicated in schizophrenia including serotonin and acetylcholine (Meltzer et al., 2003; Tandon, 1999) which will not be discussed.

PATHOPHYSIOLOGY OF SCHIZOPHRENIA: GLUTAMATE

Glutamate is the main excitatory neurotransmitter in the body and glutamate receptors mediate half of synaptic transmission throughout the central nervous system (Hollmann and Heinemann, 1994). *N*-methyl-D-aspartate (NMDA) receptor hypofunction has gained attention when receptor antagonists were shown to induce positive, negative and cognitive deficits in healthy humans and rats and exacerbates symptoms in schizophrenics (Luby et al., 1959; Pearlson, 1981; Javitt and Zukin, 1991; Krystal et al., 1994; Verma and Moghaddam, 1996; Malhotra et al., 1997b; Adler et al., 1999; Newcomer et al., 1999; Coyle, 2004). Phencyclidine and ketamine are non-competitive, open channel blockers of the NMDA receptor inhibiting influx of Ca^{2+}; these drugs have also been shown to block type 2 dopamine receptors. However, it is thought that the psychotomimetic effects are due to inhibition of the NMDA receptor (Javitt and Zukin, 1991; Vollenweider and Geyer, 2001). Further support for NMDA receptor hypofunction in schizophrenia are elevated levels of kynurenic acid and N-acetyl aspartyl glutamate (NAAG) which are endogenous NMDA receptor antagonists although NAAG is also an agonist at the metabotropic glutamate 3 receptor (Schwarcz et al., 2001; Tsai et al., 1995; Berger et al., 1999; Hakak et al., 2001). Additionally, the NMDA receptor co-agonists D-serine and glycine, necessary for receptor activity, are decreased

in schizophrenics (Sumiyoshi et al., 2004; Neeman et al., 2005; Hashimoto et al., 2003a). Lastly, there are reports of altered NMDA receptor expression. However, these findings are not present in all schizophrenics and not always replicated across studies; based on this controversy, NMDA receptor expression will not be discussed in detail (Akbarian et al., 1996; Mirnics et al., 2000; Dracheva et al., 2001; Kristiansen et al., 2006; Kristiansen et al., 2007). It is likely that NMDA receptor hypofunction in schizophrenics results from a combination of altered levels of co-agonists or antagonists, altered redox state (discussed below in the glutathione section), altered expression and abnormal trafficking of the NMDA receptor. However, further investigations are necessary to establish the nature of NMDA receptor hypofunction.

It is hypothesized that phencyclidine and ketamine target NMDA receptors located on GABAergic interneurons leading to pyramidal cell disinhibition (Jones and Buhl, 1993; Lei and McBain, 2002; Maccaferri and Dingledine, 2002; Homayoun and Moghaddam, 2007; Lewis et al., 2012). This leads to subsequent disinhibition of pyramidal cell firing elevating synaptic neurotransmitter release shown in rodents by electrophysiological methods (Jackson et al., 2004), metabolic imaging methods (Vaisanen et al., 2004; Sharp et al., 2001) and increased glutamate concentrations (Moghaddam et al., 1997; Homayoun et al., 2005). Elevated glutamate levels will over stimulate non-NMDA glutamate receptors including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors shown by AMPA receptor antagonists normalizing dopamine levels and behavioral measures induced by NMDA receptor antagonists (Moghaddam et al., 1997). Thus NMDA receptor antagonists result in

abnormal firing in the prefrontal cortex and cognitive deficits associated with schizophrenia.

Metabotropic glutamate receptors (mGluR) have also been implicated in schizophrenia. Of the eight known receptor types divided into three groups based on G protein coupling specificity and sequence homology, group I mGluRs, particularly mGluR5, and group II mGluRs constituting mGluR2 and mGlur3, have been identified as potential targets for the treatment of schizophrenia. mGlurR5 is coupled to $G_{q/11}$ to increase intracellular Ca^{2+} (for review see De Blasi et al., 2001; Ribeiro et al., 2010) and physically interacts with and potentiates the NMDA receptor through scaffolding proteins to enhance synaptic transmission (Attucci et al., 2001; Ehlers, 1999; Doherty et al., 2000; Mannaioni et al., 2001; Marino and Conn, 2002). Metabotropic group II glutamate receptors (mGluR2/3) are located on both presynaptic and postsynaptic neurons in the synaptic and extrasynaptic space; mGluR3 is also expressed on astrocytes (Phillips et al., 2000; Tamaru et al., 2001). Further, mGlur2/3 couples to $G_{i/o}$ leading to reduced intracellular Ca^{2+} and inhibition of Ca^{2+} mediated synaptic neurotransmitter release (Flor et al., 1995; Conn and Pin, 1997). Interestingly, preclinical studies provide evidence of mGluR2/3 agonist normalizing NMDA antagonist-induced increase in glutamate (Moghaddam and Adams, 1998; Lorrain et al., 2003) and reducing many behavioral abnormalities induced by NMDA receptor antagonism (Moghaddam and Adams, 1998; Cartmell et al., 1999; Krystal et al., 2003; Baker et al., 2008). Further, clinical trials have yielded positive results with mGluR2/3 agonists in schizophrenia treatment (Patil et al., 2007; Mosolov et al., 2010). However, one study did not find a significant benefit with mGluR2/3 agonist but they also failed to see improvements with antipsychotic

medication (Kinon et al., 2011) suggesting it was a failed trial. Thus stimulating metabotropic glutamate receptors may represent a novel target in the development of schizophrenia treatments.

When studying glutamatergic activity it is important to not only consider glutamate receptors but also the location of receptors and glutamate itself. Vesicular glutamate is released into the synapse, activates postsynaptic receptors and is quickly and efficiently cleared by astrocytes through excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) (Rothstein et al., 1994; Rothstein et al., 1996). The non-synaptic pool of glutamate is in part supplied by the non-vesicular cystine-glutamate antiporter or system x_c^- (Baker et al., 2002b; Massie et al., 2011; De Bundel et al., 2011). System x_c^- exchanges one intracellular glutamate for an extracellular cystine (Baker et al., 2002b). This has been shown by blocking system x_c^- or genetic knockouts of active subunit of system x_c^- having significantly reduced extrasynaptic glutamate in multiple brain regions (Baker et al., 2002b; Moran et al., 2005; De Bundel et al., 2011; Massie et al., 2011). Extrasynaptic glutamate can activate extrasynaptic glutamate receptors including mGluR2/3 and NMDA receptors. Further, xCT expression is altered in the dorsolateral prefrontal cortex of schizophrenics (Baker et al., 2008). Taken together, glutamate neurotransmission is a complex regulated network which has the ability to produce behavioral deficits associated with schizophrenia and other neuropsychiatric disorders when glutamate transmission is disturbed or altered.

PATHOPHYSIOLOGY OF SCHIZOPHRENIA: GABA

Numerous studies implicate abnormal GABAergic interneuron activity in the dorsolateral prefrontal cortex in schizophrenia. First, two isoforms of glutamic acid decarboxylase of 65 and 67 kDa (GAD_{65} and GAD_{67}) synthesize GABA; GAD_{67} synthesizes the majority of GABA (Asada et al., 1997; Waagepetersen et al., 1999). GAD_{67} expression is activity dependent (Jones, 1990; Akbarian and Huang, 2006); decreased cortical activity reduces GAD_{67} levels (Benson et al., 1994; Jiao et al., 2006) whereas increased cortical activity increases GAD_{67} expression (Liang and Jones, 1997; Esclapez and Houser, 1999). In schizophrenics, GAD_{67} is decreased in the PV expressing subset of GABAergic interneurons (Akbarian et al., 1995b; Volk et al., 2000; Hashimoto et al., 2003b). Vesicular GABA release activates postsynaptic receptors ($GABA_A$ and $GABA_B$) typically generating inhibitory postsynaptic potentials (IPSPs) on postsynaptic targets (Gonzalez-Burgos and Lewis, 2008). GABA diffuses out of the synaptic cleft and is taken up by GABA transporters (GAT) (for review see Misgeld et al., 1995; Bak et al., 2006). Of the number of different subclasses of GABAergic interneurons, fast-spiking PV expressing neurons are altered in schizophrenics (for review see Lewis et al., 2012). First, PV containing GABAergic interneurons have either basket or chandelier cell morphology (for review see DeFelipe, 1997). Basket cell PV containing interneurons synapse on pyramidal cell bodies and proximal dendrites thereby having a large impact on pyramidal neuronal activity (Jones and Buhl, 1993; Bartos et al., 2007). Further, one interneuron synapses on each pyramidal neuron multiple times and each interneuron can contact multiple pyramidal cells and other similar interneurons (Gonzalez-Burgos and

Lewis, 2008). GABAergic interneurons also contain gap junctions allowing for fast communication between cells and for synchronization between interneurons (Tamas et al., 2000). This enables interneurons to generate oscillations by synchronizing a network of pyramidal neurons. PV is a calcium-binding protein that buffers Ca^{2+} in the presynaptic terminal impacting Ca^{2+} regulated neuronal processes such as excitation and synaptic activity (Chard et al., 1993; Pauls et al., 1996; Savic et al., 2001). Schizophrenics have decreased PV mRNA in the dorsolateral prefrontal cortex (Hashimoto et al., 2003b; Mellios et al., 2009; Fung et al., 2010) although the number of PV neurons appears to be unchanged (Woo et al., 1997).

Experiments have demonstrated the capacity of PV neurons to inhibit pyramidal neuron activity. PV GABAergic interneurons have also been shown necessary for network oscillations (Cobb et al., 1995; Bartos et al., 2007; Gulyas et al., 2010; Lewis et al., 2012). PV neurons receive excitatory input from surrounding pyramidal neurons and interneurons as well as projecting neurons (Behrens et al., 2007). Excitatory synapses contain both NMDA and AMPA receptors. Studies have shown that NMDA receptor antagonists binding within the pore preferentially block receptors on GABA interneurons due to the increased activation and firing rate of these cells in comparison to surrounding neurons. This results in disinhibition of pyramidal cells, increased pyramidal neuron activity and elevated glutamate release (Jones and Buhl, 1993; Moghaddam and Adams, 1998; Lorrain et al., 2003; Homayoun and Moghaddam, 2007; Amitai et al., 2011). Loss of inhibition also leads to network desynchronization and reduced gamma oscillations contributing to behavioral deficits (Sohal et al., 2009; Cobb et al., 1995; Cardin et al., 2009). Interestingly, chronic NMDA receptor antagonism results in a loss of PV and

GAD_{67} expression (Braun et al., 2007; Amitai et al., 2011) lending further support for the link between NMDA receptor antagonists and GABAergic interneurons.

Schizophrenic GABA system alterations appear to result from abnormal activity and not from a change in number (Benes et al., 1996; Woo et al., 1997). Further, changes in PV basket cells are thought to contribute to abnormal dorsolateral prefrontal activity (for review see Lewis et al., 2012). As stated above, GAD_{67} and PV mRNA is reduced in PV cells (Akbarian et al., 1995b; Volk et al., 2000; Hashimoto et al., 2003b; Curley et al., 2011; Mellios et al., 2009; Fung et al., 2010) with no change in GAT1 expression in the dorsolateral prefrontal cortex of schizophrenics (Woo et al., 1998). Also, mRNA for $GABA_A$ α1 receptor, thought to mediate basket cell inputs onto pyramidal soma, are significantly reduced in prefrontal cortex (Akbarian et al., 1995a; Hashimoto et al., 2008a; Hashimoto et al., 2008b; Beneyto et al., 2011) although no changes in receptor expression has also been observed (Duncan et al., 2010). Decreased $GABA_A$ α1 on pyramidal cells could indicate lower basket cell synaptic inputs since each PV neuron typically innervates one postsynaptic targe↓t many times which is also supported by

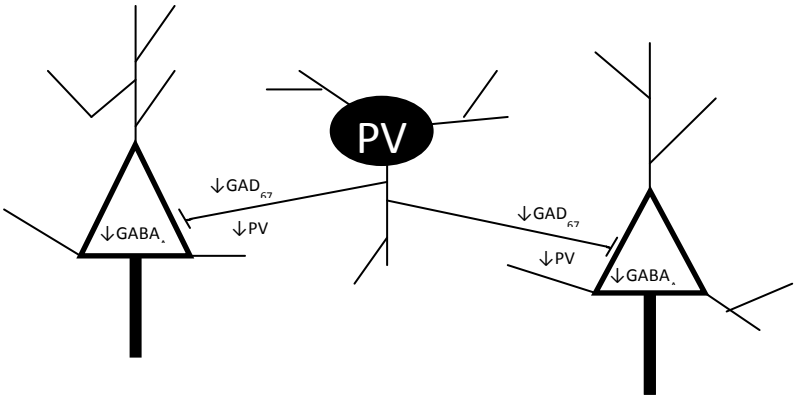


Figure 1.1 Schematic summary of alterations in GABAergic neural circuitry in layer 2-3 of the dorsolateral prefrontal cortex of schizophrenics. Pyramidal neurons (PN) have reduced expression of $GABA_A$ α1 on the cell body. PV positive basket cells (PVBC) have less GAD_{67} and PV.

decreased PV presynaptic expression.

Figure 1.1 depicts altered GABAergic dysfunction in schizophrenics. As shown, disinhibition of PV basket cells is suggested by reduced PV and GAD_{67} in interneurons with lower pyramidal $GABA_A$ α1 expression. Further, evidence supports weaker inhibitory basket cell inputs onto pyramidal neurons contributing to loss of hyperpolarization which is necessary for pyramidal cell synchronization and gamma oscillations (Bartos et al., 2007; Lewis et al., 2012). Taken together, there is strong support for abnormal GABAergic interneuron activity contributing to the loss of gamma oscillations necessary for cognition in schizophrenics.

ASTROCYTES: CONTRIBUTION TO NETWORK SYNCHRONIZATION

A less well studied regulator of network synchronization is the contribution of astrocytes. Astrocytes have minimal overlapping areas (Ogata and Kosaka, 2002; Bushong et al., 2002) and one astrocyte contacts approximately 100,000 synapses in rodents and up to one million synapses in humans (Bushong et al., 2003; Oberheim et al., 2006). As shown in Figure 1.2, astrocytes communicate with pyramidal-pyramidal synapses, GABAergic-pyramidal synapses, neuron cell bodies and dendritic processes impacting network activity within the entire domain of the astrocyte. Further, astrocytes communicate with neurons by releasing factors which regulate ionotropic receptor expression, synaptic activity and neuronal activation. Brain-derived neurotrophic factor (BDNF) is released by astrocytes and regulates $GABA_A$ receptor expression and the frequency of IPSCs (Elmariah et al., 2005; Barker and Ullian, 2010). Astrocytes also

release tumor necrosis factor-alpha (TNF-α) to increase AMPAR trafficking to synapses (Beattie et al., 2002; Barker and Ullian, 2010). Additionally, astrocytes have the capacity to control the number and strength of synapses through the release of glutamate, D-serine which is a co-agonist of the NMDA receptor and ATP which can depress synaptic activity (for review see Kondziella et al., 2006; Barker and Ullian, 2010; Reichenbach et al., 2010). Of particular importance to this work is the expression of system x_c^- which is capable of regulating glutathione concentrations and extrasynaptic mGluR2/3 activity and will be discussed in the following section. Based on these findings, astrocytes are uniquely positioned and contain the necessary transporters to have a profound influence on network activity and contribute to network synchronization leading to cognitive processes associated with schizophrenia.

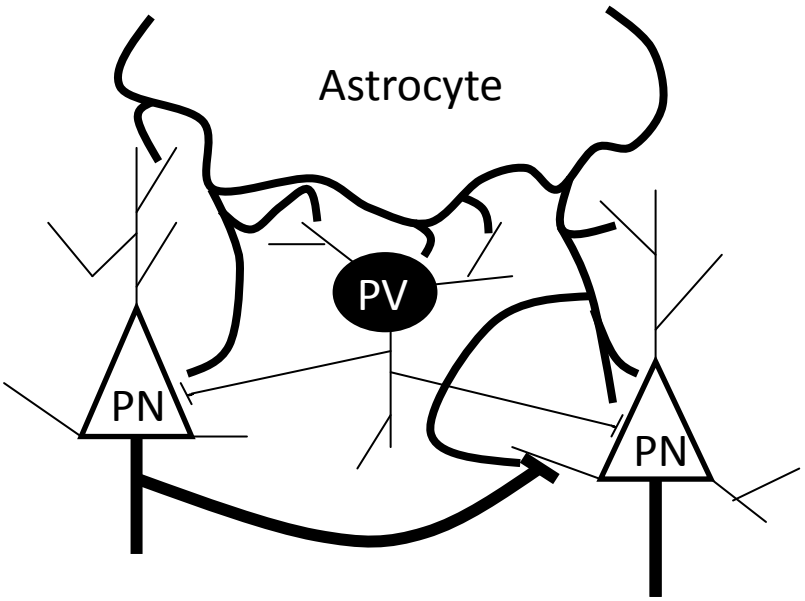


Figure 1.2 Astrocytes are capable of communicating with neurons including pyramidal neurons (PN) and GABAergic PV containing basket interneurons (PV) near and away from the synapse thereby impacting the excitiatory environment

CYSTINE- GLUTAMATE EXCHANGE BY SYSTEM X_C^-

System x_c^- or the cystine-glutamate antiporter is a critical transporter within the central nervous system capable of regulating both oxidative stress and neurotransmission (Baker et al., 2002b; Shih et al., 2006). It is a Na^+-independent Cl^--dependent transporter capable of exchanging intracellular glutamate for extracellular cystine in a 1:1 ratio (Baker et al., 2002b). As shown in Figure 1.3, evidence indicates system x_c^- is located on astrocytes (Allen et al., 2001; Pow, 2001) although there is also evidence of immature neuronal expression (Murphy et al., 1989; Murphy et al., 1990). Extracellular cysteine is predominantly expressed in the oxidized form of cystine which is transported by system x_c^- (Bannai and Tateishi, 1986). Once taken up, intracellular cystine is rapidly reduced into cysteine for glutathione synthesis which is then utilized by the astrocyte or transported back into the extrasynaptic space to serve as substrates for neuronal uptake for glutathione synthesis (Meister, 1988; Sies, 1999; Dringen and Hirrlinger, 2003). Thus, astrocytic cysteine is essential for both astrocytic and neuronal glutathione levels.

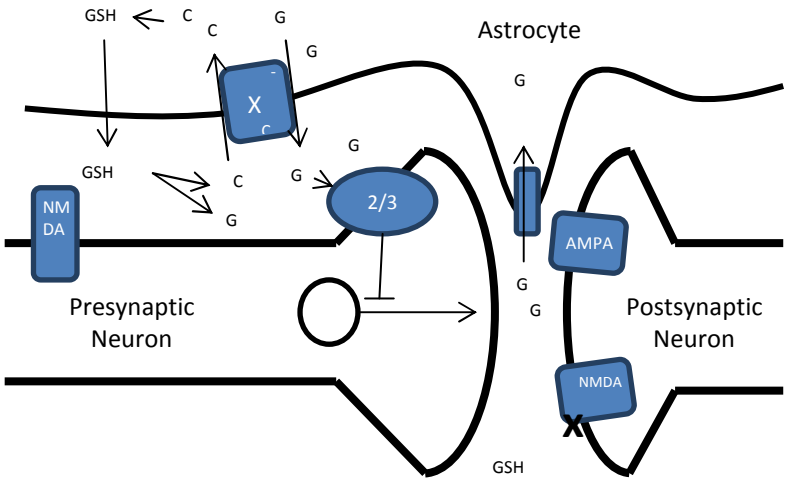


Figure 1.3 System x_c^- exchanges one intracellular glutamate (G) for an extracellular cystine (C). Glutamate can activate mGluR2/3 (2/3) receptors, cystine uptake is the rate limiting step in glutathione (GSH) synthesis.

Externalized glutamate by system x_c^- is critical for nonvesicular glutamate concentrations in the nucleus accumbens and capable of stimulating group II metabotropic glutamate receptors (Baker et al., 2002a; Mohan et al., 2011). Group II mGluRs are G-protein coupled heteroreceptors which inhibits intracellular Ca^{2+} accumulations necessary for vesicular neurotransmitter release such as glutamate and dopamine thereby regulating neurotransmission (Conn and Pin, 1997; Flor et al., 1995). Interestingly, these receptors serve as a potential therapeutic target in schizophrenia treatments highlighting abnormal functioning of this circuit in schizophrenics (Patil et al., 2007; Mosolov et al., 2010; although see Kinon et al., 2011). In addition, enhanced efflux of glutamate via system x_c^- may induce excitotoxicity through increased extrasynaptic NMDA receptor activation cascades (for review see Bridges, 2011).

Due to the unique regulation of both cystine and glutamate cycling, system x_c^- has already been linked to many central nervous system processes including oxidative stress and protection (Shih et al., 2006), regulating synaptic transmission (Baker et al., 2002b), blood brain barrier regulation (Hosoya et al., 2002), synaptic organization (Augustin et al., 2007), viral pathology (Espey et al., 1998), drug addiction (Kalivas, 2009), and brain tumor growth (Chung et al., 2005; Lyons et al., 2007; Chen et al., 2009). Given this, it is imperative to understand the structure and regulation of the cystine-glutamate antiporter. System x_c^- is a membrane bound protein of the heteromeric amino acid transporter (HAT) family (Broer and Wagner, 2002; Verrey et al., 2004). It is composed of a hydrophobic non-glycosylated light chain (xCT) linked to a type II N-glycosylated heavy chain (4F2hc) via a disulfide bond. While 4F2hc is required for trafficking and cell surface expression, xCT is the functioning unit that exchanges glutamate for cystine (Gasol et al.,

2004; Jimenez-Vidal et al., 2004; Bassi et al., 2001). Whether full length xCT or splice variants are the functional membrane-bound proteins *in vivo* remains unclear and requires further exploration as does the regulation of cystine-glutamate exchange. Evidence suggests that oxidative stress is capable of up regulating system x_c^- transcription through Nuclear factor erythroid-2-related factor (Nrf2) binding to the Antioxidant Response Element (ARE) promoter on xCT gene (Bannai, 1984; Ishii et al., 2000) as does depleted glutathione concentrations (Seib et al., 2011). There is a high probability of additional factors within the intra and extracellular milieu having an as yet unidentified contribution to system x_c^- regulation which will require future studies.

While cystine-glutamate exchange has not been extensively studied in schizophrenia, there is increasing evidence of altered antiporter activity. First, there is evidence of increased expression of xCT, the active subunit of the cystine-glutamate antiporter, in the dorsolateral prefrontal cortex of schizophrenic patients that is not present in other brain regions (Baker et al., 2008). Increased expression may result from an inefficient glutathione supply since decreased glutathione has been shown to elevate system x_c^- expression (Seib et al., 2011). In support, studies have shown an overall reduction in glutathione levels in the central nervous system with a 50% reduction in the dorsolateral prefrontal cortex of schizophrenics (Do et al., 2000; Gawryluk et al., 2010; Raffa et al., 2011). Whether altered glutathione or system x_c^- expression occurs first remains unclear. Lastly, the cysteine prodrug, N-acetylcysteine, has shown clinical efficacy in treating symptoms of schizophrenia that currently do not have effective treatments (Berk et al., 2008c; Lavoie et al., 2008; Carmeli et al., 2012). Accordingly, these data have the potential to identify the cystine-glutamate antiporter as both a novel

mechanism contributing to the pathophysiology of schizophrenia and as a potential therapeutic target.

Interestingly, xCT knockout mice have been generated and they have a normal appearance, normal lifespan and no changes in cortical or hippocampal width (De Bundel et al., 2011; Sato et al., 2005). Not surprisingly, decreased extracellular glutamate was observed through microdialysis studies in the hippocampus and striatum suggesting that system x_c^- is one of the main contributors to this pool and cannot be replaced (De Bundel et al., 2011; Massie et al., 2011). Surprisingly, there were no changes in tissue glutathione concentrations in these regions or other non-CNS organs including the liver and pancreas (Sato et al., 2005; Massie et al., 2011; De Bundel et al., 2011) likely due to a compensatory increase in other cysteine transporters although this has yet to be explored in these animals. Behavioral measures included locomotion in open field, spatial reference memory in Morris water maze and delayed alternation in Y-maze task measuring spatial working memory. No changes in spontaneous locomotor activity to a novel environment corresponds with the methylazoxymethanol acetate (MAM) neurodevelopmental model which requires a stimulant such as amphetamine or phencyclidine to reveal deficits from control animals (Flagstad et al., 2004; Moore et al., 2006; Lodge and Grace, 2007). Intact spatial reference memory also corresponds to the neurodevelopmental MAM model where deficits emerge after reversal learning challenge in the Morris water maze (Flagstad et al., 2005). Interestingly, xCT knockouts have deficits in spatial working memory and make significantly less correct alternations compared to control (De Bundel et al., 2011). Since this occurs in the absence of a glutathione deficit, it suggests that spatial working memory tasks require extracellular

glutamate which can activate extrasynaptic mGluR2/3 receptors. This is a very interesting mutant model and will require a great amount of further research to understand the widespread impact of system x_c^- on homeostatic mechanisms and behavior.

GLUTATHIONE AND SCHIZOPHRENIA

Glutathione is a critical antioxidant necessary for the scavenging of free radicals, protecting cells from oxidative stress and regulating redox potentials (Cooper and Kristal, 1997; Sies, 1999; Soltaninassab et al., 2000; Dringen, 2000). Total glutathione is decreased by up to 50% in the dorsolateral prefrontal cortex and by 27% in the cerebral spinal fluid of schizophrenic patients compared to matched controls (Do et al., 2000; Yao et al., 2006; Gawryluk et al., 2010; Raffa et al., 2011). Secondary to reduced glutathione concentrations is an increase in reactive oxygen species and impaired antioxidant defense system in schizophrenics (Olney et al., 1999; Yao et al., 2006; Bitanihirwe and Woo, 2011; Yao and Reddy, 2011). Further, glutathione reduces the extracellular redox site on the NMDA receptor causing potentiation of NMDA receptor currents (Kohr et al., 1994; Lipton et al., 2002). Therefore, reduced glutathione levels leads to NMDA receptor hypoactivity (Steullet et al., 2006); NMDA receptor hypofunction is hypothesized to contribute to the pathophysiology of schizophrenia (Olney et al., 1999; Coyle, 2006). While these reports implicate glutathione, it is necessary to also understand glutathione cycling and how it is altered in schizophrenia.

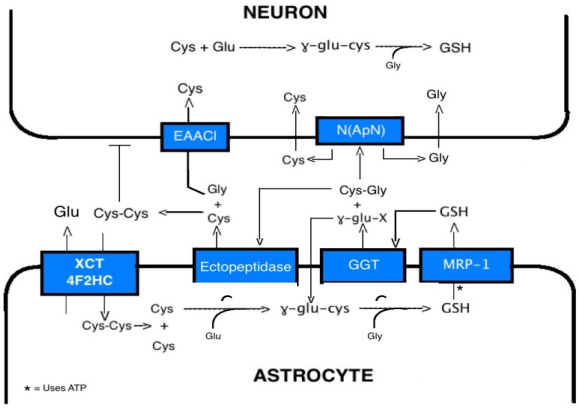


Figure 1.4 Glutathione cycling. Cystine uptake by system x_c^- is the rate limiting step in glutathione synthesis. Glutamate cysteine ligase (GCL) is the rate limiting enzyme in glutathione synthesis. Glutathione synthetase (GSS), multidrug resistance protein-1 (MRP-1) exports glutathione out of the astrocyte for degradation by ɣ-glutamyl transpeptidase (GGT) and can undergo further degradation to cystine which will drive cystine-glutamate exchange.

Glutathione cycling and concentrations are tightly regulated by a number of proteins and checkpoints, some of which have been implicated in schizophrenia (Dringen and Hirrlinger, 2003). First, the rate limiting step of glutathione synthesis is cystine in astrocytes and cysteine uptake in neurons (Sagara et al., 1993; Kranich et al., 1998; Dringen and Hirrlinger, 2003). In astrocytes, system x_c^- is a key transporter for cystine (Bannai, 1984; Bridges, 2011) and studies have shown altered protein expression of xCT, the active subunit of the cystine-glutamate antiporter in schizophrenics (Baker et al., 2008). As indicated in Figure 1.4, intracellular cystine is rapidly reduced to cysteine and synthesized into glutathione by the addition of glutamate and glycine in two enzymatic processes involving glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS) (Meister, 1988; Sies, 1999). GCL is composed of a catalytic subunit (GCLC) necessary for glutathione synthesis and a modifier subunit (GCLM) to increase efficiency (Yang et al., 2007). In schizophrenics, research has shown a decrease in GCLM gene expression, reduced GCLC protein expression and GCL activity in fibroblasts (Tosic et al., 2006; Gysin et al., 2007) although GCLM genetic linkage remains controversial (Butticaz et al., 2009). Further, GSS mRNA levels are also reduced (Tosic et al., 2006). After

completion of glutathione synthesis, it is utilized intracellularly or exported into the extracellular space by multidrug resistance protein 1 (Mrp1). Glutathione undergoes extracellular catabolism by the membrane ectoenzyme γ-glutamyl transpeptidase (GGT) into CysGly and γ-glutamylX. "X" represents an acceptor of the γ-glutamyl moiety. CysGly is hydrolyzed into cysteine and glycine by aminopeptidase N (N(ApN)) and transported into neurons for neuronal glutathione synthesis (Dringen and Hirrlinger, 2003). Additionally, extracellular cysteine is readily oxidized to cystine thus driving cystine-glutamate exchange and glutathione cycling (Lu, 1999; Dringen, 2000). Taken together, whether altered cystine-glutamate exchange limits glutathione synthesis or altered glutathione synthesis reduces cystine-glutamate exchange, the end result is reduced glutathione and altered cell homeostasis potentially contributing to symptoms of schizophrenia.

A promising treatment for schizophrenia is restoration of glutathione levels. Clinical trials have administered the cysteine precursor N-acetylcysteine to drive glutathione synthesis (Berk et al., 2008c; Lavoie et al., 2008; Carmeli et al., 2012). Results show improvements with N-acetylcysteine as adjunctive therapy. Whether N-acetylcysteine restores glutathione by driving system x_c^- in schizophrenics remains unclear and will be discussed later.

Given the reports of altered glutathione homeostasis in schizophrenia, studies have examined animal models of reduced glutathione concentrations and its impact to schizophrenic-like symptoms. One model is a genetic glutamate-cysteine ligase modifier (GCLM) knockout mouse. First, while GCLC knockout mouse is embryonic lethal, GCLM -/- reduces glutathione in all regions including the prefrontal cortex, striatum and

liver to around 30% of control levels throughout the lifespan (Steullet et al., 2010; Duarte et al., 2011). These animals have a subset of symptoms associated with schizophrenia including positive symptoms (increased locomotion to a novel environment and in response to amphetamine), negative symptoms (altered social behavior) and cognitive deficits (novel object recognition). Additionally, these animals show deficits in prepulse inhibition, increased oxidative stress and reduced parvalbumin expression, the marker for fast-spiking interneurons, in the ventral hippocampus. However it should be noted that these animals did not display spatial or short term working memory deficits (Morris water maze or Y-maze) or elevated baseline locomotor responses in home cage (Steullet et al., 2010; Cole et al., 2011; Kulak et al., 2012). Interestingly, while N-acetylcysteine has not been given as a behavioral treatment, it has been shown to normalize some neurochemical indices of knockout mice including glutamate, glutamine, alanine and myo-inositol (Duarte et al., 2011) suggesting the therapeutic beneficial effects of N-acetylcysteine may be independent of glutathione concentrations.

Glutathione is essential particularly in the central nervous system to maintain cellular redox systems and to protect cells from oxidative damage and cell death. Many labs have shown a significant reduction in dorsolateral prefrontal cortical glutathione concentrations in schizophrenia patients. This accounts for NMDA receptor hypoactivity and potentially reduced cystine-glutamate exchange causing dysregulation of GABAergic and glutamatergic signaling. This results in abnormal oscillations and desynchronization of cortical activity and symptoms of schizophrenia.

NEURODEVELOPMENT

Neurogenesis is a highly regulated process that relies on growth factors and neurotransmitters for normal development and migration. Neuroepithelial cells destined to become neocortical pyramidal neurons undergo mitosis in the germinal layers lining the ventricular system and migrate to their final destination (Sidman and Rakic, 1973; Hatten, 1993). The first cells to proliferate on rat embryonic day 14 are the Cajal-Retzius neurons; these cells become cortical layer I. Thereafter neurons are generated in an inside to out pattern; layers IV-II are born primarily on embryonic days 17-19 (for review see Bayer et al., 1993). Of importance is the contribution of glutamate and GABA signaling prior to synapse formation. Functional ionotropic glutamate receptors are expressed during terminal cell division and differentiation in neuroepithelial cells (Maric et al., 2000). Post-mitotic neurons undergo radial migration along radial glial to reach their final destination (Parnavelas, 2000; Sidman and Rakic, 1973) usually within 1-2 days following birth (Bayer et al., 1993). Interneurons originate in the medial and caudal ganglionic eminence in the ventral forebrain and must migrate tangentially to reach the cortex; cells undergo radial migration once reaching the cortex (for review see Marin and Rubenstein, 2003; Metin et al., 2006; Huang, 2009). Among many signaling pathways required for normal migration, interneurons rely on AMPA mediated receptor signaling for migration (Manent et al., 2006).

In contrast to interneurons, pyramidal cells require NMDA and $GABA_A$ receptor signaling to promote radial migration. In support, immature neurons express NMDA and GABA receptors and glutamate and GABA are detected early in the microenvironment of

migrating neurons (Nguyen et al., 2001; Lujan et al., 2005; Manent and Represa, 2007). Further, non-vesicular glutamate and GABA activate migratory neuronal receptors since synapses have yet to develop at this time (Behar et al., 1998; Behar et al., 1999; Behar et al., 2000; Behar et al., 2001; Hirai et al., 1999; Simonian and Herbison, 2001; Kihara et al., 2002). In support, animals with a genetic deletion of munc18-1 and munc13-1/2, proteins necessary for vesicle priming, are incapable of vesicular neurotransmitter release yet have normal cortical layering and synapse formation (Verhage et al., 2000; Varoqueaux et al., 2002). Finally, work in cerebellar granule cells suggests that $GABA_A$ receptor activity depolarizes immature neurons allowing NMDA receptor activation allowing for Ca^{2+} influx which is necessary for cell migration (Ben-Ari et al., 1997; Komuro and Rakic, 1993; Kumada and Komuro, 2004). While the non-vesicular source of glutamate has yet to be established, it is possible that system x_c^- could contribute to this pool and have a critical role in neurodevelopment.

Synaptogenesis: Synaptogenesis is another regulated process requiring numerous signaling cascades to develop first an immature then mature synapse. Non-vesicular glutamate release, specifically from genderblind encoded from 'genderblind' gene which encodes the xCT subunit in *Drosophila,* has been shown to modulate postsynaptic glutamate receptor clustering at the neuromuscular junction. Specifically, loss of cystine-glutamate exchange in genderblind knockouts have a 50% decrease in extracellular glutamate resulting in a large increase in postsynaptic glutamate receptors due to reduced constitutive desensitization (Featherstone et al., 2002; Augustin et al., 2007). Further, these mutants have behavior deficits including abnormal adult copulation (Grosjean et al., 2008). Given the role of cystine-glutamate exchange in *Drosophila* and the possible

contribution to neurodevelopment in rodents, abnormal cystine-glutamate exchange has the capacity of producing profound neurodevelopmental abnormalities that could lead to abnormal behavior.

ANIMAL MODELS OF SCHIZOPHRENIA

Phencyclidine: While there is evidence for both an environmental and genetic component for the etiology of schizophrenia, the contributions from each on the manifestation of the disorder remain largely unknown. Therefore, a main hurdle in studying the pathophysiology and therapeutic potential for schizophrenia research is developing an animal model. Non-competitive NMDA receptor antagonists, such as ketamine phencyclidine and MK801, have been shown to produce a schizophrenic-like state with positive, negative and cognitive deficits in healthy humans and rodents and exacerbate symptoms in schizophrenic patients (Luby et al., 1959; Pearlson, 1981; Javitt and Zukin, 1991; Krystal et al., 1994; Verma and Moghaddam, 1996; Malhotra et al., 1997b; Adler et al., 1999; Newcomer et al., 1999; Coyle, 2004). Phencyclidine acts as a non-competitive NMDA receptor antagonist with evidence suggesting it targets PV positive GABAergic interneuron NMDA receptors (Jones and Buhl, 1993; Lei and McBain, 2002; Maccaferri and Dingledine, 2002; Homayoun and Moghaddam, 2007; Lewis et al., 2012). Interestingly, repeated administration of PCP has been shown to decrease GABAergic markers, specifically parvalbumin and the GABA synthesizing agent GAD_{67} (Amitai et al., 2011). NMDA receptor antagonists have been shown to increase extracellular glutamate likely due to disinhibition of GABA interneurons in the mPFC which

interestingly, is blocked by an mGluR2/3 agonist (Moghaddam et al., 1997; Homayoun et al., 2005; Katayama et al., 2007; Baker et al., 2008; Amitai et al., 2011).

Methylazoxymethanol acetate (MAM): MAM is a DNA methylating agent which targets cells undergoing active mitosis producing an antimitotic effect (Nagata and Matsumoto, 1969; Cattabeni and Di Luca, 1997; Hoareau et al., 2006). However, there is also evidence that MAM effects proteins necessary for cell growth (Singh, 1980; Dambska et al., 1982) and impairs neuronal organization (Matricon et al., 2010). Based on a narrow time window for effect (12-24 hours) (Matsumoto et al., 1972), timing of administration is critical. Administration on gestational day 15 leads to whole brain size reductions presumably due to antimitotic effects of MAM (Dambska et al., 1982; Jongen-Relo et al., 2004) whereas gestation day 17 treatment selectively targets regions implicated in schizophrenia including the prefrontal cortex, hippocampus and thalamus since neurogenesis for these structures is occurring at this time point (Flagstad et al., 2004; Moore et al., 2006; Le Pen et al., 2006; Bayer et al., 1993). Further, gestational day 17 treatment parallels schizophrenia symptoms including deficits in sensorimotor gating, social interaction, hypersensitivity to amphetamines and NMDA receptor antagonists and cognitive impairments including executive functions (Flagstad et al., 2004; Flagstad et al., 2005; Moore et al., 2006; Le Pen et al., 2006; Featherstone et al., 2007; Featherstone et al., 2009; Gill et al., 2011). These deficits are thought to arise, in part, by abnormal prefrontal cortex and hippocampal activity (Lavin et al., 2005; Goto and Grace, 2006; Lena et al., 2007; Lodge and Grace, 2007; Lodge et al., 2009; Chin et al., 2011) including reduced PV expression (Penschuck et al., 2006; Lodge et al., 2009) and abnormal oscillatory activity (Lavin et al., 2005; Goto and Grace, 2006; Phillips et al., 2012).

Taken together, MAM models the neurodevelopmental hypothesis of schizophrenia and has many morphological, molecular and behavioral aspects that parallel those seen in schizophrenia making it a useful model to study.

Sulfasalazine: Abnormal system x_c^- activity is implicated in schizophrenia. First, externalized glutamate is capable of activating extrasynaptic mGluR2/3 receptors which modulates neurotransmission (Baker et al., 2002b; Mohan et al., 2011); schizophrenics have abnormal glutamatergic activity (for review see Goff and Coyle, 2001; Carlsson, 2006; Coyle, 2006; Lewis, 2009). Second, internalized cystine is necessary for glutathione synthesis (Bannai and Tateishi, 1986; Meister, 1995; Dringen and Hirrlinger, 2003); schizophrenics have up to a 50% reduction in dorsolateral prefrontal cortical glutathione concentrations (Do et al., 2000; Yao et al., 2006; Gawryluk et al., 2010; Raffa et al., 2011). Further, nonvesicular release of glutamate of unknown origins is necessary for normal neurodevelopment (Behar et al., 1999; Hirai et al., 1999; Simonian and Herbison, 2001; Kihara et al., 2002). Therefore, it is necessary to evaluate the contribution system x_c^- activity to schizophrenic-like symptoms by directly modulating the cystine-glutamate antiporter. The xCT genetic knockout mouse evaluated a few behavioral measures and found some cognitive deficits however these animals likely have compensatory mechanisms for absent cystine-glutamate exchange (De Bundel et al., 2011). Instead, a knockdown of xCT or acute inhibition of system x_c^- is needed to evaluate the contribution of altered cystine-glutamate exchange.

Sulfasalazine is marketed as an anti-inflammatory prodrug which is metabolized in the large intestines into sulfapyridine and 5-aminosalicylic acid; the anti-inflammatory effect is mediated by 5-aminosalicylic acid (Peppercorn, 1984). Importantly,

sulfasalazine and not the metabolites are capable of potently inhibiting system x_c^- (Gout et al., 2001; Chung and Sontheimer, 2009). Evidence suggests that sulfasalazine crosses the blood brain barrier since intraperitoneal administration limits primary brain tumor growth *in vivo* (Gout et al., 2001; Chung et al., 2005; Lyons et al., 2007). Further, it has been shown to penetrate the placental barrier (Peppercorn, 1984). Based on the ability of sulfasalazine to potently and acutely inhibit cystine-glutamate exchange it is an ideal drug to test the contribution of system x_c^- in behavioral measures of schizophrenia-like symptoms.

Since the etiology of schizophrenia remains unknown and there are many inconsistencies across populations there is no perfect schizophrenia model. Therefore, using a range of approaches with similarities in molecular and behavioral symptoms of schizophrenia is necessary to examine the contribution of system x_c^- to schizophrenia.

THERAPEUTIC IMPLICATIONS FOR SYSTEM X_C^-

Antipsychotic medications have been around since the 1950's and all possess some D_2 receptor antagonism effects (Hill et al., 2010). All have shown to be effective at reducing positive symptoms however most, with the exception of clozapine, show small if any improvements in negative or cognitive deficits (for review see Hill et al., 2010). Additionally all have serious side effects with low compliance rates (Lieberman et al., 2005). Unfortunately, this results in many unmediated schizophrenics or schizophrenics with only modest improvements in symptom severity. Current research seeks to find more efficacious and more tolerable treatments for schizophrenia.

N-acetylcysteine: N-acetylcysteine has shown efficacy in clinical trials of a number of disorders including gambling (Grant et al., 2007), nicotine addiction (Knackstedt et al., 2009), cocaine addiction (LaRowe et al., 2006; LaRowe et al., 2007; Mardikian et al., 2007), bipolar disorder (Berk et al., 2008b) and schizophrenia (Berk et al., 2008c; Lavoie et al., 2008; Carmeli et al., 2012). First, preclinical studies have shown that N-acetylcysteine stimulates system x_c^- (Baker et al., 2008; Kau et al., 2008). Clinical schizophrenia studies have shown improvements on Positive and Negative Symptoms Scale (PANSS) total, PANSS negative, PANSS general and Clinical Global Impression-Severity (CGI-S) scales with N-acetylcysteine (Berk et al., 2008c). Further, mismatch negativity, a measure of auditory sensory processing dependent on NMDA receptor activity was improved in schizophrenics receiving N-acetylcysteine compared to placebo (Lavoie et al., 2008). Lastly, N-acetylcysteine treatment increases synchronization measured by EEG over the left parieto-temporal, the right temporal, and bilateral prefrontal regions (Carmeli et al., 2012). The authors of these studies propose that M-acetylcysteine increases glutathione to improve symptom severity in schizophrenics. While N-acetylcysteine does increase blood glutathione levels in schizophrenics (Lavoie et al., 2008), it is likely through stimulation of system x_c^- to increase astrocytic cysteine for glutathione synthesis. This would also lead to increased extracellular glutamate, enhanced mGluR2/3 tone and modulation of synaptic activity. In support, N-acetylcysteine normalizes neurochemical deficits in genetic mouse models with chronic glutathione deficiency knockout mouse in the absence of glutathione concentration restoration (Duarte et al., 2011). These studies suggest N-acetylcysteine treatment improves the severity of negative and cognitive deficits in schizophrenia, symptoms

poorly treated with current antipsychotics. Further, there is support that N-acetylcysteine stimulates system x_c^- for improvements although further preclinical and clinical research is necessary.

Group II Metabotropic Glutamate Receptor Agonists: Many preclinical studies have shown significant improvements in NMDA receptor antagonist schizophrenia models including elevated glutamate and behavioral deficits associated with schizophrenia (Moghaddam and Adams, 1998; Cartmell et al., 1999; Homayoun et al., 2005; Patil et al., 2007; Baker et al., 2008; Hackler et al., 2010). This is interesting since it implicates insufficient extrasynaptic glutamate concentrations reducing tone on mGluR2/3 receptors which could be a result from altered cystine-glutamate exchange. Clinical studies have also shown improvements in PANSS total, PANSS positive and negative and CGI-S scales with the mGluR2/3 agonist LY2140023 without side effects typically seen with antipsychotics (Patil et al., 2007; Mosolov et al., 2010). However, a different study did not find any therapeutic potential with the mGluR2/3 agonist LY2140023 in PANSS total score but they also did not see any benefit from the atypical antipsychotic olanzapine which should have an effect resulting in inconclusive findings (Kinon et al., 2011). In conclusion, while the beneficial effects of mGluR2/3 agonists in clinical trials are inconclusive, the results in preclinical and clinical studies suggest that stimulation of the mGluR2/3 receptor may lead to improvements in schizophrenic symptoms as would increased extrasynaptic glutamate potentially through system x_c^-.

The primary goal of this body of work is to characterize the contribution of cystine-glutamate exchange to symptoms of schizophrenia. To determine the

importance of system x_c^- dysregulation, the impact of increased or decreased system x_c^- activity to glutamate levels in the prefrontal cortex and behaviors produced by the prefrontal cortex that are used to model schizophrenia was examined. First, system x_c^- activity was assessed in the acute phencyclidine and the neurodevelopmental MAM model of schizophrenia and the ability of increased cystine-glutamate exchange to reverse behavioral deficits was investigated. Further, the contribution of decreased system x_c^- activity by sulfasalazine administration and behavioral measures of schizophrenia was assessed. Collectively, these data position system x_c^- as a key regulator of behavioral output from the prefrontal cortex and indicate that system x_c^- dysregulation in schizophrenia may be an important component of the pathology of the disease.

II. TARGETING SYSTEM X_C^- IN THE PREFRONTAL CORTEX TO REVERSE SENSORIMOTOR GATING DEFICITS PRODUCED BY ACUTE PHENCYCLIDINE

Introduction

Aspects of schizophrenia, including negative symptoms and cognitive deficits, are thought to arise as a result of abnormal signaling in cortical structures such as the dorsolateral prefrontal cortex and the hippocampus (Weinberger, 1987; Javitt, 1987; Bunney and Bunney, 2000; Chavez-Noriega et al., 2002). Attempts to understand the cellular basis of schizophrenia will be aided by advances in our understanding of the mechanisms that regulate cortical activity. Cystine-glutamate exchange by system x_c^- may be a key component of altered excitatory signaling in the prefrontal cortex observed in schizophrenia (Baker et al., 2008; Krystal, 2008; Bridges, 2012).

System x_c^- appears to play an important role in normal and pathological brain functioning. First, system x_c^- exchanges one extracellular molecule of cystine for one intracellular molecule of glutamate resulting in an extracellular concentration that is sufficient to activate glutamate receptors (Baker et al., 2002b; Moran et al., 2005). Extrasynaptic glutamate provides tone on group II metabotropic glutamate receptors thereby inhibiting synaptic neurotransmitter release (Battaglia et al., 1997; Moran et al., 2005). Inside the cell, cystine is reduced into cysteine, which is the rate-limiting precursor in the synthesis of the antioxidant glutathione (Bannai, 1984; Sies, 1999). In astrocytes, cystine uptake is predominantly achieved by cystine-glutamate exchange, making system x_c^- a key mechanism in regulating oxidative stress (Cho and Bannai, 1990). Thus, changes in system x_c^- activity could contribute to diseased states involving either abnormal glutamate signaling or oxidative stress. Extant data suggest that the activity of system x_c^- may be altered in the dorsolateral prefrontal cortex of individuals

with schizophrenia (Baker et al., 2008). Specifically, schizophrenia is associated with a significant reduction in glutathione levels (Do et al., 2000; Yao et al., 2006; Gawryluk et al., 2010; Raffa et al., 2011) coupled with a modest but significant change in the protein levels of xCT, which is the active subunit of system x_c^-, in the dorsolateral prefrontal cortex (Baker et al., 2008). These changes may contribute to the disease since the decrease in glutathione has been shown to contribute to negative symptoms of schizophrenia (Steullet et al., 2006; Matsuzawa et al., 2008).

The NMDA receptor antagonist phencyclidine is used as a model of schizophrenia based on its ability to create a broad range of schizophrenic-like symptoms in humans and rats and exacerbation of symptoms in schizophrenics (Luby et al., 1959; Pearlson, 1981; Javitt and Zukin, 1991; Krystal et al., 1994; Malhotra et al., 1997b). In rodents, phencyclidine produces a hyperlocomotive state recapitulating positive symptoms of schizophrenia (Sturgeon et al., 1979; Enomoto et al., 2007; Young et al., 2010), negative symptoms including social interaction deficits (Sams-Dodd, 1999) and cognitive deficits including novel object recognition, reversal learning and attentional set shift (for review see Coyle et al., 2003; Javitt, 2007; Neill et al., 2010). Additionally, NMDA receptor antagonists have been shown to increase prefrontal glutamate levels generating an increase in cortical neuron firing rate which is essential for the psychotomimetic effects of phencyclidine (Moghaddam and Adams, 1998; Lorrain et al., 2003; Homayoun et al., 2005). Furthermore, group II metabotropic glutamate receptor activation has been shown to reverse NMDA receptor antagonist induced disruptions in activity (Moghaddam and Adams, 1998; Homayoun et al., 2005).

N-acetylcysteine is a cysteine prodrug capable of driving cystine-glutamate exchange and preclinical and clinical studies indicate that administration has therapeutic potential for the treatment of schizophrenia (Berk et al., 2008d; Lavoie et al., 2008; Bulut et al., 2009; Baker et al., 2008). The therapeutic efficacy of N-acetylcysteine in sensorimotor gating has yet to be established. Sensorimotor gating, a measure of executive functioning, reflects the ability of the central nervous system to inhibit irrelevant sensory information and allow focus on salient information from the environment (Braff et al., 1978). Schizophrenic patients and rodents treated with the non-competitive NMDA receptor antagonist phencyclidine have been shown to produce deficits in prepulse inhibition (Mansbach and Geyer, 1989; Geyer et al., 1990; Swerdlow et al., 1994; Braff et al., 2001a; Geyer et al., 2001; Swerdlow et al., 2006; Kumari et al., 2007). Further, executive functioning deficits are core features of schizophrenia (Szoke et al., 2008; Eisenberg and Berman, 2010) and remains poorly treated with antipsychotics (for review see Hill et al., 2010).

The primary goal of this chapter was to assess the ability of system x_c^- stimulation by N-acetylcysteine in the prefrontal cortex to attenuate phencyclidine-induced deficits in sensorimotor gating.

Materials and Methods

Animals and Surgeries: Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300-400 grams were individually housed in a temperature controlled room with a 12-h light/dark cycle with food and water *ad libitum*. The housing conditions and care of the rats were in accordance with the Animal Welfare Act, and all procedures were approved by the Marquette University IACUC Committee. Rats used in the microdialysis studies were anesthetized using pentobarbital (50 mg/kg, IP) with atropine sulfate (1 mg/kg, IP) pretreatment to limit tracheobronchial secretions. Bilateral guide cannula (20 gauge, 14 mm; Plastics One, Roanoke, VA) were implanted using coordinates +3.1 mm anterior, ±1.0 mm mediolateral to Bregma, and -0.75 mm ventral from the surface of the skull at a 6° angle from vertical derived from Paxinos and Watson (Paxinos and Watson, 1986). The placement of the active region of the microdialysis probe, which began two mm beyond the ventral tip of the guide cannulae, was primarily in the prelimbic cortex, the rat equivalent of the human dorsolateral prefrontal cortex (Uylings et al., 2003), although regions immediately ventral to this were also likely sampled. Following surgery, rats were provided acetaminophen (480 mg/L) in their drinking water for 2 days and were given at least six days to recover prior to testing.

Drug Treatments: Phencyclidine (0-3 mg/kg; NIDA Drug Supply Program, Research Triangle, NC) was dissolved in isotonic saline. N-acetylcysteine (Sigma Chemical Co., St Louis, MO) was dissolved in saline or microdialysis buffer and brought to a pH of 7.0 using NaOH. (*S*)-4-carboxyphenylglycine (CPG, 0.5μM; Tocris-Cooksin, Ellisville,

MO) was dissolved in dialysis buffer. All treatments were administered according to the experimental design.

Prepulse Inhibition: Rats were placed on a platform in a sound attenuating chamber (10.875"x14"x19.5"; Hamilton Kinder, CA) that rested on a motion sensing plate. A matching session was conducted to determine the magnitude of the startle response for each rat. This session consisted of a five minute habituation period followed by 20 trials; 17 trials involved the presentation of a single auditory stimulus (pulse stimulus; 50 dB above the 60 dB background noise) and three trials in which a prepulse stimulus (12 dB above background) was presented 100 ms before the pulse stimulus. Rats were then assigned into the various treatment groups based on the magnitude of their startle response. At least one day later, an experimental session was conducted to assess sensorimotor gating. On this day, rats received a 5-10 min habituation period followed by 58 discrete trials; eight background trials with only background noise, 26 trials with only the pulse stimulus (50 dB above background), and 24 trials with the pulse stimulus being preceded by one of three prepulse stimuli (2, 6, or 15 dB above background). The percent of prepulse inhibition was determined as 100-(average prepulse startle response/average startle stimulus alone)*100.

***In Vivo* Microdialysis:** Microdialysis probes, constructed as previously described (Baker et al., 2003), were inserted into indwelling guide cannula. Dialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.2 mM KCl, and 0.15% phosphate buffer saline, pH 7.4) was pumped through the probes at a rate of 1 μl/min for at least three hours prior to sampling in order for neurotransmitter levels to stabilize.

Afterwards, twenty minute samples were collected as described below for each experiment.

***In situ* hybridization:** Brains were rapidly removed and frozen in OCT (Sakura, Torrance, CA) in a dry ice and ethanol bath and then cut into 12 um coronal sections. Standard *in vitro* transcription methods were used to generate riboprobes against xCT (Choi, Milwaukee, WI) which was subsequently diluted in hybridization cocktail (Amresco, Solon, OH) and tRNA. Sections were hybridized overnight at 55 ºC in a mixture of tRNA, formamide, dextran, NaCl, EDTA, Denhardt's solution and ^{33}P-labeled riboprobe. After hybridization, slides were rinsed in 2x SSC buffer (pH 7.0). They were treated with RNase A in a 0.5 M sodium chloride, 10 mM Tris, 1 mM EDTA buffer for 30 min at 37 ºC and then washed in the same buffer without RNase A for 30 min at 37 ºC. Slides were stringently washed in 0.5x SSC for 30 min at 6 ºC and then exposed to autoradiographic film (3 days) and then subsequently coated with Kodak autoradiographic emulsion NTB (Rochester, NY) and exposed for 9 days to produce silver grains. Following standard autoradiography development, NTB emulsion-dipped sections were counterstained with 0.5% cresyl violet. Photodocumentation of silver grains was achieved using dark field microscopy (Axioskop-2, Zeiss, Thornwood, NY) and Axiovision image analysis software (Zeiss, Thornwood, NY).

Histology: Rats included in the microdialysis studies were given an overdose of pentobarbital (60 mg/kg, IP), and the brains were fixed by intracardiac infusion of 0.9% saline followed by 2.5% formalin solution. Brains were removed and stored in 2.5% formalin for at least seven days prior to sectioning. The tissue was then blocked and coronal sections (100 μM) were cut and stained with cresyl violet to verify probe

placements. Rats determined to have misplaced guide cannula were excluded from all analyses.

Statistics: Data was analyzed using analysis of variance (ANOVA) with drug treatment (e.g., phencyclidine, CPG, N-acetylcysteine) as between-subjects factors and prepulse intensity as a repeated measure. Significant interactions and main effects were further analyzed using Tukey HSD.

Experimental Design

Experiment 1: The purpose of this experiment was to select a dose of phencyclidine for subsequent experiments. Rats received an acute injection of phencyclidine (0-3 mg/kg, SC) 10 minutes before being placed in the startle chamber and testing was conducted as described above and shown in Figure 2.1.

Experiment 2: The purpose of this experiment was to assess the capacity of N-acetylcysteine to reverse phencyclidine-induced deficits in prepulse inhibition when given orally, and thus subject to metabolism in the GI tract and liver. N-acetylcysteine (0-600 mg/kg, PO) was given 50 minutes prior to phencyclidine (0 or 1.25 mg/kg, SC). Rats were placed in the startle chamber 10 minutes after the phencyclidine injection and testing was conducted as described above and shown in Figure 2.2. Rats were given 1.25 mg/kg phencyclidine since this dose produced robust and reproducible deficiencies in prepulse inhibition.

Experiment 3: This experiment was designed to identify the expression pattern of xCT using *in situ* hybridization in the medial prefrontal cortex and shown in Figure 2.3a and b.

Experiment 4: The purpose of this experiment was to determine the maximal effect that N-acetylcysteine would exert on phencyclidine-induced deficits in prepulse inhibition absent systemic metabolism or poor blood brain barrier permeability. To do this, we reverse dialyzed N-acetylcysteine (0-100 μM) directly into the prefrontal cortex for one hour prior to phencyclidine (0 or 1.5 mg/kg, SC) administration. One hour later, the probes were removed and rats underwent prepulse inhibition testing as described above and shown in Figure 2.3c. Phencycldine was administered at 1.5 mg/kg dose since similar to 1.25 mg/kg, 1.5 mg/kg also produced robust and reproducible deficiencies in prepulse inhibition but the higher dose was administered since animals underwent behavioral testing for a longer period after phencyclidine administration.

Experiment 5: This experiment was designed to verify that N-acetylcysteine reverses phencyclidine-induced deficits in prepulse inhibition by increasing cystine-glutamate exchange by system x_c- in the prefrontal cortex. To do this, the system x_c- inhibitor (*S*)-4-carboxyphenylglycine (CPG; 0-0.5 μM), was infused into the prefrontal cortex. Twenty min later, N-acetylcysteine (0-30 μM) was added to the dialysis buffer such that CPG and NAC were co-infused for one hour prior to phencyclidine administration (0-1.5 mg/kg, SC). One hour later, the probes were removed and rats underwent prepulse inhibition testing as described above and shown in Figure 2.4a and b.

Results

Acute phencyclidine creates a range of schizophrenic-like symptoms (Javitt, 2007) including deficits in executive function (Swerdlow et al., 1994; Geyer et al., 2001). Figure 2.1 depicts the impact of phencyclidine across a range of doses on sensorimotor gating. An ANOVA used to compare different phencyclidine doses in prepulse inhibition with drug treatment as a between subjects factor and prepulse intensity as a repeated measure yielded a significant interaction ($F_{(12,102)} = 5.77$ $p < 0.001$). Analysis of the simple main effects revealed a significant main effect of drug treatment at prepulse intensities of six ($F_{(6,57)} = 9.20$ $p < 0.001$) and 15 dB ($F_{(6,57)} = 14.79$ $p < 0.001$), but not at two dB ($F_{(6,57)} = 0.40$ $p > 0.05$). Post hoc analyses indicated that phencyclidine produced dose-dependent deficits in prepulse inhibition (Tukey HSD, $p < 0.05$).

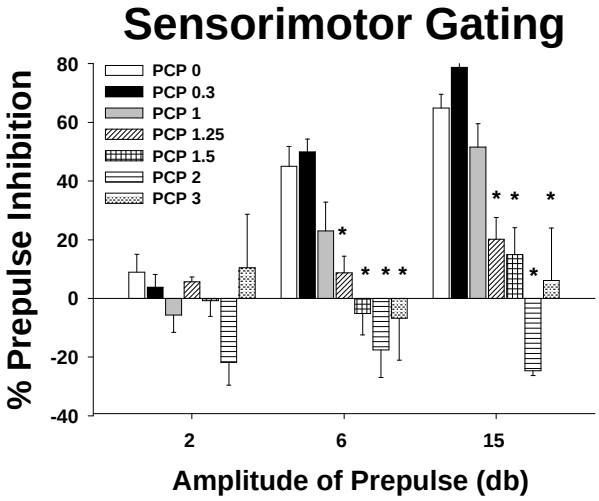


Figure 2.1 Phencyclidine produces a dose-dependent disruption of prepulse inhibition. The data is expressed as the mean (+ SEM) percent inhibition Behavior depicted was obtained from rats receiving an acute injection of phencyclidine (0-3 mg/kg; SC; *N* = 6-40/group) ten minutes prior to testing. * indicates a difference from controls receiving 0 mg/kg phencyclidine (Tukey HSD, $p < 0.05$).

The ability of pretreatment with N-acetylcysteine on phencyclidine-induced prepulse inhibition deficit was assessed. Figure 2.2 illustrates the impact of acute N-acetylcysteine pretreatment on phencyclidine-induced deficits in prepulse inhibition with oral administered (Figure 2.2). An ANOVA comparing a range of N-

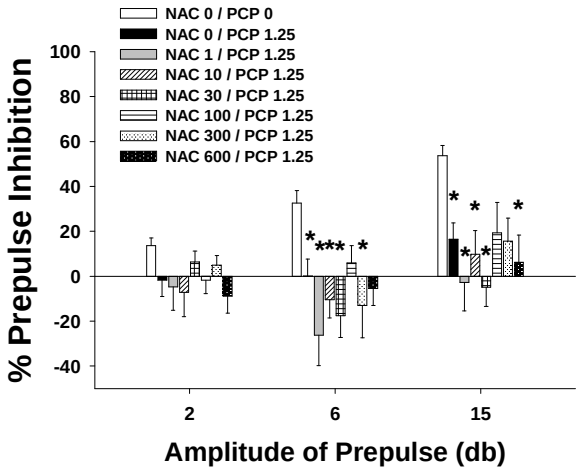


Figure 2.2 The effect of oral N-acetylcysteine pretreatment on phencyclidine-induced disruption of prepulse inhibition. The data is expressed as the mean (+ SEM) prepulse inhibition displayed by rats receiving oral (0-600 mg/kg, *N* = 6-19/group) 50 minutes prior to phencyclidine (0 or 1.25 mg/kg, SC) * indicates a significant difference from control rats receiving only saline (NAC 0 / PCP 0; Tukey HSD, $p < 0.05$).

acetylcysteine doses on phencyclidine-induced deficits included drug treatment as a between subjects factor and prepulse intensity as a repeated measure resulted in a significant interaction ($F_{(14,136)} = 2.96$ $p < 0.001$). Analysis of the simple main effects revealed a significant main effect at prepulse intensities of 6 ($F_{(7,75)} = 5.64$ $p < 0.001$) and 15 dB $F_{(7,75)} = 5.87$ $p < 0.001$) but not at 2 dB ($F_{(7,75)} = 1.89$ $p > 0.05$). Post hoc analyses indicated that phencyclidine produced deficits in prepulse inhibition that were not altered by oral administration of N-acetylcysteine even at doses up to 600 mg/kg (Tukey HSD, $p < 0.05$). This could indicate that 1) N-acetylcysteine is ineffective at reducing phencyclidine-induced deficits in prepulse inhibition or 2) that it is not penetrating the blood brain barrier it produce a therapeutic effect.

To test these possibilities, N-acetylcysteine was infused directly into an area rich in system x_c^-. *In situ* hybridization revealed a particularly prominent dorsal-ventral linear band located primarily in the prelimbic cortex to be richly innervated with xCT, the active subunit of the cystine-glutamate antiporter (Figure 2.3a left panel). Further, the

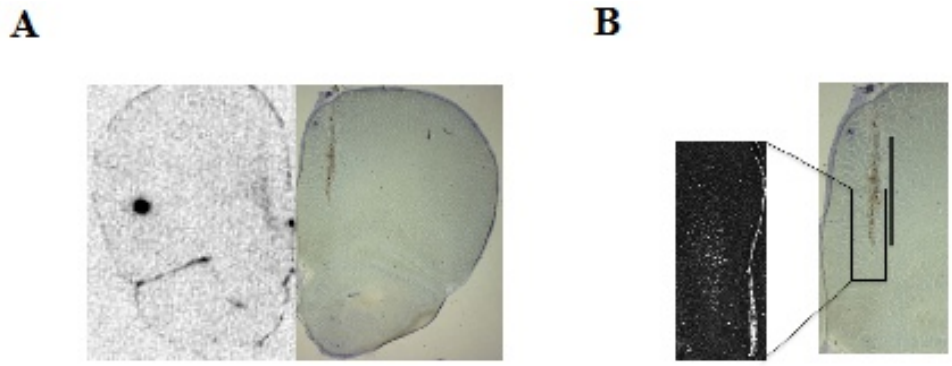


Figure 2.3 (A) Representative coronal section displaying expression of xCT mRNA via *in situ* hybridization (left, dark field photomicrograph) and subregion targeted in direct infusion studies (right). Note, the ventral two mm of the tract, which is primarily contained in the prelimbic cortex, represents the site of drug perfusion since this is the portion of the probe containing the active membrane. (B) Expression of xCT mRNA and tract placement using a 5X objective. The rectangle denotes the portion of the tract created by the microdialysis probe that is illustrated in the dark field photomicrograph. The bar represents 1mm.

medial prefrontal cortex has been identified as a main integration center for sensorimotor gating (Swerdlow et al., 2001). N-acetylcysteine was therefore administered via reverse microdialysis into the medial prefrontal cortex as shown in Figure 2.3a right panel. Figure 2.3b shows dark field photomicrograph of xCT mRNA and the representative tract created by the insertion of a microdialysis probe in the vicinity of the xCT mRNA linear band within the medial prefrontal cortex.

Figure 2.4 depicts the impact of N-acetylcysteine infused via reverse dialysis directly into the prefrontal cortex on phencyclidine-induced deficits in sensorimotor gating. The effect of infused N-acetylcysteine on phencyclidine deficits was measured using an ANOVA with drug treatment as a between subjects factor and prepulse intensity as a repeated measure yielded a significant interaction ($F_{(8,54)} = 2.26$ $p < 0.05$). Analysis of the simple main effects revealed a significant main effect at every prepulse intensity (2 dB: $F_{(4,31)} = 4.12$ $p = 0.010$; 6 dB: $F_{(4,31)} = 10.70$ $p < 0.001$; 15 dB $F_{(4,31)} = 7.15$ $p <$

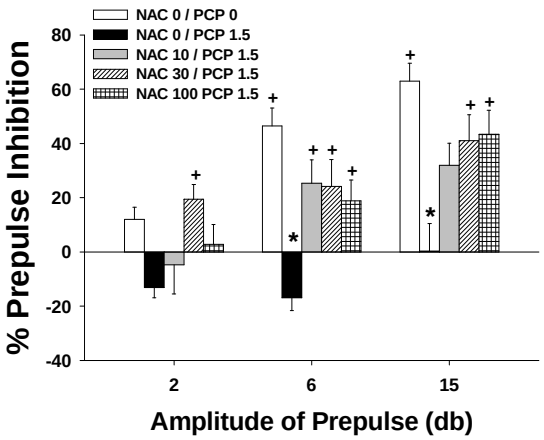


Figure 2.4 N-acetylcysteine into the prefrontal cortex via reverse dialysis reverses phencyclidine-induced deficits in prepulse inhibition deficits. Data is expressed as the mean (+ SEM) prepulse inhibition of rats receiving direct infusion of N-acetylcysteine into the prefrontal cortex (0-100 μM, *N* = 6-8/group) followed 1 hour later by an acute injection of phencyclidine (0 or 1.5 mg/kg, SC). * indicates a significant difference from control rats receiving saline (NAC 0 / PCP 0). + indicates a significant difference from rats receiving phencyclidine (NAC 0 / PCP 1.25) using Tukey HSD, $p < 0.05$.

0.001). Post hoc analyses indicated that phencyclidine produced deficits in prepulse inhibition at every prepulse intensity that were attenuated by N-acetylcysteine (Figure 2.3c, Tukey HSD, $p < 0.05$). Note, the active region of the microdialysis probe was primarily in the prelimbic cortex although regions immediately ventral to this were also likely sampled (Figure 2.3a). These results suggest the ability of N-acetylcysteine to reverse phencyclidine-induced deficits in sensorimotor gating in the prefrontal cortex. However, at this point the target of N-acetylcysteine remains unclear.

To determine whether N-acetylcysteine targets system x_c^- to reverse phencyclidine-induced prepulse inhibition deficits it was co-administered with an inhibitor of the cystine-glutamate antiporter. Figure 2.5 illustrates the impact of intra-prefrontal N-acetylcysteine on phencyclidine-induced deficits in prepulse inhibition when tested in the absence or presence of the system x_c^- inhibitor (*S*)-4-carboxyphenylglycine (CPG; 0 or 0.5 μM). An ANOVA compared CPG and N-acetylcysteine treatment and included drug treatments as between subjects variable and prepulse intensity as within subjects variable to produce a significant interaction between ($F_{(2,60)} = 3.146$ $p < 0.05$).

To further deconstruct the interaction, we compared the effect of N-acetylcysteine and phencyclidine treatment with or without CPG. In the absence of CPG (Figure 2.5a), an ANOVA with drug treatment as a between subjects and prepulse intensity as a repeated measures yielded a significant interaction between phencyclidine with and without infused N-acetylcysteine treatment ($F_{(4,60)} = 3.07$ $p < 0.05$). Analysis of the simple main effects revealed a significant main effect at every prepulse intensities (2 dB: $F_{(2,32)} = 12.48$ $p < 0.001$); 6 dB $F_{(2,32)} = 14.52$ $p < 0.001$; 15 dB $F_{(2,32)} = 12.30$ $p < 0.001$). Post hoc analyses indicated that N-acetylcysteine reversed phencyclidine-induced deficits in prepulse inhibition when tested in the absence of CPG (Tukey HSD, $p < .05$). In the presence of CPG (Figure 2.5a), an ANOVA compared the effect of CPG with and

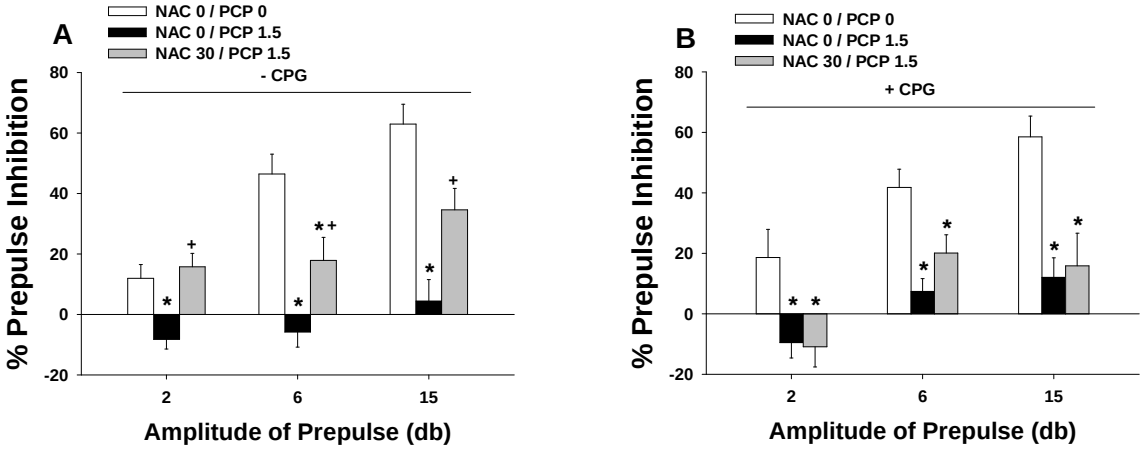


Figure 2.5 N-acetylcysteine targets system x_c- to reverse deficits in prepulse inhibition produced by phencyclidine. The data is expressed as mean (+ SEM) prepulse inhibition displayed by rats receiving intra-prefrontal N-acetylcysteine (1-30 μM, N = 6-16/group) (a) in the absence or (b) or presence of the system x_c- inhibitor (*S*)-4-carboxyphenylglycine (CPG, 0.5μM) co-infused into the prefrontal cortex. Note, CPG was infused alone for 20 min, N-acetylcysteine was then added to the microdialysis buffer for 60 min prior to the injection of phencyclidine (1.5 mg/kg, SC). * indicates a significant difference from respective saline controls (NAC 0 /PCP 0/ $\pm$ CPG; Tukey HSD, $p < 0.05$). + indicates a significant difference from respective phencyclidine controls (NAC 0 / PCP 1.5/ $\pm$ CPG; Tukey HSD, $p < 0.05$).

without phencyclidine and N-acetylcysteine administration with treatment as between subjects and prepulse intensity as repeated measures has a significant main effect of treatment ($F_{(2,30)}$= 13.64 p < 0.001) without a significant interaction ($F_{(4,60)}$ = 0.80 p > 0.05). Post hoc analyses indicated that N-acetylcysteine failed to alter phencyclidine-induced deficits in prepulse inhibition when tested in the presence of CPG (Tukey HSD, p <.05). Therefore, these data suggest that N-acetylcysteine targets system x_c^- in the prefrontal cortex to reverse phencyclidine-induced deficits in prepulse inhibition.

Table 2.1 is the startle magnitude in all prepulse inhibition testing. As shown, when comparing the acute effect of phencyclidine on prepulse inhibition in experiment 1

Table 2.1
Startle Magnitude
Drug Treatment

Exp	PCP 0	PCP 0.3	PCP 1.0	PCP 1.25	PCP 1.5	PCP 2.0	PCP 3.0	
1	1.82±0.19	1.58±0.47	2.19±0.35	**3.37±0.53***	3.18±0.31	**3.61±1.77***	1.06±0.32	
Exp	NAC 0 /	NAC 0 /	NAC 1 /	NAC 10 /	NAC 30 /	NAC 100 /	NAC 300 /	NAC 600 /
2	PCP 0	PCP 1.25	PCP 1.25	PCP 1.5	PCP 1.25	PCP 1.25	PCP 1.25	PCP 1.25
	1.77±0.26	2.37±0.29	1.83±0.36	1.88±0.34	2.64±0.18	2.60±0.45	2.66±0.42	3.02±0.70
Exp	NAC 0 /	NAC 0 /	NAC 10 /	NAC 30 /	NAC 100			
4	PCP 0	PCP 1.5	PCP 1.5	PCP 1.5	/ PCP 1.5			
	1.63±0.28	1.63±0.34	1.99±0.33	1.92±0.59	1.75±0.26			
Exp	CPG 0 /	CPG 0 /	CPG 0 /	CPG 0.5 /	CPG 0.5 /	CPG 0.5 /		
5	NAC 0 /	NAC 0 /	NAC 30 /	NAC 0 /	NAC 0 /	NAC 30 /		
	PCP 0	PCP 1.5	PCP 1.5	PCP 0	PCP 1.5	PCP 1.5		
	1.63±0.29	2.87±0.62	2.31±0.54	1.75±0.27	2.43±0.33	2.20±0.53		

[d] PCP = phencyclidine, NAC = N-acetylcysteine, CPG = (*S*)-4-carboxyphenylglycine
* P < 0.05, compared to PCP 0 group and are indicated in bold.

(Figure 2.1), an ANOVA indicated a main effect of phencyclidine treatment ($F_{(6,57)} = 5.80$ $p < 0.05$) on startle magnitude. Post-hoc analysis revealed that phencyclidine 1.25 mg/kg and 2.0 mg/kg (Table 2.1; Tukey HSD, $p < 0.05$) were significantly different from vehicle alone treated animals. However there was no effect of phencyclidine in experiment 2 with oral N-acetylcysteine treatment ($F_{(7,75)} = 1.89$ $p > 0.05$), experiment 4 ($F_{(4,33)} = 0.21$ $p > 0.05$) or experiment 5 ($F_{(5,50)} = 0.71$ $p > 0.05$).

Discussion

Abnormal glutamate signaling within cortical structures has been linked to negative symptoms and cognitive deficits present in schizophrenia (Weinberger, 1987; Bunney and Bunney, 2000). Identifying cellular mechanisms capable of regulating glutamate signaling may advance our understanding of the neurobiological basis of schizophrenia and facilitate the development of pharmacotherapies. A primary finding of this chapter is that increasing system x_c^- activity in the prefrontal cortex by locally applying the cysteine prodrug N-acetylcysteine attenuated phencyclidine-induced deficits in sensorimotor gating. Further, we found that the capacity of N-acetylcysteine to restore prepulse inhibition was dependent upon the route of administration with oral administration failing to produce an effect. Note, the dose range for N-acetylcysteine used was well within the dose range used for other experiments (Harvey et al., 2008). Collectively, these data suggest involvement of system x_c^- in phencyclidine-induced schizophrenic-like symptoms.

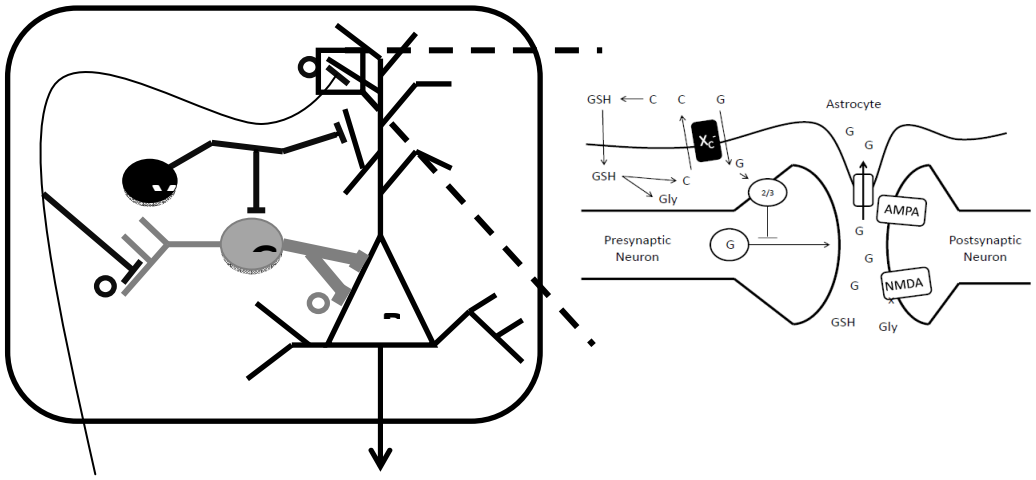
A key finding of the current studies is that N-acetylcysteine reverses phencyclidine-induced deficits in prepulse inhibition. Prepulse inhibition refers to a reduction in the magnitude of response to an acoustic stimulus when the eliciting auditory stimulus (pulse) is preceded by a lower-intensity auditory cue (prepulse); deficits in prepulse inhibition have been used to model sensorimotor gating deficits that occur in schizophrenic patients (Kumari et al., 2007; Swerdlow et al., 2006; Braff et al., 2001b). Sensorimotor gating reflects the ability of the central nervous system to inhibit irrelevant sensory information and allow focus on salient information from the environment (Braff

et al., 1978). Restoration of sensorimotor gating as assessed by reversal of phencyclidine-induced deficits in prepulse inhibition is used to establish antipsychotic activity (Geyer et al., 2001). These data, taken together with earlier findings that N-acetylcysteine reverses deficits in prepulse inhibition evident in metabotropic glutamate receptor five knockout mice (Chen et al., 2010) provide support for the involvement of cystine-glutamate exchange in schizophrenia.

The effects of N-acetylcysteine on prepulse inhibition of an acoustic startle response are likely due to increased activity of system x_c^- in the prefrontal cortex. In support, reverse dialysis of N-acetylcysteine directly into the medial prefrontal cortex produced a near complete reversal of phencyclidine-induced deficits in prepulse inhibition and this effect was prevented when the system x_c^- inhibitor (*S*)-4-carboxyphenylglycine (CPG) was co-infused into the prefrontal cortex. This is an important finding since the prefrontal cortex, specifically the dorsolateral prefrontal cortex, has been implicated in executive functions such as selective attention and task management (Smith and Jonides, 1999). Numerous neuroimaging studies have found abnormal dorsolateral prefrontal cortical activation in response to executive functioning in schizophrenic patients (for review see Eisenberg and Berman, 2010). Additionally, reductions in dorsolateral prefrontal cortical gray matter volume has been found in patients with more pronounced executive functioning dysfunction as measured by the Wisconsin card sorting task (Rusch et al., 2007) and a positive correlation between gray matter volume and prepulse inhibition (Kumari et al., 2008). Given the crucial role of the dorsolateral prefrontal cortex in normal brain function, the change in xCT expression associated with schizophrenia and the abundant expression of xCT mRNA in this region

(at least in the rodent), system x_c^- likely contributes to dorsolateral prefrontal cortical activity and tasks associated with schizophrenia.

The mechanism of N-acetylcysteine and system x_c^- to reverse phencyclidine-induced behavioral deficits has not been clearly defined although one possibility that merits further testing is that N-acetylcysteine normalizes pyramidal cell firing. Symptoms of schizophrenia including executive function are thought to arise, at least in part, to desynchronization of dorsolateral prefrontal cortical activity (Haenschel et al., 2009). GABAergic, particularly parvalbumin containing fast-spiking interneurons, have been shown to regulate network oscillations (for review see Bartos et al., 2007). Phencyclidine has been shown to desynchronize oscillatory network activity in rodents by preferentially inhibiting GABAergic interneurons thereby causing disinhibition and subsequent over-activation of pyramidal cells and increased synaptic glutamate release (Moghaddam and Adams, 1998; Homayoun and Moghaddam, 2007; Amitai et al., 2012). Further, evidence suggests that phencyclidine increases extracellular glutamate and reduces synchronous network activity potentially leading to symptoms of schizophrenia (Moghaddam et al., 1997; Moghaddam and Adams, 1998; Homayoun et al., 2005; Hakami et al., 2009). Stimulation of system x_c^- increases glutamatergic tone on metabotropic group II glutamate receptors (Baker et al., 2002b; Moran et al., 2005), inhibits phencyclidine-induced glutamate release (Baker et al., 2008), and thereby, may restore activity and synchrony of prefrontal cortical outputs. Further, mGluR2/3 agonists have also been shown to inhibit phencyclidine-induced glutamate release (Moghaddam and Adams, 1998; Homayoun et al., 2005; Patil et al., 2007). Another potential mechanism of N-acetylcysteine reversal of phencyclidine-induced deficits is the ability of



N-acetylcysteine independently or through stimulation of system x_c^- to alter the activity

Figure 2.6 Schematic of neural circuitry and synaptic connections within prefrontal cortex brain. (A) Pyramidal neurons labeled with a P are the main excitatory output of the region and receives inputs from other neurons within and outside the structure (black line entering prefrontal cortex) indicated by black lines which can be stimulatory, inhibitory or modulatory. Fast spiking basket cell parvalbumin containing GABAergic interneurons (gray marked with G are uniquely positioned to inhibit the soma of pyramidal neurons to modulate neuronal activity. X indicates a nonspecific neuron within the region aiding in the synchronization of the circuit. Panel B depicts the synaptic cleft in the box from panel A. The presynaptic neuron may release neurotransmitters such as glutamate (G), dopamine and GABA. System x_c^- exchanges glutamate for cystine (C), allows for glutathione (GSH) synthesis and tonic tone on metabotropic group II glutamate receptors (2/3) to modulate synaptic neurotransmitter release.

of NMDA receptors. This could arise as a result of N-acetylcysteine-induced release of glutamate, generation of glycine following metabolism of de novo synthesized glutathione, or activation of the extracellular redox site on the NMDA receptor by cystine or glutathione (Baker et al., 2002b; Dringen et al., 2001; Steullet et al., 2006). Figure 2.6 depicts normal prefrontal cortical circuitry. Note, in the presence of phencyclidine, there would be disinhibition of pyramidal cells due to reduced GABAergic firing resulting in elevated pyramidal neuron activity and glutamate release.

Interestingly, extant data suggest that diminished system x_c^- function may contribute to the pathology of schizophrenia. In support, schizophrenia is associated with a significant change in the tissue levels of glutathione, which is dependent upon the

uptake of cystine/cysteine, and in the protein levels of xCT, the active subunit for system x_c-, in the dorsolateral prefrontal cortex (Do et al., 2000; Raffa et al., 2011; Baker et al., 2008). Interestingly, a decrease in glutathione or reduced system x_c^- activity inversely correlates with the severity of negative symptoms and is sufficient to produce hypoactivity of NMDA or group II metabotropic glutamate receptors, as well as reduced expression of parvalbumin (Steullet et al., 2006; Matsuzawa et al., 2008) all of which are thought to be central to the pathology of schizophrenia (Javitt, 2004; Lewis and Moghaddam, 2006). Thus, efforts to increase cystine-glutamate exchange represent an approach that would be expected to produce a number of therapeutically relevant effects in cell functioning including the restoration of glutathione levels and increased activity of NMDA and group II metabotropic glutamate receptors.

These experiments indicate that in the acute phencyclidine model of schizophrenia, there are deficits in sensorimotor gating that can be reversed by stimulating system x_c^- in the prefrontal cortex. Oral treatment was without effect, possibly due to the poor bioavailability of oral N-acetylcysteine which has been estimated to range between four and ten percent (Borgstrom et al., 1986; Olsson et al., 1988). Involvement of system x_c^- needs to be further investigated to determine whether it is involved in the neuropathology of schizophrenia by utilizing a more accurate representation of schizophrenia. The following chapters will examine system x_c^- in a neurodevelopmental approach to model the neurodevelopmental hypothesis of schizophrenia.

III. EXAMINATION OF CYSTINE-GLUTAMATE EXCHANGE IN METHYLAZOXYMETHANOL ACETATE TREATED OFFSPRING; A NEURODEVELOPMENTAL MODEL OF SCHIZOPHRENIA

Introduction

Accumulating evidence supports the neurodevelopmental hypothesis of schizophrenia suggesting pre or postnatal environmental complications lead to abnormal neurodevelopment and symptoms of schizophrenia (for review see Weinberger, 1987; Marenco and Weinberger, 2000; Rapoport et al., 2005). The neurodevelopmental hypothesis posits that a type of insult occurs in the pre or postnatal period leading to abnormal neurodevelopment of critical circuits causing the behavioral manifestation of schizophrenia later in life. In support, several prenatal insults including viral, stress or delivery complications increase the likelihood of the offspring developing schizophrenia; there is also evidence of postnatal complications leading to elevated chance of schizophrenia (Brown, 2011; for review see Marenco and Weinberger, 2000; Lewis and Levitt, 2002; Brown and Patterson, 2011). Further, social abnormalities and cognitive impairments are often observed for several years prior to diagnosis (Chua and Murray, 1996; Elvevag and Goldberg, 2000; Blanchard and Cohen, 2006; Lewis and Moghaddam, 2006) which typically occurs after positive symptoms emerge in late teens to early 20's (Andreasen, 1995; Chua and Murray, 1996; Lewis and Lieberman, 2000) suggesting neurodevelopmental manifestation of symptoms of the disorder. Unlike acute disruption models such as phencyclidine, neurodevelopmental models of schizophrenia attempt to mimic the lifelong span of symptoms observed in schizophrenics; this chapter seeks to determine whether system x_c^- is involved in the pathophysiology of one of these models.

Methylazoxymethanol acetate (MAM) is a DNA methylating agent which selectively targets neuroepithelial proliferating cells (Matsumoto and Higa, 1966;

Cattabeni and Di Luca, 1997) and when given on gestational day 17 will target select brain regions undergoing neurogenesis including the prefrontal cortex, hippocampus and thalamus (Cattabeni and Di Luca, 1997; Matricon et al., 2010), areas that have also been implicated in schizophrenia (Bunney and Bunney, 2000; Heckers and Konradi, 2010; Swerdlow, 2010). MAM administration generates behaviors consistent with symptoms of schizophrenia including hyperstimulation in locomotor studies to amphetamine and NMDA receptor antagonists (Le Pen et al., 2006; Lodge and Grace, 2007; Flagstad et al., 2004; Phillips et al., 2012), social interaction deficits (Le Pen et al., 2006; Flagstad et al., 2004; Lieberman et al., 2001), sensorimotor gating deficits (Moore et al., 2006) and cognitive deficits (Gourevitch et al., 2004; Flagstad et al., 2005; Le Pen et al., 2006; Featherstone et al., 2007). Similar to schizophrenia, positive symptoms (humans: hallucinations, delusions, paranoia; rat: hyperlocomotion) emerge in adults whereas negative symptoms (humans and rats: social withdrawal) occur before puberty (Lewis and Levitt, 2002; Le Pen et al., 2006), cognitive deficits, while occurring prior to puberty in schizophrenics has not been tested in rats (Lewis and Levitt, 2002). Furthermore, both schizophrenics and MAM offspring have lower prefrontal cortical volumes with no change in neuronal density (Selemon et al., 1995; Thune and Pakkenberg, 2000; Moore et al., 2006; Penschuck et al., 2006). Also, abnormal inhibitory circuitry has been shown in schizophrenics by a decrease in GAD_{67}, the main GABA synthesizing protein, and reduced GABAergic parvalbumin expression in the prefrontal cortex of schizophrenics and MAM offspring (Hashimoto et al., 2003b; Lodge et al., 2009) although no change in MAM parvalbumin in the prefrontal cortex has also been reported (Penschuck et al.,

2006). Collectively, these and other data support and establish the rodent MAM neurodevelopmental model of schizophrenia.

To date, system x_c^- is poorly studied in neurodevelopmental models with no reports of cystine-glutamate exchange or glutathione in MAM treated offspring which is interesting since both are changes reported in schizophrenia. Schizophrenics have reduced glutathione by as much as 50% in the dorsolateral prefrontal cortex (Do et al., 2000; Yao et al., 2006; Gawryluk et al., 2010; Raffa et al., 2011). The rate limiting step in glutathione synthesis is cystine uptake (Sagara et al., 1993; Kranich et al., 1998; Dringen and Hirrlinger, 2003) and system x_c^- is a key transporter for astrocytic cystine (Bannai, 1984; Bridges, 2011). Additionally, expression of xCT, the active subunit of the cystine-glutamate antiporter, is elevated in the dorsolateral prefrontal cortex (Baker et al., 2008). Further, system x_c^- is capable of regulating synaptic neurotransmitter release through released extrasynaptic glutamate providing tone on extrasynaptic group II metabotropic glutamate receptors (Baker et al., 2002b) thereby serving as a heteroreceptor regulating synaptic neurotransmitter release (Baskys and Malenka, 1991; Conn and Pin, 1997; Xi et al., 2002; Chaki and Hikichi, 2011). Interestingly preclinical and clinical studies have shown that group II agonists have been shown to significantly improve symptoms of schizophrenia (Moghaddam and Adams, 1998; Patil et al., 2007; Mosolov et al., 2010) although see (Kinon et al., 2011) who did not find improvements from placebo for mGluR2/3 agonists however they also failed to see improvements with atypical antipsychotic medication. Additionally, N-acetylcysteine, which preclinically was shown to stimulate cystine-glutamate exchange (Baker et al., 2002b; Kau et al., 2008; Baker et al., 2008; Chapter 2), has also improved clinical symptoms of

schizophrenia (Berk et al., 2008c; Lavoie et al., 2008). Collectively, system x_c^- is implicated in the pathophysiology of schizophrenia. However, the extent of its involvement in neurodevelopmental models and its direct effect on behavior is not yet known.

Cognitive deficits are a core feature of schizophrenia and are the single best predictor of long-term outcome (Liddle, 2000; Kurtz et al., 2005; Holthausen et al., 2007). Behavioral flexibility tests the ability to shift attention in response to changes in one's environment; schizophrenics have difficulty shifting attention between different rules or strategies as measured by the Wisconsin Card Sorting task (Morice, 1990; Gold et al., 1997; Prentice et al., 2008; Waford and Lewine, 2010). Further, schizophrenics have also shown deficits in reversal learning (Waltz and Gold, 2007; Murray et al., 2008). Deficits in set shifting and reversal learning have been found in multiple models of schizophrenia including with NMDA receptor antagonists (Stefani and Moghaddam, 2005; Abdul-Monim et al., 2006) and neurodevelopmental models including prenatal immune challenge (Meyer et al., 2005) and MAM (Featherstone et al., 2007). The attentional set shifting protocol in this chapter examines MAM offspring with a visual discrimination task, an attentional set shift and reversal learning while using the same stimuli across the different tasks making it similar to the Wisconsin Card Sorting task (Floresco et al., 2009).

The primary goal of this chapter is to assess the status of system x_c^- activity in the MAM neurodevelopmental model of schizophrenia and to determine whether

stimulation of system x_c^- by N-acetylcysteine is sufficient to treat MAM-induced cognitive deficits.

Materials and Methods

Methylazoxymethanol Treatment: Timed pregnant Sprague-Dawley rats were given an acute injection of saline or methylazoxymethanol (22 mg/kg, IP; MRIGlobal Chemical Carcinogen Repository, Kansas City, MO) on gestational day 17. Following vaginal birth all mothers and offspring were left undisturbed until weaning on postnatal day 22. Male offspring were individually housed in a temperature controlled room with a 12-h light/dark cycle with food and water *ad libitum*. The housing conditions and care of the rats were in accordance with the Animal Welfare Act, and all procedures were approved by the Marquette University IACUC Committee. Offspring were tested as juveniles (PND 28-40) or adults (PND 60+).

Drug Treatments: N-acetylcysteine (Sigma Chemical Co., St Louis, MO), when given, was dissolved in isotonic saline with NaOH to bring the pH to 7.0 and given acutely or

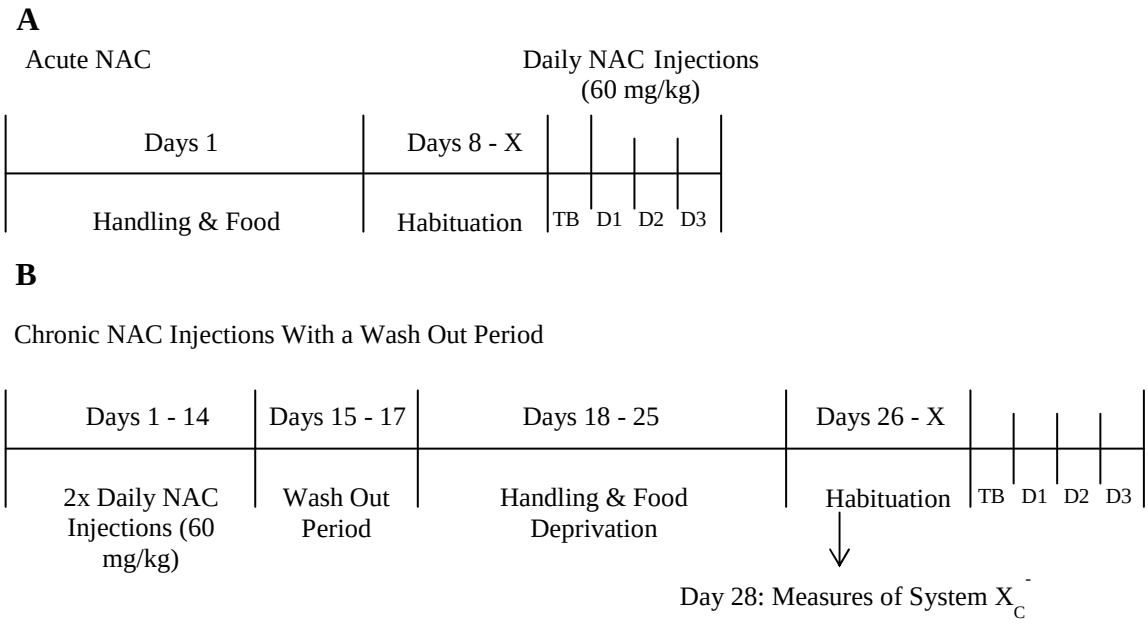


Figure 3.1. Acute (A) and chronic with a wash out period (B) administration of N-acetylcysteine (NAC) in behavior and measures of system x_c^-. Note, habituation can take anywhere from 2 to 10 days.

chronically with a wash out period. With acute administration, N-acetylcysteine (0-60 mg/kg, IP) was given one hour prior to start of testing in set shifting or measures of system x_c^- (see Figure 3.1a). Chronic administration involved 14 days of twice daily injections (0-60 mg/kg, IP) followed by a 14 day wash out period before behavioral testing or measures of system x_c^- were conducted (see Figure 3.1b) to determine whether chronic treatment induced lasting changes in system x_c^- activity and behavior even after cessation of treatment.

Attentional Set Shifting: The attentional set shifting task was based on work from the Floresco lab (Floresco et al., 2006a; Floresco et al., 2008; Floresco et al., 2009). The maze consisted of four-arms (60 x 20 x 12 cm) in the shape of an X connected by a center area (12 x 12 cm) constructed of black Plexiglas with a movable wall to block an arm to form a "T" configuration. Dividers at the end of each arm concealed the sugar pellet reward such that the rat was unable to see it from the center of the maze. The visual cue, when used, was laminated with black and white diagonal stripes and placed on the floor of an arm. Prior to habituation, rats were handled for five minutes each day for seven days and food deprived to 85% of their free-feeding weight. The day before habituation, each rat was given approximately 30 sugar pellets in their home cage to acquaint them with the pellets.

Habituation: A habituation period was conducted in order to familiarize the animal to the maze and train him to find and eat the food reward within the maze. Day one, 5 pellets were placed in each arm, 3 throughout the length of the arm and 2 behind the divider at the end. The rat was placed in the center of the maze and allowed to freely explore for up to 15 minutes. On the second day, 3 pellets were placed in each arm, one in the center,

the other 2 behind the divider and the rats freely explored for up to 15 minutes. On the third day and all remaining days till criteria was achieved, one pellet was placed behind the divider in each arm. After the rat consumed a pellet behind the divider on any day, he was picked up and placed at the entrance to another baited arm to familiariaze him to handling within the maze. If the rat consumed all sugar pellets on any day, the maze was rebaited with the following habituation day's configuration or with 4 pellets, one behind each divider after habituation day 2. Criteria was met after the maze was rebaited 3 times and all pellets were consumed within 15 minutes; turn bias was run the following day. If the rat did not consume all sugar pellets within 15 minutes, he continued habituation daily until he met criteria.

Turn bias: Turn bias introduced the animal to the "T" maze configuration and the visual cue. During turn bias and testing, one arm was blocked with the movable wall to form a "T" configuration with the visual cue in one of the side arms. In turn bias, both side arms were baited with sugar pellets behind the dividers. The rat was placed in the stem arm at the bottom of the "T" and allowed to enter either arm; after consuming the pellet he was placed back in the stem arm and again allowed to enter either arm. If he entered the same arm again, he was returned to the stem arm until he turned into the opposite arm and ate the remaining sugar pellet. When he successfully ate both sugar pellets, he was placed back in his home cage signaling the end of the trial. The next trial, the rat began from a different arm such that over 7 trials, the rat started from each of 3 arms 2 times and the remaining arm once and the visual cue was placed in either the right or left arm as equally as possible. Inter-trial time was about 10 seconds to rebait the maze. The

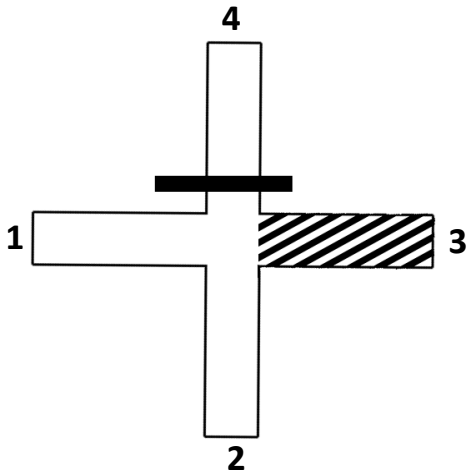


Figure 3.2 Attentional set shifting diagram

direction the rat turned first the most out of 7 trials was considered his turn bias for the rest of testing.

Testing: As in turn bias, one arm was blocked with the movable wall, either the right or left arm contained the visual cue and the rat began in the stem arm. Trials were grouped into a block of 12 with the stem arm in arms 1-3 as shown in Figure 3.2. In the probe trial, arm 4 was the stem arm and the visual cue was placed in the turn bias arm. Within treatment groups, the probe arm switched such that at least one animal in each group used each arm as the stem arm for the probe trial. Each testing day required the following:

- 10 consecutive correct trials using the 12 trial block in stem arms 1-3
- 1 correct probe trial from stem arm 4
- If correct probe trial, testing was complete
    - If incorrect probe trial
        - 5 consecutive correct trials using the 12 trial blocks
        - 1 correct probe trial from stem arm 4
        - This continues till correct probe trial

Day 1: Visual-cue learning: On day one, the rat was trained in a simple discrimination task to attend to the visual cue such that the food reward was placed in each arm with the visual cue 50% of the time. Turing towards the visual cue and attaining the reward was a correct choice. In the probe trial the rat turned towards the visual cue.

Day 2: Attentional Set Shift: On day 2 the complex task of an attentional set shift required the rat to switch from a visual to a spatial cue with the new strategy for reward turning into the arm opposite of his turn bias. The probe trial required the rat to turn in the opposite direction of the visual cue.

Day 3: Reversal learning: Reversal learning required the rat to enter the arm opposite the strategy from day 2 such that the rats turn bias arm now contained the food reward and the rat turned towards the visual cue on the probe trial.

Tissue Glutathione Analysis: Animals were rapidly decapitated (PND 100+) and punches taken from the medial prefrontal cortex. The tissues were homogenized in a 4:1 dialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.2 mM KCl, and 0.15% phosphate buffer saline, pH 7.4): mobile phase solution (75 mM $NaH_2PO_4 \cdot H_2O$, 1.8 mM $C_8H_{17}O_3Sna$, 25 μM EDTA, 0.1% TEA and CH_3CN pH to 3.0 with phosphoric acid). One fraction was analyzed in the BCA method to determine protein content. The other fraction analyzed thiol content using HPLC with EC detection (Decade II, MagicDiamond electrode set at +1.89V, Antec Leyden, Netherlands).

^{14}C Cystine Uptake: Animals were rapidly decapitated and brains extracted. All tissue punches of the prefrontal cortex were treated with TBOA (100 μM) and in the presence ^{14}C-L-cystine (approximately 0.3 μM). Experimental groups were MAM or control rat's male offspring (PND 100+) in the presence or absence of (*S*)-4-carboxyphenylglycine (CPG, 1 mM; Tocris-Cooksin, Ellisville, MO). The punches were washed and solubilized with 1% SDS. One fraction (200 uL) of the solubilized tissue was used for determining [14C]-L-cystine uptake by a scintillation counter. The other fraction (25 uL)

was used to determine protein content by the BCA method. Uptake was measured in counts/µg protein.

Surgeries: Adult male offspring of control or MAM treated animals weighing 300-400 grams were anesthetized using pentobarbital (50 mg/kg, IP) with atropine sulfate (1 mg/kg, IP) pretreatment to limit tracheobronchial secretions. Bilateral guide cannula (20 gauge, 14 mm; Plastics One, Roanoke, VA) were implanted using coordinates +3.1 mm anterior, ±1.0 mm mediolateral to Bregma, and -0.75 mm ventral from the surface of the skull at a 6° angle from vertical derived from Paxinos and Watson (Paxinos and Watson, 1986). Rats were given at least 6 days to recover from surgery prior to testing.

***In Vivo* Microdialysis:** Microdialysis experiments were conducted as described previously (Baker et al, 2002). Briefly, after a minimum of six days post surgery, removable probes extending 2 mm past the guide cannula were inserted through the cannulae into the prefrontal cortex and microdialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.15% phosphate-buffered saline, pH 7.4) was pumped through the probes at one µl/min for at least three hours prior to collecting baseline samples to allow for neurotransmitters to stabilize. Afterwards, 20-min samples were collected for 4 baseline samples followed by 3 samples at each cystine concentration in ascending order: 0, 0.01, 0.05, 0.1, 0.15, 1.5µM. Samples were run for on HPLC for thiol and glutamate content.

Thiol HPLC: Thiol content was analyzed on HPLC with electrochemical detection. Thiols were separated using a reversed-phase column (Kinetex 2.6u XB-C18 100A 75x4.6mm, Phenomenex, Torrence, CA) and a mobile phase consisting of 25mM H3PO4, 25mM Citric Acid, 500mg/L OSA, 0.35% ACN, pH 3.3. Thiols were detected

using a Decade II electrochemical detector (Antec Leyden; The Netherlands) with a single cell MagicDiamond electrode set at +1.80V.

Glutamate HPLC: Baseline samples were run on glutamate HPLC coupled to fluorescence detection. Precolumn derivatization of glutamate with o-pthalaldehyde was performed using a Shimadzu LC10AD VP autosampler. The mobile phase consisted of 13% acetonitrile, 100 mM Na_2HPO_4 and 0.1 mM EDTA, pH = 5.90. Glutamate was separated using a reversed-phase column (3 µM; 100 x 4.2 mm; Bioanalytical Systems, West Lafayette, IN), and detected using a Shimadzu 10RF-AXL fluorescence detector with an excitation and emission wavelength of 320 and 400 nm, respectively (Baker et al., 2002).

Western Blotting: Animals were rapidly decapitated (PND 100+) and punches taken from the medial prefrontal cortex. Tissue was homogenized in RIPA + protease inhibitor cocktail and protein concentration was determined by the BCA assay method. 20 µg of homogenized denatured protein was run on 10% tris-gly PAGE gel and transferred to PVDF membrane. The membrane was blocked in 5% non-fat dry milk solution for one hour and subsequently incubated with a custom antibody against the C-terminal of xCT (Prosci, rabbit anti-xCT 1:500) overnight at 4° C. The blots were washed and incubated with the secondary antibody (Omega anti-rabbit 1:10,000) and the signal was detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Bands were quantified using Kodak Molecular Imaging software (Eastman Kodak Company) and normalized with GAPDH control.

Histology: Tissue slices obtained from rats included in the microdialysis studies were stained with cresyl violet to verify probe placements. Rats determined to have misplaced guide cannula were excluded from all analyses.

Statistics: MAM treated offspring were compared to vehicle treated offspring given the same treatment as adults using *t* test.

Results

Attentional set shifi: Evidence supports MAM as a neurodevelopmental model of schizophrenia since it produces a range of symptoms similar to those observed in schizophrenia. The contribution of system x_c^- to MAM-induced cognitive deficits has not been established. Attentional set shifting is a complex cognitive task that tests three types of learning and memory: visual cue learning (day 1), attentional set shift (day 2) and reversal learning (day 3). The task presented to the animals is shown in Figure 3.3, day one the arm with the visual cue contains the reward (Figure 3.3a), day two requires a shift in attention from a visual cue to a spatial cue for reward (Figure 3.3b) and reversal learning on day three is when the opposite arm of day 2 contains the reward (Figure 3.3c). Acute and chronic with a wash out period treatment of MAM treated offspring is shown in Figure 3.1.

The impact of MAM treatment in offspring in a complex cognitive task is illustrated in Figure 3.4. MAM treated animals showed a significant deficit in visual cue learning (Figure 3.4a, $T = 2.96$, $p < 0.05$) and not in the attentional set shift task (Figure

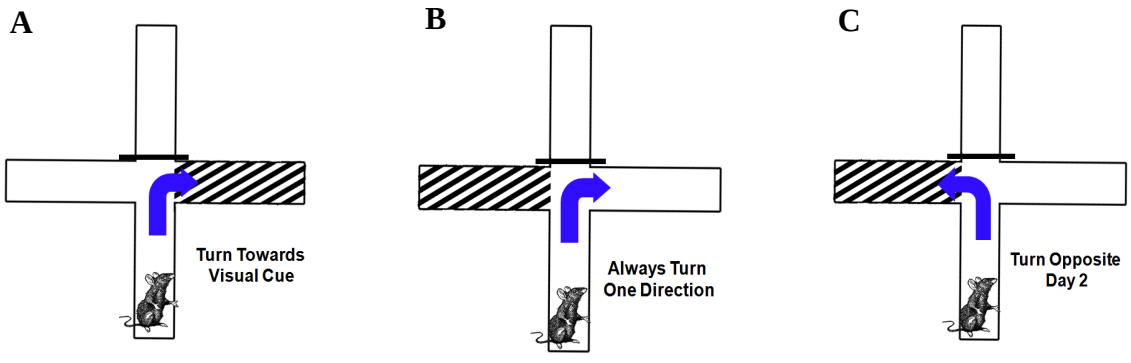


Figure 3.3 Attentional set shifting task. A) Response discrimination (day 1); animals are trained to turn towards the visual cue. B) Attentional set shift (day 2); animals are trained to turn in one direction. C) Reversal learning (day 3); animals must turn opposite the direction they went on day 2.

3.4b, T = 0.21, p > 0.05). Further, reversal learning deficits were also found in MAM treated offspring on day 3 (Figure 3.4c, T = 2.57, p < 0.05). To test the involvment of system x_c^- in attentional set shifting, direct manipulations of the transporter are necessary.

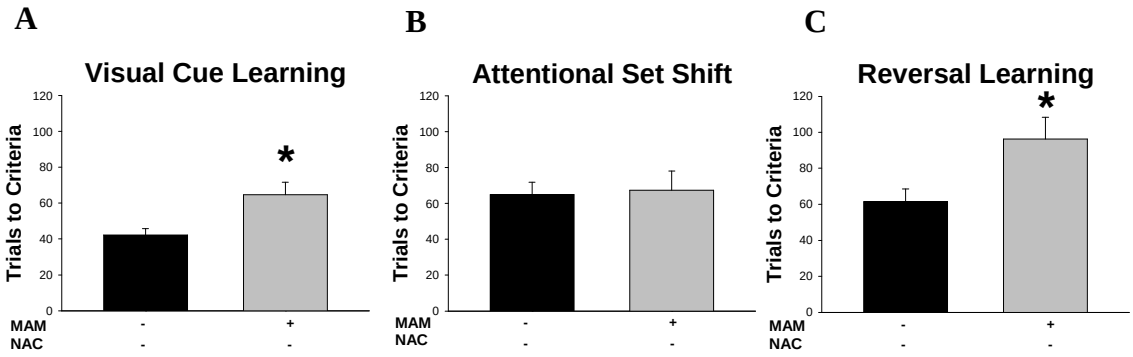


Figure 3.4 Attentional set shift in MAM treated offspring. MAM induced deficits (*N* = 10-11) in attentional set shift on day 1 (visual cue learning) (A), day 2 (attentional set shift) (B) and day 3 (reversal learning) (C). * indicates significant difference from MAM 0 / NAC 0 treated controls using *t* test.

Next, the impact of system x_c^- on attentional set shifting were analyzed. Chapter 5 will examine the impact of reduced cystine-glutamate exchange; here the effects of system x_c^- stimulation were measured in control and MAM treated offspring by N-acetylcysteine treatment which has been shown to drive cystine-glutamate exchange. Figure 3.5 depicts the effect of N-acetylcysteine administration either acutely one hour before each testing day or after chronic treatment with a wash out period (as shown in Figure 3.1). Whereas MAM treated offspring show a deficit in the simple discrimination task of visual cue learning (Figure 3.4a), there was no significant difference of MAM compared to vehicle treated offspring with acute or chronic with a wash N-acetylcysteine treatment (Figure 3.5 a and d, T = 0.74 and T = 0.56 respectively, p > 0.05). Similarily, day 2 attentional set shift had no effect of treatment (Figure 3.5 b and e, T = 0.36, T =

0.21 respectively, $p > 0.05$). Interestingly, N-acetylcysteine acute and chronic treatment attenuated MAM-induced deficits in reversal learning on day 3 shown by no significant difference from control animals with same treatments (Figure 3.5c and f, T = 1.51, T = 1.43 respectively, $p > 0.05$) using *t* test. These data suggest that system x_c^- may be altered in MAM animals since targeting system x_c^- is capable ofattenuating cognitive deficits .

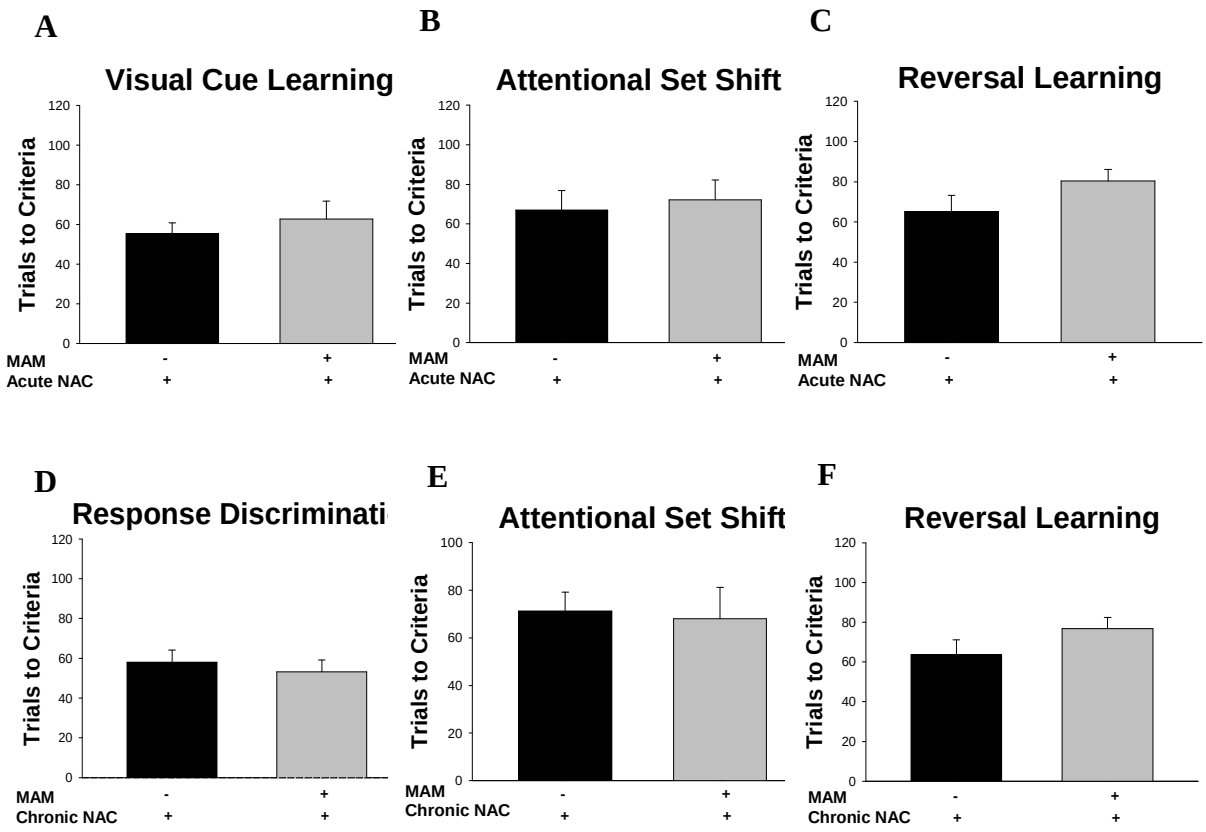


Figure 3.5 Attentional set shift in MAM with N-acetylcysteine. Attentional set shifting as shown on day 1 (visual cue learning), day 2 (attentional set shift) and day 3 (reversal learning). Panels A-C are the effects of acute N-acetylcysteine on each day of testing (60 mg/kg, IP; *N* = 8-9) and D-F are chronic N-acetylcysteine with a wash-out period on MAM induced deficits in attentional set shifting (60 mg/kg, IP; *N* = 7-8). As shown, there are no evident MAM-induced deficits on visual cue learning or reversal learning with N-acetylcysteine treatment.

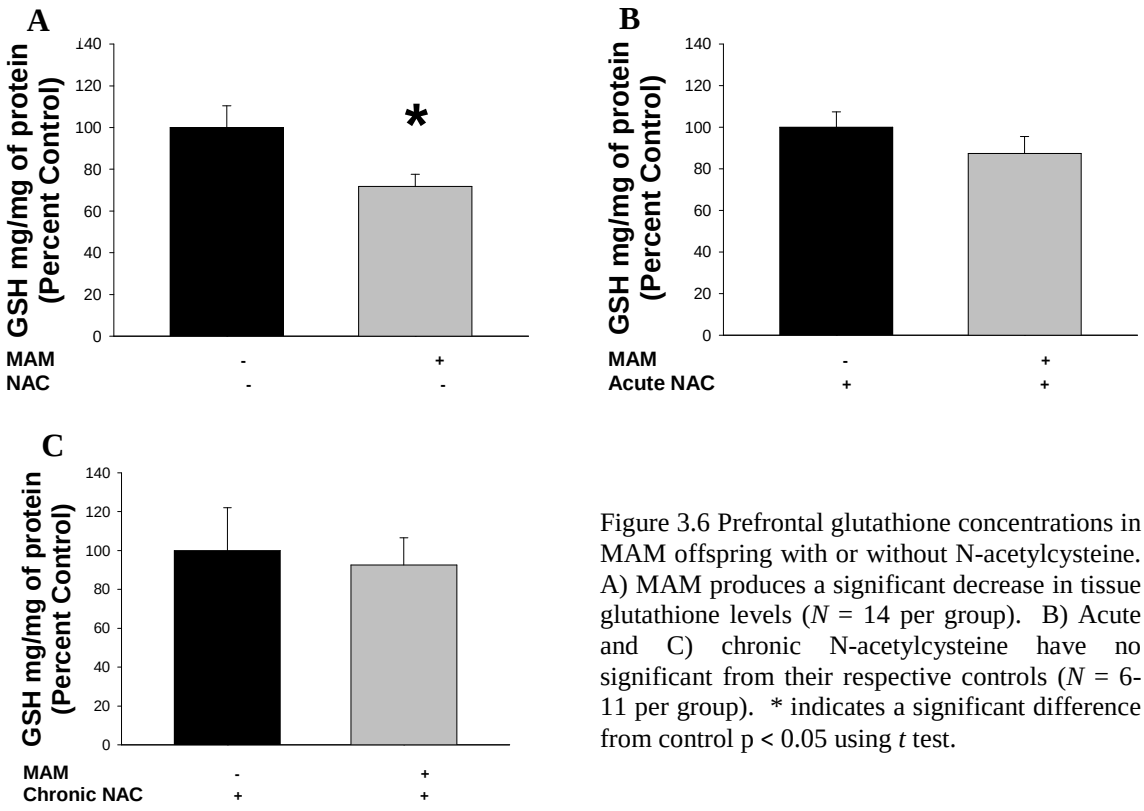


Figure 3.6 Prefrontal glutathione concentrations in MAM offspring with or without N-acetylcysteine. A) MAM produces a significant decrease in tissue glutathione levels (*N* = 14 per group). B) Acute and C) chronic N-acetylcysteine have no significant from their respective controls (*N* = 6-11 per group). * indicates a significant difference from control $p < 0.05$ using *t* test.

Measures of system x_c^- in MAM treated offspring: Since N-acetylcysteine attenuated MAM-induced deficits in reversal learning, the next step was to investigate the status of system x_c^- activity in MAM offspring. Glutathione levels are sensitive to cystine-glutamate exchange since cystine uptake is the rate limiting step in glutathione synthesis (Sagara et al., 1993; Dringen and Hirrlinger, 2003). Figure 3.6a depicts MAM-induced reductions in tissue glutathione content in the prefrontal cortex ($T = 2.36$, $p < 0.05$). Interestingly, both acute (Figure 3.6b, $T = 0.30$, $p > 0.05$) and chronic N-acetylcysteine with a wash (Figure 3.6c, $T = 1.15$, $p > 0.05$) showed no significant differences from their respective controls suggesting that N-acetylcysteine is able to restore tissue gltuathione levels in the prefrontal cortex. Deficits in glutathione levels suggest abnormal glutathione cycling and or reduced cystine-glutamate exchange.

The ability of N-acetylcysteine to improve MAM-induced deficits in glutathione concentrations suggests abnormal cystine-glutamate exchange since N-acetylcysteine has been shown to drive activity (Baker et al., 2008; Kau et al., 2008). An *ex vivo* approach was used to measure system x_c^- activity using ^{14}C cystine uptake with and without a system x_c^- inhibitor (CPG) to measure cystine-glutamate antiporter dependent activity within tissue punches taken from the prefrontal cortex. Suprisingly, MAM treated offspring showed a significant increase in *ex vivo* system x_c^- dependent uptake from punches of the prefrontal cortex (Figure 3.7a, $T = 2.62$, $p < 0.05$) that does not return to baseline with acute N-acetylcysteine treatment (Figure 3.7b, $T = 1.88$ $p = 0.09$). Interestingly, Figure 3.7c illustrates that chronic N-acetylcysteine with a wash out period

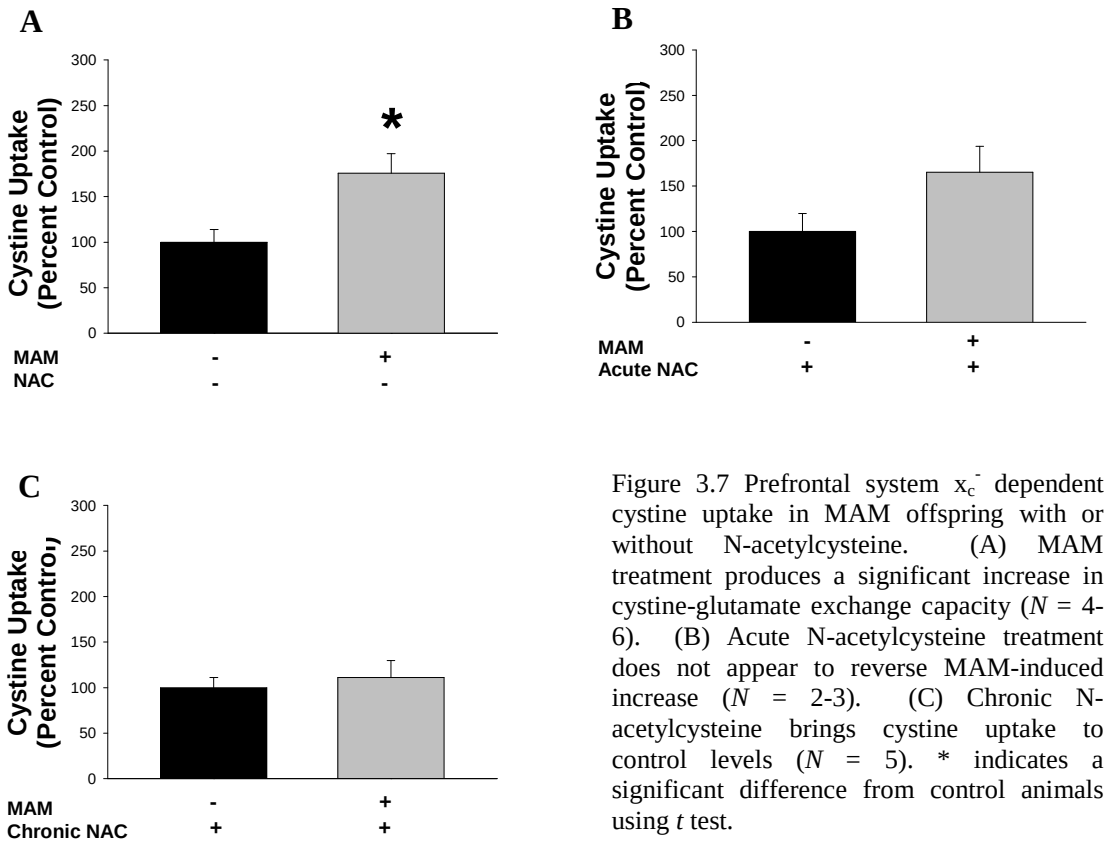


Figure 3.7 Prefrontal system x_c^- dependent cystine uptake in MAM offspring with or without N-acetylcysteine. (A) MAM treatment produces a significant increase in cystine-glutamate exchange capacity (*N* = 4-6). (B) Acute N-acetylcysteine treatment does not appear to reverse MAM-induced increase (*N* = 2-3). (C) Chronic N-acetylcysteine brings cystine uptake to control levels (*N* = 5). * indicates a significant difference from control animals using *t* test.

inhibited the MAM-induced increased capacity in system x_c^- activity (T = 0.11, p = 0.91). An outstanding question of the data showing deficient glutathione in the presence of increased potential for system x_c^- activity is whether this represents an *in vivo* increase in cystine-glutamate exchange as a compensatory effect of reduced glutathione concentrations.

To examine this question a cystine no-net flux experiment was done to measure extracellular cystine concentrations and to determine whether the apparent increase in cystine-glutamate exchange suggested by the *ex vivo* data occurs *in vivo*. Figure 3.8a illustrates cystine no-net flux microdialysis tartgeting the prefrontal cortex of control and MAM offspring. Both the x-intercept, where the trend line crosses the x-axis which is the point with no net flow between the probe and extracellular space which indicates the extracellular cystine concentration (control = 0.44 ± 0.13, MAM = 0.40 ± 0.07) and extraction fraction indicating cystine clearance rate (control = 0.86 ± 0.05; MAM = 0.96

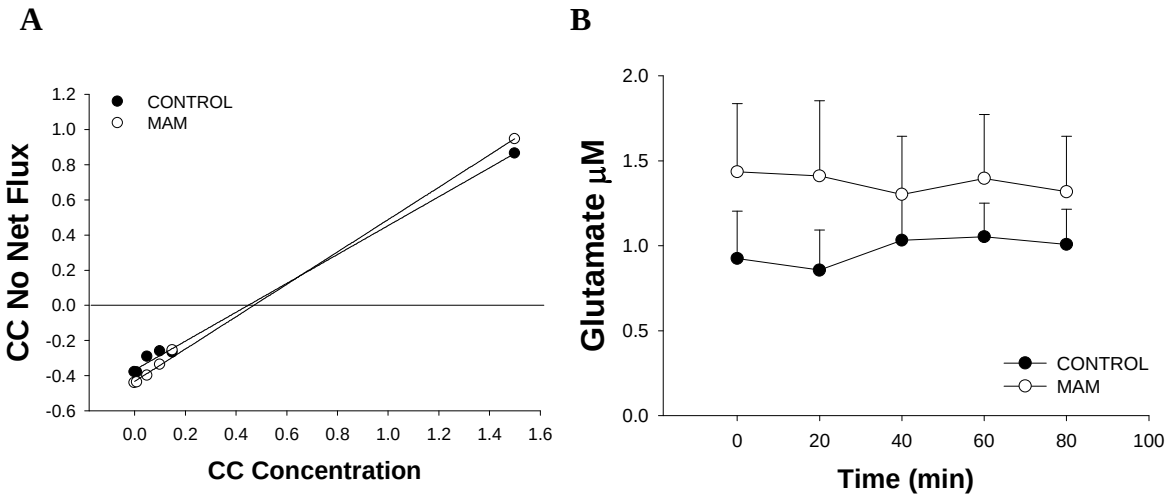


Figure 3.8 Prefrontal cystine no-net flux microdialysis in MAM offspring. (A) indicates cystine (CC) no-net flux in the prefrontal cortex and shows no significant differences between extracellular cystine levels or extraction fraction (*N* = 8). Additionally, there is no change in baseline glutamate levels (B) in the same animals in the prefrontal cortex of MAM offspring compared to control (*N* = 8).

± 0.03) showed no significant differences between the groups (Figure 3.7a; T = 1.78 and 0.23 respectively, p > 0.05). Additionally, baseline glutamate levels from this experiment also indicate no signficant differences between groups shown in Figure 3.8b. A repeated measure with treatment as a between subjects factor and time as a repeated measure shows no interaction ($F_{(4, 52)} = 0.23$, p > 0.05), main effect of treatment ($F_{(1, 13)} = 0.53$, p > 0.05) or time ($F_{(4, 52)} = 0.37$, p > 0.05). These results suggest no change in *in vivo* cystine-glutamate exchange.

An additional measure of in vivo system x_c^- is measuring protein expression of xCT, the active subunit of the cystine-glutamate antiporter. Using an anti-xCT antibody and measuring the projected 55 kDa band which others have used to measure xCT, MAM treated offspring have no differences in xCT expression, however there is not a consensus regarding anti-xCT antibodies or even which band represents functional xCT expression. Figure 3.9a is a representative blot with quantitative values of the 55 kDa band in Figure 3.9b. Taken together, these data suggest that adult MAM offspring have decreased glutathione concentrations and a potential increase in system x_c- activity *ex vivo* yet no

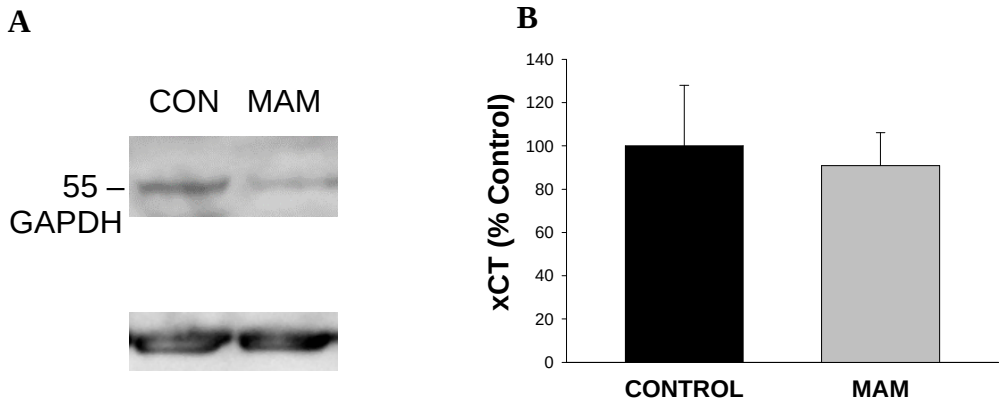


Figure 3.9 Western blot of xCT, the active subunit of system x_c^-, in the medial prefrontal cortex of control and MAM treated offspring. (A) representation of western blot from control and MAM treated animal. Note, the 55 kDa band was used for quantitative measures of xCT expression (B) using the ProSci antibody however the other bands could represent splice variants, degradation products or different cellular localization of system x_c^-.

apparent differences in cystine-glutamate exchange *in vivo*.

Juvenile measures of system x_c^- in MAM treated offspring: Adult MAM-treated offspring exhibit diminished glutathione levels (Figure 3.6a) in the prefrontal cortex with an apparent compensatory increase in the capacity of system x_c^- activity (Figure 3.7a), yet *in vivo* evidence of increased cystine-glutamate exchange was not obtained. Measures of juvenile system x_c^- activity were conducted in an attempt to learn when these changes emerged. To determine this, juvenile (PND 28-40) measures of glutathione and cystine-glutamate exchange were examined. Interestingly, glutathione, as shown in Figure 3.10a is not altered compared to controls in prefrontal tissue concentrations ($T = 0.79$, $p > 0.05$). However, similar to adults, juveniles also have a significant increase in system x_c^- dependent cystine uptake in the prefrontal cortex (Figure 3.10b, $T = 2.67$, $p < 0.05$). One possible interpretation of this data is that system x_c^- is upregulated in response to an earlier deficit in glutathione and, during adolescence, is sufficient to normalize glutathione concentrations. Potentially, as the brain continues to mature and develop, elevated system x_c^- activity is no longer sufficient and the glutathione deficit emerges.

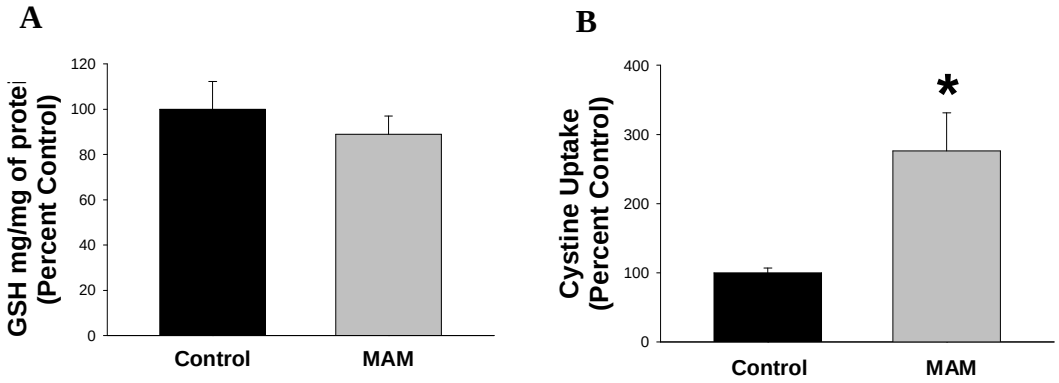


Figure 3.10 Measures of system x_c^- activity in juvenile MAM offspring. (A) Prefrontal glutathione in MAM offspring (*N* = 5-9). (B) Illustrates a significant increase in ex vivo prefrontal cystine uptake in MAM offspring compared to control (*N* = 7-8 per group). * denotes significance from control animals using *t* test.

Discussion

Chapter 2 illustrates the ability of system x_c^- in the prefrontal cortex to reverse phencyclidine-induced deficits in sensorimotor gating. This chapter seeks to establish the activity of the cystine-glutamate antiporter in the MAM neurodevelopmental model of schizophrenia. MAM-induced cognitive deficits in reversal learning, as measured in the attentional set shifting task, are attenuated by stimulation of system x_c^- with N-acetylcysteine either acutely and even after a two week wash out period from chronic dosing. Furthermore, both acute and chronic N-acetylcysteine treatment restores a MAM-induced prefrontal tissue glutathione deficit, and interestingly, chronic N-acetylcysteine also normalizes the significant increase in *ex vivo* system x_c^- activity in MAM animals. However, MAM animals do not show *in vivo* evidence of increased system x_c^- activity as shown in the cystine no-net flux study and xCT expression. Lastly, juvenile studies of MAM treated offspring have normal prefrontal glutathione tissue concentration in the presence of a significant increase in system x_c^- activity. Taken together, these results indicate abnormal cystine-glutamate exchange in the MAM model of schizophrenia.

MAM-induced behavioral deficits: Many of the diverse range of symptoms associated with schizophrenia are induced by prenatal MAM treatment and follow similar manifestations. For example, MAM treated offspring show hyperstimulation to amphetamine and phencyclidine in locomotor studies (Le Pen et al., 2006; Lodge and Grace, 2007; Flagstad et al., 2004; Phillips et al., 2012) simulating positive symptoms of

schizophrenia (Sturgeon et al., 1979; Enomoto et al., 2007; Young et al., 2010). Interestingly, similar to schizophrenia, this effect does not emerge until adulthood (Lieberman et al., 2001; Le Pen et al., 2006). Further, negative symptoms are modeled with social interaction deficits; MAM treated offspring show deficits both pre and post-pubertal, also similar to emergence of social deficits prior to diagnosis (Le Pen et al., 2006; Flagstad et al., 2004; Lieberman et al., 2001). Finally, there have been numerous cognitive deficits reported including sensorimotor gating and behavioral flexibility (Le Pen et al., 2006; Moore et al., 2006; Flagstad et al., 2005; Featherstone et al., 2007). The behavioral deficits in the MAM model summarized here and morphological and neurochemical data described elsewhere parallels many features of schizophrenia.

Executive functioning deficits including behavioral flexibility are core features of schizophrenia and are the best predictors of social dysfunction (Kurtz et al., 2005; Liddle, 2000). Behavioral flexibility is the ability to shift attention between different rules or strategies; the Wisconsin Card Sorting task measures deficits in schizophrenics, attentional set shifting measures deficits in rodents (Morice, 1990; Prentice et al., 2008; Floresco et al., 2009). This chapter examines MAM-induced deficits in attentional set shifting. There is a significant increase in trials to criteria in MAM offspring compared to control on day one of visual cue learning indicating impairments in a simple discrimination task. It is important to note that control animals that received N-acetylcysteine show similar trials to criteria with MAM offspring suggesting that while interesting, more investigations will need to be conducted to fully understand simple discrimination deficits in MAM offspring. Surprisingly, there was no effect of MAM or N-acetylcysteine treatment on day 2 for the attentional set shift. This was unexpected

since attentional set shifting is a complex task mediated by prefrontal cortical activity, an area with a significant amount of xCT mRNA (Floresco et al., 2008; see Chapter 2).

Reversal learning tests the flexibility of the animal when reinforcement strategies are reversed, schizophrenics also have difficulties with reversal learning (Waltz and Gold, 2007; Murray et al., 2008). MAM treated offspring require significantly more trials to reach criteria in the reversal learning phase of attentional set shifting (Figure 3.4c). This is consistent with reversal learning impairments in MAM offspring in other tasks (Flagstad et al., 2005; Featherstone et al., 2007; Gastambide et al., 2012). Reversal learning deficits have been linked to the orbitofrontal cortex in both humans and rodents (Waltz and Gold, 2007; Ghods-Sharifi et al., 2008) a region also shown to have lower PV expression in MAM treated offspring (Gastambide et al., 2012). What is unknown is the ability of system x_c^- to regulate reversal learning.

Interestingly, stimulation of system x_c^- with N-acetylcysteine treatment was able to attenuate MAM-induced deficits in reversal learning. N-acetylcystine has been shown in preclinical studies to exert its therapeutic potential by stimulation of cystine-glutamate exchange (Kau et al., 2008; Baker et al., 2008). During acute administration, animals received N-acetylcysteine injections (60 mg/kg, IP) one hour before testing began on all three days. Whereas acute treatment tests the effect of direct manipulation of cystine-glutamate exchange, chronic treatment with a wash out period of at least 14 days examined the ability of N-acetylcysteine to have therapeutic efficacy even after termination of treatment. This could be due to the normalization of glutathione leading to normal glutathione cycling for long term stabilization of system x_c^- which is driven by extrasynaptic cystine. Interestingly, both treatment regimens attenuate MAM-induced

deficits in reversal learning (Figure 3.5c and f). Of note, N-acetylcysteine had no effect in the absence of behavioral deficits in control animals on all three phases of testing or on MAM offspring in the absence of deficit (attentional set shift). This suggests that system x_c^- is working at full capacity and an increase in extracellular cystine is insufficient to improve performance or there are compensatory mechanisms within the circuitry to prevent a change in system x_c^- activity in the absence of a deficit. In order to determine the potential effects of N-acetylcysteine, it is important to understand the state of the antiporter in MAM treated offspring.

System x_c^- in MAM treated offspring: One way to examine system x_c^- activity is by measuring glutathione concentrations. Glutathione is dependent on astrocytic cystine uptake for synthesis; system x_c^- is a key transporter for astrocytic cystine (Dringen and Hirrlinger, 2003; Bannai, 1984; Bridges, 2012). Further, neurons lack the machinery to transport cystine and rely on astrocytic glutathione release and breakdown into cysteine which is taken up by neurons for glutathione synthesis (for review see Dringen and Hirrlinger, 2003). As such, glutathione levels are a functional measure of *in vivo* system x_c^- activity and reductions in concentrations suggest decreased cystine-glutamate exchange. Additionally, up to 50% reduction in dorsolateral prefrontal cortical glutathione concentrations has been found in schizophrenics (Do et al., 2000; Gawryluk et al., 2010; Raffa et al., 2011). The medial prefrontal cortex was examined since a) there is strong mRNA expression of xCT in the area (Chapter 2), b) abnormal dorsolateral prefrontal cortical activity is indicated in schizophrenics and prelimbic prefrontal cortex is the rodent analog (Callicott et al., 2000; Bunney and Bunney, 2000; Uylings et al.,

2003) and c) other labs have found morphological differences in MAM treated offspring in the prefrontal cortex including volume, neuronal size and disruptions in network synchrony (Flagstad et al., 2004; Moore et al., 2006; Goto and Grace, 2006). Similar to human schizophrenics, *MAM treated offspring have reduced prefrontal tissue glutathione concentrations* (Figure 3.6a). The questions now are why are glutathione levels decreased and when do levels change compared to control levels?

Deficient glutathione concentrations may result from compromised cystine uptake or conversely, cause an increase in cystine transporters. The results presented in this chapter support the latter with a significant increase in *ex vivo* system x_c^- dependent cystine uptake (Figure 3.7a). This is not unexpected since depleted glutathione has been shown to increase the active subunit of system x_c^- (xCT) and cystine-glutamate exchange as a compensatory response to increase glutathione synthesis (Seib et al., 2011). Further, similar to MAM, schizophrenics have increased expression of xCT in the dorsolateral prefrontal cortex (Baker et al., 2008). However, for some as yet unknown reason, in adult MAM treated offspring this response fails to normalize levels of glutathione. What is shown is no evidence for increased cystine-glutamate exchange *in vivo* in the cystine no-net flux study.

Microdialysis no-net flux studies are useful experiments to measure *in vivo* concentrations and clearance efficiency (Smith and Justice, 1994). Cystine no-net flux in MAM treated offspring show no changes in baseline extrasynaptic cystine levels nor changes in extraction fraction which measures cystine clearance compared to control animals (Figure 3.8) even with the potential increase in *ex vivo* capacity of system x_c^-. Further, there is no change in basal extrasynaptic glutamate levels. This raises an

interesting question as to why an apparent need (decreased glutathione) and ability (increased *ex vivo* potential) for increased cystine uptake does not result in increased *in vivo* cystine-glutamate exchange compared to controls. Endogenous regulation of system x_c^- is poorly understood (for review see Bridges, 2012) and likely dependent on a multitude of neurotransmitters and protein kinases making it difficult to assess. What is known is the ability of N-acetylcysteine to drive cystine-glutamate exchange (Kau et al., 2008; Baker et al., 2008; Chapter 2).

A western blot analyzed expression of xCT, the active subunit of the cystine-glutamate antiporter in control and MAM treated offspring. There is controversy surrounding all available antibodies about the specificity to xCT and whether the various bands represent different isoforms, expression patterns or degradation products. Using the ProSci antibody against xCT and measuring the 55 kDa band which others have used (Knackstedt et al., 2010), there was no difference in MAM expression of xCT compared to controls in the medial prefrontal cortex. So while there does not appear to be a change in xCT expression, it is quite possible that a different antibody or a different band would give different results and is therefore inconclusive.

Glutathione and *ex vivo* cystine-glutamate exchange were also measured in the presence of N-acetylcysteine. Acute N-acetylcysteine (60 mg/kg, IP) was given one hour before animals were euthanized and tissue analyzed. Treatment normalized prefrontal tissue glutathione concentrations to control levels and decreased *ex vivo* cystine uptake by system x_c^- without returning to control N-acetylcysteine treated animal baseline. This is not an indication of the inability of acute N-acetylcysteine to drive cystine-glutamate exchange since some of N-acetylcysteine is likely washed out during the course of the

assay. Interestingly, the ability of chronic N-acetylcysteine treatment with a 14 day wash out period similar to the behavioral experiment normalized system x_c^- activity. Both glutathione and *ex vivo* system x_c^- dependent cystine uptake were normalized to control N-acetylcysteine animals even after the wash out period suggesting plasticity within the circuit to normalize cystine-glutamate exchange that extends beyond the termination of treatment. What is most important is the behavioral translation. Indeed, both acute and chronic with a wash out period attenuated reversal learning deficits in MAM treated offspring. Taken together, N-acetylcysteine treatment normalizes MAM-induced deficits in glutathione, *ex vivo* measures of cystine-glutamate exchange and attenuates MAM-induced deficits in reversal learning. A remaining question is when do changes in system x_c^- occur during neurodevelopment?

Juvenile measures of system x_c^- in MAM treated offspring: Juvenile testing of system x_c^- was examined to determine when altered glutathione concentrations and cystine-glutamate exchange emerge during development. While there was a modest decrease that did not reach significance in tissue prefrontal glutathione levels in juveniles (Figure 3.10a), there was a significant increase in *ex vivo* potential for cystine-glutamate exchange similar to adults (Figure 3.10b). This may suggest an earlier developmental glutathione deficit causing a compensatory upregulation of system x_c^-. Indeed depleted glutathione has shown to increase xCT and functional cystine-glutamate exchange (Seib et al., 2011). Further, this may be sufficient to restore glutathione concentrations at this age but after puberty, with increased antioxidant demand as a result of aging (for review see Hybertson et al., 2011) or another factor preventing elevated cystine-glutamate

exchange, is unable to do so. Interestingly, other labs have reported shifts in behavior between juveniles and adults of MAM treated offspring. Locomotor hyperstimulation to amphetamine and NMDA receptor antagonists only occurs after puberty while social interaction deficits are evident prior to puberty, similar to human schizophrenia development (Le Pen et al., 2006; Lieberman et al., 2001). Attempts at juvenile set shifting were conducted however the animals had difficulties in attending to the task. This suggests some process is occurring during puberty allowing additional symptoms to emerge. Evidence presented here indicates a neurodevelopmental shift between adolescence and adulthood that may contribute to behavioral manifestation of symptoms associated with schizophrenia in MAM treated offspring.

Contribution of glutathione and glutamate to neurotransmission: An remaining question is: how does N-acetylcysteine improve MAM-induced deficits in reversal learning? As described above, acute and chronic with a wash treatment normalizes glutathione and chronically restores *ex vivo* cystine-glutamate exchange. Glutathione is the main antioxidant in the central nervous system and regulates redox potentials (Cooper and Kristal, 1997; Sies, 1999; Dringen, 2000). NMDA receptor currents are potentiated when glutathione reduces the NMDA receptor extracellular redox site (Kohr et al., 1994; Lipton et al., 2002). Interestingly, reduced glutathione levels leads to oxidation and hypoactivity of the NMDA receptor (Steullet et al., 2006); this is important given the critical role NMDA receptor activity has in neurotransmission. N-acetylcysteine also stimulates system x_c^- elevating extrasynaptic glutamate to a level capable of activating mGluR3/2 receptors thereby also regulating synaptic neurotransmission (Kau et al., 2008;

Baker et al., 2008; Baker et al., 2002b; Moran et al., 2005). This is important since studies in the medial prefrontal cortex have shown low or absent slow and fast local field potential oscillations in MAM treated offspring (Goto and Grace, 2006) which suggest disruption of network synchrony; schizophrenics have evidence of abnormal network synchrony as well (Steriade et al., 1993; Traub et al., 1998). Further, studies in the hippocampus have shown increased neuronal excitability (Lodge and Grace, 2007; Sanderson et al., 2012). This is hypothesized to occur by reduced GABAergic interneuron activity leading to disinhibition of pyramidal cells in MAM treated offspring (Lodge et al., 2009; Gill et al., 2011; Sanderson et al., 2012) which interestingly is also observed in schizophrenics (for review see Lewis et al., 2012). Disinhibition of pyramidal neurons results in network desynchronization which is necessary for gamma oscillations and cognition (Cobb et al., 1995; Sohal et al., 2009; Cardin et al., 2009; Howard et al., 2003; Tallon-Baudry et al., 2004). While GABA interneurons regulate pyramidal neuron activity, system x_c^- also has the capacity to regulate pyramidal neurons through modulation of neurotransmission by stimulation of mGluR2/3 (Conn and Pin, 1997; Baker et al., 2002b; Baker et al., 2003; Baskys and Malenka, 1991; Chaki and Hikichi, 2011).

Figure 3.11 proposes circuitry in a "normal" (A) and MAM (C) prefrontal cortex with a typical (B) and atypical (D) synapse onto a pyramidal dendritic spine. Note the positioning of basket cell parvalbumin positive GABAergic interneurons on soma body modulating pyramidal neuron activity and system x_c^- on astrocytes modulating input onto pyramidal neurons. Both systems regulate pyramidal neuron excitability allowing for synchronization and modulation of projections from the region. GABAergic interneurons

are necessary to regulate pyramidal neurons for network oscillations (for review see Lewis et al., 2012) and system x_c^- has been shown to modulate neurotransmitter release (Baker et al., 2002b).

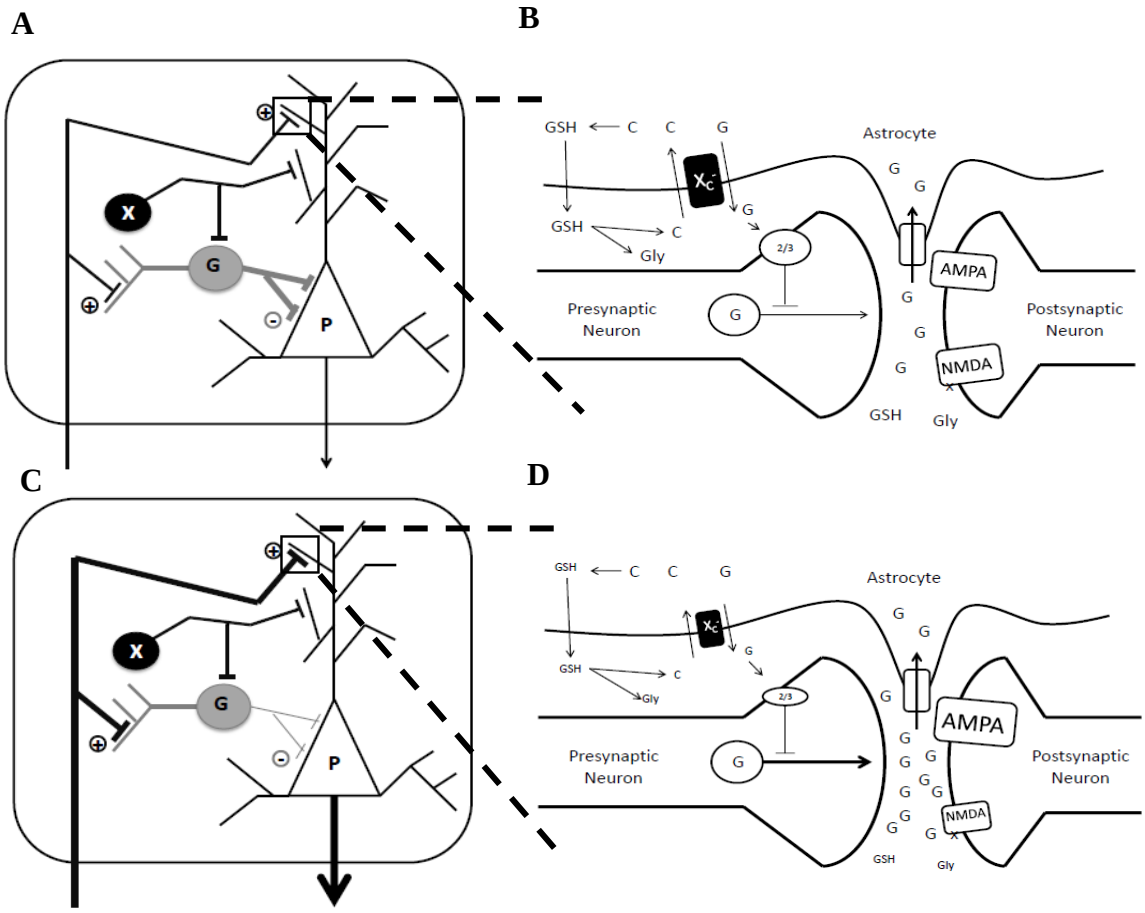


Figure 3.11 Neural circuitry and synaptic connections within brain regions implicated in schizophrenia such as the hippocampus and prefrontal cortex. (A, C) Pyramidal neurons labeled with a P are the main excitatory output of the region and receives inputs from other neurons within and outside the structure indicated by black lines which can be stimulatory, inhibitory or modulatory. Fast spiking basket cell parvalbumin containing GABAergic interneurons (dark gray marked with G are uniquely positioned to inhibit the soma of pyramidal neurons to modulate neuronal activity. X indicates a nonspecific neuron within the region aiding in the synchronization of the circuit. Normal circuitry is indicated in panel A whereas schizophrenic or MAM hypothesized abnormal circuitry is in C. Notice a decrease in GABAergic activity subsequently leading to enhanced pyramidal cell output and desynchronization. Panels B and D depict the synaptic cleft in the box from panels A and C respectively. The presynaptic neuron may release neurotransmitters such as glutamate (G), dopamine and GABA. System x_c^- exchanges glutamate for cystine (C), allows for glutathione (GSH) synthesis and tonic tone on metabotropic group II glutamate receptors (2/3) to modulate synaptic neurotransmitter release. Panel B indicates a typical synapse whereas panel D is the hypothesized schizophrenic and MAM synapse.

Conclusion: One interpretation of the data is that some yet unknown event or process prevents normal glutathione levels early in development. A compensatory response is upregulation of cystine-glutamate exchange to elevate intracellular cystine promoting glutathione synthesis. While this appears to compensate for juvenile glutathione levels, it is insufficient in adulthood as evidence from decreased glutathione in the presence of increased potential for cystine-glutamate exchange. Further, N-acetylcysteine restores glutathione when administered in adulthood and alleviates MAM-induced cognitive deficits in reversal learning. This is possible due to restored glutathione potentiating NMDA receptor activity or through increased tone on mGluR2/3 receptors modulating synaptic neurotransmitter release likely both aiding in synchronization of pyramidal cells necessary for cognitive function.

IV. SIMULTANEOUS STIMULATION OF CYSTINE-GLUTAMATE EXCHANGE AND METHYLAZOXYMETHANOL ACETATE ADMINISTRATION: A TWIST ON A NEURODEVELOPMENTAL MODEL OF SCHIZOPHRENIA

Introduction

While the exact etiology of schizophrenia remains elusive, the neurodevelopmental hypothesis which posits an environmental insult during pre or postnatal neurodevelopment contributes to the symptoms of schizophrenia has emerged as likely contributing to the pathophysiology of schizophrenia (Weinberger, 1987; Marenco and Weinberger, 2000; Rapoport et al., 2005). Neurodevelopment is a highly regulated process relying on specific sequences of events and molecular machinery for normal development (Lewis and Levitt, 2002). Additionally, the neurotransmitters glutamate and GABA have been shown to have a crucial modulatory effect on migrating neuroblasts (Behar et al., 1998; Behar et al., 1999) (Behar et al., 2000; Hirai et al., 1999; Manent et al., 2005), importantly, nonvesicular neurotransmitter release drives neuronal migration. In support, munc18-1 and munc13-1/2 knockout mice which are incapable of vesicular neurotransmitter due to loss of vesicle priming, display normal cortical layering and synapse formation (Verhage et al., 2000; Varoqueaux et al., 2002).

Interestingly, the cystine-glutamate antiporter, which exchanges one extracellular cystine for one intracellular glutamate is capable of nonvesicular glutamate release (Baker et al., 2002b) and is thought to be expressed on immature neurons (Murphy et al., 1989; Murphy et al., 1990). While there are few if any studies identifying the specific source of nonvesicular glutamate or the contribution of system x_c^- in neuronal migration, research has shown the critical role system x_c^- has in postsynaptic development in *Drosophila*. Specifically, system x_c^- has been shown to impact postsynaptic glutamate receptor expression (Featherstone et al., 2002; Augustin et al., 2007).

This chapter utilizes the DNA methylating agent methylazoxymethanol acetate (MAM) (Nagata and Matsumoto, 1969) which has been shown to be an antimitotic agent (Dambska et al., 1982; Jongen-Relo et al., 2004), effect neuronal migration (Hoffman et al., 1996; Dehmelt and Halpain, 2004) and neuronal organization (Matricon et al., 2010). Furthermore, when given on gestational day 17 offspring have positive, negative and cognitive deficits similar to schizophrenia (Flagstad et al., 2004; Gourevitch et al., 2004; Flagstad et al., 2005; Le Pen et al., 2006; Moore et al., 2006; Featherstone et al., 2007); and specifically, as shown in Chapter 3, deficits in reversal learning. Also, while MAM-induced deficits in reversal learning are attenuated by adult stimulation of system x_c^- by N-acetylcysteine, complete reversal of the symptoms was not observed. Since evidence suggests MAM inhibits neuronal migration and extrasynaptic glutamate stimulates migration, the goal of this chapter is to ascertain whether stimulation of system x_c^- at the time of MAM insult is capable of reversing behavioral and neurochemical changes observed in MAM treated offspring.

The primary goal of this chapter is to determine whether in utero stimulation of system x_c^- by N-acetylcysteine is capable of reversing MAM-induced deficits in adult offspring.

Materials and Methods

Methylazoxymethanol Treatment: Pregnant Sprague-Dawley rats were given twice daily injections of N-acetylcysteine (0 or 60 mg/kg, IP; Sigma Chemical Co., St Louis, MO) dissolved in isotonic saline with NaOH to bring the pH to 7.0, beginning gestational day 17 and continuing until day 20. On gestational day 17 an acute injection of methylazoxymethanol acetate was also administered (22 mg/kg, IP; MRIGlobal Chemical Carcinogen Repository, Kansas City, MO). Following vaginal birth all mothers and offspring were left undisturbed until weaning on postnatal day 22. Male offspring were single caged for testing as adults (PND 60+).

Attentional Set Shifting: The maze consisted of four-arms (60 x 20 x 12 cm) in the shape of an X connected by a center area (12 x 12 cm); constructed of black Plexiglas with a movable wall to block an arm to form a "T" configuration. Dividers at the end of each arm concealed the sugar pellet reward such that the rat was unable to see it from the center of the maze. The visual cue, when used, was laminated with black and white diagonal stripes and placed on the floor of an arm. Prior to habituation, rats were handled for five minutes each day for seven days and food deprived to 85% of their free-feeding weight. The day before habituation, each rat was given approximately 30 sugar pellets in their home cage to acquaint them with the pellets.

Habituation: Day one, 5 pellets were placed in each arm, 3 throughout the length of the arm and 2 behind the divider at the end. The rat was placed in the center of the maze and allowed to freely explore for up to 15 minutes. On the second day, 3 pellets were placed in each arm, one in the center, the other 2 behind the divider and the rats freely explored

for up to 15 minutes. On the third day and all remaining days till criteria was achieved, one pellet was placed behind the divider in each arm. After the rat consumed a pellet behind the divider on any day, he was picked up and placed at the entrance to another baited arm to habituate him to handling within the maze. If the rat consumed all sugar pellets on any day, the maze was rebaited with the following habituation day's configuration or with 4 pellets, one behind each divider after habituation day 2. After the maze was rebaited 3 times and all pellets consumed within 15 minutes, criteria was met and turn bias was run the following day. If the rat did not consume all sugar pellets within 15 minutes, he continued habituation daily until he met criteria.

Turn bias: During turn bias and testing, one arm was blocked with the movable wall to form a "T" configuration with the visual cue in one of the side arms. In turn bias, both side arms were baited with sugar pellets behind the dividers. The rat was placed in the stem arm at the bottom of the "T" and allowed to enter either arm; after consuming the pellet he was placed back in the stem arm and again allowed to enter either arm. If he entered the same arm again, he was returned to the stem arm until he turned into the opposite arm and ate the remaining sugar pellet. When he successfully ate both sugar pellets, he was placed back in his home cage signaling the end of the trial. The next trial, the rat began from a different arm such that over 7 trials, the rat started from each of 3 arms 2 times and the remaining arm once and the visual cue was placed in either the right or left arm as equally as possible. Inter-trial time was about 10 seconds to rebait the maze. The direction the rat turned first the most out of 7 trials was considered his turn bias for the rest of testing.

Testing: As in turn bias, one arm was blocked with the movable wall, either the right or left arm contained the visual cue and the rat began in the stem arm. Trials were grouped into a block of 12 with the stem arm in arms 1-3 as shown in Figure 3.2. In the probe trial, arm 4 was the stem arm and the visual cue was placed in the turn bias arm. Within treatment groups, the probe arm switched such that at least one animal in each group used each arm as the stem arm for the probe trial. Each testing day required the following:

- 10 consecutive correct trials using the 12 trial block in stem arms 1-3
- 1 correct probe trial from stem arm 4
- If correct probe trial, testing was complete
    - If incorrect probe trial
        - 5 consecutive correct trials using the 12 trial blocks
        - 1 correct probe trial from stem arm 4
        - This continues till correct probe trial

Day 1: Visual-cue learning; see Figure 3.3a: On day one, the rat was trained in a simple discrimination task to attend to the visual cue such that the food reward was placed in each arm with the visual cue 50% of the time. Turing towards the visual cue and attaining the reward was a correct choice. In the probe trial the rat turned towards the visual cue.

Day 2: Attentional Set Shift; see Figure 3.3b: On day 2 the complex task of an attentional set shift required the rat to switch from a visual to a spatial cue with the new strategy for reward turning into the arm opposite of his turn bias. The probe trial required the rat to turn in the opposite direction of the visual cue.

Day 3: Reversal learning; see Figure 3.3c: Reversal learning required the rat to enter the arm opposite the strategy from day 2 such that the rats turn bias arm now contained the food reward and the rat turned towards the visual cue on the probe trial.

Tissue Glutathione Analysis: Animals were rapidly decapitated (PND 100+) and punches taken from the medial prefrontal cortex. The tissues were homogenized in a 4:1 dialysis buffer(5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.2 mM KCl, and 0.15% phosphate buffer saline, pH 7.4): mobile phase solution (75 mM $NaH_2PO_4 \cdot H_2O$, 1.8 mM $C_8H_{17}O_3Sna$, 25 μM EDTA, 0.1% TEA and CH_3CN pH to 3.0 with phosphoric acid). One fraction was analyzed in the BCA method to determine protein content. The other fraction analyzed thiol content using HPLC with EC detection (Decade II, MagicDiamond electrode set at +1.89V, Antec Leyden, Netherlands).

^{14}C Cystine Uptake: Animals were rapidly decapitated and brains extracted. All tissue punches of the prefrontal cortex were treated with TBOA (100 μM) and in the presence ^{14}C-L-cystine (approximately 0.3 μM). Experimental groups are MAM or control rat's male offspring with or without N-acetylcysteine treatments as described in each experiment (PND 130) in the presence or absence of (*S*)-4-carboxyphenylglycine (CPG, 1mM; Tocris-Cooksin, Ellisville, MO). The punches were washed and solubilized with 1% SDS. One fraction (200 uL) of the solubilized tissue was used for determining [^{14}C]-L-cystine uptake by a scintillation counter. The other fraction (25 uL) was used to determine protein content by the BCA method. Uptake was measured in counts/μg protein.

Statistics: MAM vehicle treated offspring were compared to MAM NAC treated offspring when tested as adults using *t* test.

Results

As shown in chapter 3, MAM treatment produced cognitive deficits and changes in system x_c^- in adult offspring. N-acetylcysteine treatment attenuated the behavioral deficits and, in the case of chronic N-acetylcysteine, normalized *ex vivo* ^{14}C cystine uptake. The animals presented in this chapter received N-acetylcysteine in utero and simultaneously with MAM treatment. Figure 4.1 depicts the ability of in utero N-acetylcysteine treatment to reverse MAM-induced deficits in reversal learning on day 3 in attentional set shifting. Similar to results observed in Chapter 3, there is no significant difference of N-acetylcysteine in MAM offspring on day one (Figure 4.1a; T = 1.12, p >

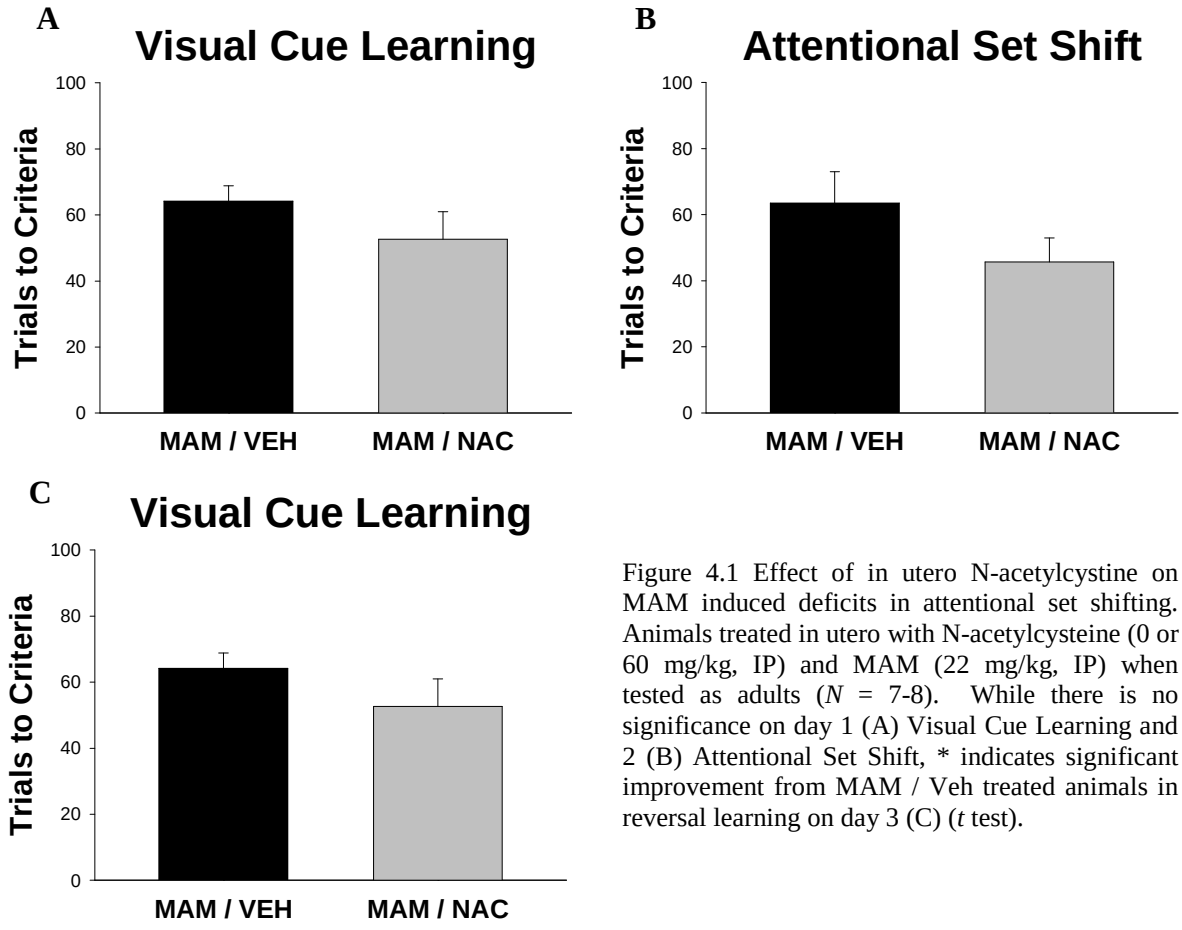


Figure 4.1 Effect of in utero N-acetylcystine on MAM induced deficits in attentional set shifting. Animals treated in utero with N-acetylcysteine (0 or 60 mg/kg, IP) and MAM (22 mg/kg, IP) when tested as adults (*N* = 7-8). While there is no significance on day 1 (A) Visual Cue Learning and 2 (B) Attentional Set Shift, * indicates significant improvement from MAM / Veh treated animals in reversal learning on day 3 (C) (*t* test).

0.05) or two (Figure 4.1b; T = 1.53, p > 0.05). Interestingly, whereas adult administration of N-acetylcysteine attenuated MAM-induced deficits in reversal learning, in utero N-acetylcysteine treatment completely reversed MAM-induced deficits (Figure 4.1c; T = 2.44 p < 0.05) using *t* test.

Measures of system x_c^- including glutathione and *ex vivo* ^{14}C cystine uptake with in utero N-acetylcysteine treatment are depicted in Figure 4.3. Figure 4.2a illustrates prefrontal cortical tissue glutathione concentrations with no difference between MAM vehicle treated animals and MAM-NAC treated animals (T = 0.57 p > 0.05). Similarly, there is no statistical difference between groups when measuring *ex vivo* prefrontal ^{14}C cystine uptake (Figure 4.2b, T = 1.69 p > 0.05).

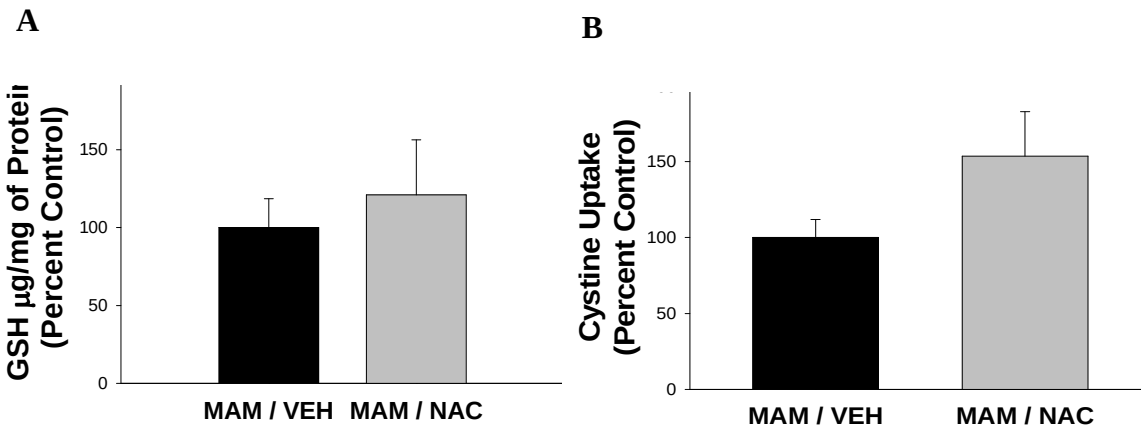


Figure 4.2 Effect of in utero N-acetylcystine on MAM-induced modifications in system x_c^- activity. N-acetylcysteine in utero does not alter adult MAM-induced tissue prefrontal glutathione concentrations (*N* = 14 and 5) but shows a slight but non-significant increase in *ex vivo* ^{14}C cystine uptake (*N* = 5 per group) using *t* test.

Discussion

There is a great amount of evidence supporting the neurodevelopmental hypothesis of schizophrenia (Weinberger, 1987; Chua and Murray, 1996; Marenco and Weinberger, 2000; Lewis and Levitt, 2002; Rapoport et al., 2005; Brown, 2011). This chapter investigates whether in utero stimulation of system x_c^- is capable of reversing behavioral deficits induced by the antimitotic agent MAM. As shown in Chapter 3 and here, MAM induces cognitive deficits in the attentional set shifting reversal learning task. As shown in Chapter 3, acute and subchronic adult administration of N-acetylcysteine attenuates MAM-induced cognitive deficits (Figure 3.4). Interestingly, N-acetylcysteine in conjunction with MAM administration in utero at the time of cortical neurogenesis and neuronal migration is able to completely reverse MAM-induced deficits in reversal learning. This occurs without significant differences in glutathione and system x_c^- activity measures. These data raise an interesting question, *what is N-acetylcysteine doing in utero to reverse MAM-induced behavioral deficits in reversal learning?*

To address this, it is necessary to understand the neurodevelopmental effects of MAM; it remains unclear the precise neuropathology induced by MAM. Studies conducted as early as the 60's identified the DNA methylating properties of MAM (Nagata and Matsumoto, 1969). Further research showed that MAM targets and inhibits cells undergoing active mitosis (Cattabeni and Di Luca, 1997; Hoareau et al., 2006). Following studies investigated timing of MAM administration and found that when given on gestational day 14 or 15, which coincides with early neuronal mitosis, MAM targets neuroepithelial cells undergoing active mitosis without effect on neuronal precursors or

post-mitotic migrating cells (Cattaneo et al., 1995). Further, it has a 12-24 hour window of activity emphasizing the importance of timing of injection, also, there is no known effect on other organs and it does not alter gestational parameters of the dam including gestation time and litter size (Matsumoto et al., 1972; Balduini et al., 1991). Before or on gestational day 15, treatment was found to produce broad reductions in brain weight presumably due to anti-proliferative actions of MAM (Dambska et al., 1982; Jongen-Relo et al., 2004). In the last decade, more labs administer MAM on gestational day 17; whereas day 15 affected many structures, day 17 treatment selectively targets regions that have been associated with schizophrenia including the prefrontal cortex, hippocampus and mediodorsal thalamus (Flagstad et al., 2004; Moore et al., 2006; Le Pen et al., 2006). Further, day 17 treatment produces similarities in behavioral symptoms to schizophrenia including deficits in sensorimotor gating, social interaction, hypersensitivity to amphetamines and NMDA receptor antagonists and cognitive impairments in adult offspring (Moore et al., 2006; Le Pen et al., 2006; Featherstone et al., 2007; Flagstad et al., 2005).

It is widely accepted that MAM is a DNA methylating agent and targets cells undergoing mitosis. Another less widely studied effect of MAM is the ability to affect neurite outgrowth, possibly by methylating the DNA of proteins necessary for growth. In support, MAM treated offspring have smaller dendrites with fewer branches and spines (Singh, 1980; Dambska et al., 1982) although it should be noted that MAM was administered on gestational day 15. However, when MAM was administered on cultured embryonic day 18 post mitotic hippocampal slices there were significant reductions in neurite outgrowth leading to decreased dendrite and axon length with no change in cell

viability. They showed a nonspecific process for depleting microtubule proteins including microtubule-associated protein 1B (MAP1B) and MAP2 which are required for neurite outgrowth (Hoffman et al., 1996; Dehmelt and Halpain, 2004). This post mitotic effect of MAM explains why there is no change in neuronal number with a corresponding decrease in volume and increase in cell density seen in gestation day 17 treated offspring in the prefrontal cortex and areas of the hippocampus (Moore et al., 2006; Matricon et al., 2010). Indeed, soma size correlates with neuropil length (Gilbert and Kelly, 1975; Lund et al., 1975) although MAM gestational day 17 effects on dendrites should be confirmed. An additional explanation for decrease in structure size is a change in glial cell number or morphology by MAM. While this has not been investigated *in vivo*, MAM seems unlikely to effect astrocytes; astrocyte cultures showed no effect with MAM application (Cattaneo et al., 1995).

Lastly, MAM has been shown to lead to abnormal neuronal migration or failure of neurons to migrate into their proper position. In support, pyramidal neuron bodies are typically arranged in a well defined columnar organization with axon hillocks in parallel orientation (Matricon et al., 2010). Conversely, MAM treatment on gestational day 17 results in neuronal disorganization, neuronal clusters and axon hillocks in random directions as seen in the hippocampus (Matricon et al., 2010) although the prefrontal cortex remains to be examined. Further, administration on gestational day 15 is used to study neuronal migration disorders including epilepsy since MAM induces hippocampal neuronal clusters, increased susceptibility to seizures and impaired synaptic plasticity (Tschuluun et al., 2005; Paredes et al., 2006; Ramakers et al., 1993). It is important to emphasize the importance of timing of injections. Earlier MAM exposure on day 15

produces a more severe phenotype including greater reductions in brain volume and behavioral deficits. Day 17 treatment is a more accurate representation of schizophrenia however whether MAM methylating DNA acts as an antimitotic agent, neurite outgrowth inhibitor, causes abnormal neuronal organization or a combination leading to deficits has yet to be confirmed.

To answer the question of the possible effects of N-acetylcysteine on MAM-induced deficits, an understanding of the possible effects of in utero N-acetylcysteine is also necessary. N-acetylcysteine is a cysteine prodrug capable of stimulating system x_c^- (Baker et al., 2008; Kau et al., 2008) thereby increasing extracellular glutamate through a nonvesicular mechanism (Baker et al., 2002b). Studies have also shown that nonvesicular glutamate plays a central role in cortical neuronal migration during development (Manent et al., 2005; Manent and Represa, 2007). In support, migrating neurons express NMDA receptors and application of the NMDA receptor antagonists MK801 or APV inhibited neuronal migration in vitro (Hirai et al., 1999; Manent et al., 2005; Behar et al., 1999). Also, munc18-1 and munc13-1/2 mutants which lack the machinery necessary for vesicular priming and are incapable of synaptic release have typical neuronal development and organization (Verhage et al., 2000; Varoqueaux et al., 2002). Further, these animals do not survive after birth presumably due to loss of synaptic activity indicating that nonvesicular and not vesicular neurotransmitter release is necessary for development and organization. While glutamate derived from system x_c^- has not yet been directly linked to neuronal migration, additional evidence indicates that system x_c^- is a key regulator of postsynaptic development in *Drosophila*, specifically the expression of postsynaptic glutamate receptors (Featherstone et al., 2002; Augustin et al.,

2007). Taken together, gestational nonvesicular glutamate impacts neurodevelopment and abnormal signaling can effect neuronal migration and synaptic formation, similar to organizational deficits observed in MAM treated offspring (Matricon et al., 2010).

A potential answer to why N-acetylcysteine is reversing cognitive deficits can now be put forth. MAM appears to be disrupting cortical neuronal migration and or cellular organization leading to a decrease in soma and dendritic size in the absence of changes in neuronal expression (as indicated by changes in density and total volume without reductions in expression (Moore et al., 2006)) although there have been no reports examining cellular organization in MAM treated offspring in the prefrontal cortex, neuronal disorganization was observed in the hippocampus (Matricon et al., 2010). This is likely occurring via the known mechanism of MAM on DNA methylation of proteins involved in neuronal migration. N-acetylcysteine, through stimulation of system x_c^- elevating extracellular glutamate, either counteracts, restores or in general promotes neuronal migratory pathways. The resulting effect of N-acetylcysteine before, during and after MAM administration partially restores neuronal migration and cortical organization to normalize behavioral deficits. Since glutathione and system x_c^- dependent uptake in the prefrontal cortex are not normalized to control levels, it is unlikely that N-acetylcysteine completely reverses the effects of MAM. Of note is the upregulation depicted by no difference in MAM alone and MAM N-acetylcysteine treated offspring in *ex vivo* tissue punches of system x_c^- dependent cystine uptake is only capable of maintaining what is considered normal rates of cystine-glutamate exchange in MAM treated offspring. Without this upregulation, there would likely be further

depression of glutathione levels. Additional research is needed to fully understand the ability of N-acetylcysteine to reverse MAM-induced changes in behavior and system x_c^-.

This chapter raises a very interesting question, is *in vivo* gestational stimulation of system x_c^- sufficient to reverse behavioral deficits in a neurodevelopmental model of schizophrenia? These studies examined reversal learning and suggest that it is capable; however, further research of additional MAM-induced behavioral abnormalities will need to be conducted. Furthermore, studies examining the structure and function of all cortical regions contributing to behavioral symptoms of schizophrenia will identify the impact of altered system x_c^- activity.

V. ACUTE SULFASALAZINE TREATMENT PRODUCES DEFICITS SIMILAR TO THOSE OBSERVED IN SCHIZOPHRENIA

Introduction

Schizophrenia is a lifelong disorder with positive symptoms (hallucinations, delusions, paranoia), negative symptoms (social deficits, anhedonia) and cognitive or executive functioning deficits (behavioral flexibility, learning, memory, attention). Additionally, most patients have anxiety and depression in conjunction with the core symptoms (Huppert et al., 2001; Braga et al., 2005). Historically, dopamine was hypothesized to underlie schizophrenia since traditional antipsychotics are group two dopamine receptor antagonists (Snyder, 1976) however these medications effectively treat the positive symptoms with little to no therapeutic effect on negative or cognitive deficits (Coyle, 2006). Accumulating evidence has since implicated abnormal glutamatergic activity, particularly a hyperglutamatergic state, contributing to symptoms of schizophrenia. In support, research has shown that NMDA receptor antagonists which produce a schizophrenic like state in healthy humans and rodents and exacerbation of symptoms in schizophrenics (Luby et al., 1959; Pearlson, 1981; Javitt and Zukin, 1991; Krystal et al., 1994; Malhotra et al., 1997a) primarily targets and suppresses activity of GABAergic interneurons due to their fast spiking properties requiring a greater frequency of NMDA receptor activiation resulting in unregulated pyramidal cell activation and glutamate release (Moghaddam et al., 1997; Moghaddam and Adams, 1998; Kinney et al., 2006; Homayoun and Moghaddam, 2007; Lewis et al., 2012).

System x_c^- appears to be a key component in the regulation of excitatory signaling in the normal and pathological brain. Cystine uptake in exchange for glutamate export is important for the antioxidant glutathione synthesis (Bannai, 1984; Sies, 1999). Further,

extrasynaptic glutamate provides tone on group II metabotropic glutamate receptors thereby inhibiting synaptic neurotransmitter release (Battaglia et al., 1997; Moran et al., 2005). Data suggest that the activity of system x_c^- may be altered in the dorsolateral prefrontal cortex of individuals with schizophrenia. Specifically, schizophrenia is associated with a significant reduction in glutathione levels coupled with a modest but significant change in the protein levels of xCT, which is the active subunit of system x_c^-, in the dorsolateral prefrontal cortex (Do et al., 2000; Gawryluk et al., 2010; Raffa et al., 2011; Baker et al., 2008). System x_c^- is positioned to regulate postsynaptic activity, and when modulating synapses on dendritic spines of pyramidal neurons, is able to modulate pyramidal cell excitability. Reduced cystine-glutamate exchange would therefore decrease group II metabotropic glutamate activity and cause a hyperexcitable postsynaptic pyramidal neuron resulting in elevated pyramidal cell activity and a hyperglutamatergic state potentially leading to schizophrenic-like symptoms.

To test this theory, the system x_c^- inhibitor sulfasalazine was administered and behavioral measures were performed. *In vitro* studies have demonstrated that sulfasalazine potently inhibits system x_c^- thereby depleting extracellular glutamate and reducing glutathione (Gout et al., 2001; Chung et al., 2005). Further, the sulfasalazine metabolites sulfapyridine and 5-aminosalicylic acid do not inhibit system x_c^-. With oral administration, sulfasalazine is rapidly metabolized in the gut. However, extant data suggest that interperitoneal injections penetrate the blood brain barrier since it reduces glial primary tumor growth in mice (Chung and Sontheimer, 2009). This is important since other potent inhibitors of system x_c^- including (*S*)-4-carboxyphenylglycine are unable to penetrate the blood brain barrier. Therefore sulfasalazine is able to inhibit

cystine-glutamate exchange through acute or chronic administration to all examination of the contribution of system x_c^- to behavioral symptoms of schizophrenia.

The primary goal of this chapter is to assess the impact of adult central nervous system inhibition of system x_c^- by sulfasalazine on symptoms associated with schizophrenia. This is important since direct manipulation of cystine-glutamate exchange on schizophrenic-like behavioral deficits has not yet been established as well as the *in vivo* properties of the sulfasalazine's effect on behavior.

Materials and Methods

Animals and Surgeries: Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300-400 grams were individually housed in a temperature controlled room with a 12-h light/dark cycle with food and water *ad libitum*. The housing conditions and care of the rats were in accordance with the Animal Welfare Act, and all procedures were approved by the Marquette University IACUC Committee. Rats used in the microdialysis studies were anesthetized using pentobarbital (50 mg/kg, IP) with atropine sulfate (1 mg/kg, IP) pretreatment to limit tracheobronchial secretions. Bilateral guide cannula (20 gauge, 14 mm; Plastics One, Roanoke, VA) were implanted using coordinates +3.1 mm anterior, ±1.0 mm mediolateral to Bregma, and -0.75 mm ventral from the surface of the skull at a 6° angle from vertical derived from Paxinos and Watson (Paxinos and Watson, 1986). Rats were given at least 6 days to recover from surgery prior to testing.

Drug Treatments: Sulfasalazine (SSZ, 0-16 mg/kg; Sigma Chemical Co., St Louis, MO) was dissolved in isotonic saline and NaOH to get into solution; pH remained between 6.0-8.0. N-acetylcysteine (0-100 mg/kg; Sigma Chemical Co., St Louis, MO) was dissolved in saline with NaOH to bring the pH to 7.0. Treatment was given as described for each experiment below.

***In Vivo* Microdialysis:** Microdialysis experiments were conducted as described previously (Baker et al, 2002). Briefly, after a minimum of six days post surgery, removable probes extending 2 mm past the guide cannula were inserted through the cannulae into the prefrontal cortex and microdialysis buffer (5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.2 mM KCl and 0.15% phosphate-buffered saline,

pH 7.4) was pumped through the probes at one µl/min for at least three hours prior to collecting baseline samples. Afterwards, 20-min samples were collected for four baseline samples. Rats were injected with SSZ (0-16 mg/kg, IP) and samples continued to be collected for an additional four hours or 12 samples. Samples were analyzed for glutamate on HPLC coupled to fluorescent detection. Precolumn derivatization of glutamate with o-pthalaldehyde was performed using a Shimadzu LC10AD VP autosampler. The mobile phase consisted of 13% acetonitrile, 100 mM Na_2HPO_4 and 0.1 mM EDTA, pH = 5.90. Glutamate was separated using a reversed-phase column (3 µM; 100 x 4.2 mm; Bioanalytical Systems, West Lafayette, IN), and detected using a Shimadzu 10RF-AXL fluorescence detector with an excitation and emission wavelength of 320 and 400 nm, respectively (Baker et al., 2002). Samples were averaged every forty minutes as percent last 40 minute baseline.

Prepulse Inhibition: Rats were placed on a platform in a sound attenuating chamber (10.875”x14”x19.5”; Hamilton Kinder, CA) that rested on a motion sensing plate. A matching session was conducted to determine the magnitude of the startle response for each rat. This session consisted of a five min habituation period followed by 20 trials; 17 trials involved the presentation of a single auditory stimulus (pulse stimulus; 50 dB above the 60 dB background noise) and three trials in which a prepulse stimulus (12 db above background) was presented 100 ms before the pulse auditory stimulus. Rats were then assigned into the various treatment groups based on the magnitude of their startle response. Two days later, an experimental session was conducted to assess sensorimotor gating. Rats were giving SSZ (0-8 mg/kg, IP) 2 hours before testing began. After a 5 min habituation period, rats received 58 discrete trials; 26 trials during which the pulse

stimulus (50 db above background) was presented alone, 8 trials each in which the pulse stimulus was preceded by a prepulse stimulus (2, 6, or 15 db above background) and 8 background trials with no pulse and only background noise. The first 6 pulse alone trials were not included in the average startle stimulus to achieve a relatively stable level of startle reactivity. The percent of prepulse inhibition was determined as 100-(average prepulse startle response/average startle stimulus alone)*100.

Elevated Plus Maze: Rats were placed in a plus maze of 1cm thick black Plexiglas and elevated at a height of 55 cm from the floor. Two open arms (50.8 cm x 10.2 cm) were connected to two enclosed arms (50.8 cm x 10.2 cm x 30 cm) by an open square (12.7 cm x 12.7 cm). Rats were given a daily SSZ injection (0-8 mg/kg, IP) two hours before testing began for five days. A different group of rats received acute injection of SSZ (0-8 mg/kg, IP) two hours before testing and N-acetylcysteine (0-100 mg/kg, IP) one hour before testing began. After treatment, the rat was placed in the elevated plus maze for 5 minutes, alternating the starting position between facing an open arm and facing a closed arm. The session was recorded and an observer blind to treatment recorded the number of explorations as defined as the rat placing two feet onto an open arm without fully entering said arm. Entries defined as the rat placing all 4 feet in an open arm. Time of entry in the open arm was also recorded from the time the rat placed 4 feet in the open arm until two of the rats feet entered the open square.

Open Field Paradigm: Rats were given an injection of SSZ (0-16 mg/kg) two hours before being placed facing the wall at one end of a novel open field apparatus measuring 150 x 100 x 40 cm for 15 minutes. The time the animals spent in the center zone defined as 15 cm from each wall was recorded as well as total distance moved by EthoVision.

Social Interaction: Rats were habituated to an open field apparatus (150 x 100 x 40 cm) on each of two days prior to testing for 15 minutes each. On test day, rats received an injection of SSZ (0-16 mg/kg) two hours before testing and marked with either yellow or red markers. Rats with the same treatment and different colors were placed in each end of the open field and allowed to interact for 10 minutes. Social interaction was defined as the amount of time spent within 20 cm and measured with EthoVision.

Attentional Set Shifting: The maze consisted of four-arms (60 x 20 x 12 cm) in the shape of an X connected by a center area (12 x 12 cm); constructed of black Plexiglas with a movable wall to block an arm to form a "T" configuration. Dividers at the end of each arm concealed the sugar pellet reward such that the rat was unable to see it from the center of the maze. The visual cue, when used, was laminated with black and white diagonal stripes and placed on the floor of an arm. Prior to habituation, rats were handled for five minutes each day for seven days and food deprived to 85% of their free-feeding weight. The day before habituation, each rat was given approximately 30 sugar pellets in their home cage to acquaint them with the pellets.

Habituation: Day one, 5 pellets were placed in each arm, 3 throughout the length of the arm and 2 behind the divider at the end. The rat was placed in the center of the maze and allowed to freely explore for up to 15 minutes. On the second day, 3 pellets were placed in each arm, one in the center, the other 2 behind the divider and the rats freely explored for up to 15 minutes. On the third day and all remaining days till criteria was achieved, one pellet was placed behind the divider in each arm. After the rat consumed a pellet behind the divider on any day, he was picked up and placed at the entrance to another baited arm to habituate him to handling within the maze. If the rat consumed all sugar

pellets on any day, the maze was rebaited with the following habituation day's configuration or with 4 pellets, one behind each divider after habituation day 2. After the maze was rebaited 3 times and all pellets consumed within 15 minutes, criteria was met and turn bias was run the following day. If the rat did not consume all sugar pellets within 15 minutes, he continued habituation daily until he met criteria.

Turn bias: During turn bias and testing, one arm was blocked with the movable wall to form a "T" configuration with the visual cue in one of the side arms. In turn bias, both side arms were baited with sugar pellets behind the dividers. The rat was placed in the stem arm at the bottom of the "T" and allowed to enter either arm; after consuming the pellet he was placed back in the stem arm and again allowed to enter either arm. If he entered the same arm again, he was returned to the stem arm until he turned into the opposite arm and ate the remaining sugar pellet. When he successfully ate both sugar pellets, he was placed back in his home cage signaling the end of the trial. The next trial, the rat began from a different arm such that over 7 trials, the rat started from each of 3 arms 2 times and the remaining arm once and the visual cue was placed in either the right or left arm as equally as possible. Inter-trial time was about 10 seconds to rebait the maze. The direction the rat turned first the most out of 7 trials was considered his turn bias for the rest of testing.

Testing: As in turn bias, one arm was blocked with the movable wall, either the right or left arm contained the visual cue and the rat began in the stem arm. Trials were grouped into a block of 12 with the stem arm in arms 1-3 as shown in Figure 3.2. In the probe trial, arm 4 was the stem arm and the visual cue was placed in the turn bias arm. Within

treatment groups, the probe arm switched such that at least one animal in each group used each arm as the stem arm for the probe trial. Each testing day required the following:

- 10 consecutive correct trials using the 12 trial block in stem arms 1-3
- 1 correct probe trial from stem arm 4
- If correct probe trial, testing was complete
    - If incorrect probe trial
        - 5 consecutive correct trials using the 12 trial blocks
        - 1 correct probe trial from stem arm 4
        - This continues till correct probe trial

Day 1: Visual-cue learning; see Figure 3.3a: On day one, the rat was trained in a simple discrimination task to attend to the visual cue such that the food reward was placed in each arm with the visual cue 50% of the time. Turing towards the visual cue and attaining the reward was a correct choice. In the probe trial the rat turned towards the visual cue.

Day 2: Attentional Set Shift; see Figure 3.3b: On day 2 the complex task of an attentional set shift required the rat to switch from a visual to a spatial cue with the new strategy for reward turning into the arm opposite of his turn bias. The probe trial required the rat to turn in the opposite direction of the visual cue.

Day 3: Reversal learning; see Figure 3.3c: Reversal learning required the rat to enter the arm opposite the strategy from day 2 such that the rats turn bias arm now contained the food reward and the rat turned towards the visual cue on the probe trial.

ERRORS: Day 2: As stated above, trials were blocked into groups of 12 with the visual cue in each arm 50% of the time. On day two, within a group of 4 in which the visual

cue was placed in the opposite arm of the strategy for the day, until the animal made a correct choice more than 50% of the time (i.e. 2 or more correct choices within the group of 4) the errors were counted as perseverative signifying the inability to disregard a previously learned strategy. After the animal correctly choose the non-visual cue arm at least 50% of the time errors were counted as regressive meaning the inability of the animal to maintain a new strategy after perseveration had ceased. If the animal choose the incorrect arm and it did not have the visual cue, the error is a never reinforced error since the arm had not been associated with a reward. This measured how quickly the animal phased our incorrect strategies.

Errors: Day 3: Reversal learning Similar to day 2 errors, the trials were grouped into 4 Since day three tested reversal learning the animals were required to turn in the opposite arm from day two on every trial. Therefore, every 4 trials were grouped together, when the rat choose the correct arm more than 50% of the time the errors were no longer considered perseverative and instead regressive. Additionally, whether the animal went towards the visual cue or away was recorded.

Statistics: With 3 or more groups analysis was conducted with variance (ANOVA) with each drug treatment as a between-subject factor and further analyzed using Tukey HSD unless otherwise noted. Experiments comparing 2 groups were analyzed using *t* test.

Results

Sulfasalazine has been shown to inhibit system x_c^- *in vitro* and reduce extracellular glutamate concentrations in culture and surrounding brain tumors in human patients (Chung et al., 2005; Chung and Sontheimer, 2009; de Groot and Sontheimer, 2011), but there is no evidence of sulfasalazine activity in the absence of glioma tumors within the prefrontal cortex. Figure 5.1 illustrates that an acute intraperitoneal injection of sulfasalazine reduced extrasynaptic glutamate in the medial prefrontal cortex as measured through microdialysis. An ANOVA was used to analyze glutamate (% baseline) with sulfasalazine as a between subjects measure and time as a within subjects measure. A main effect of treatment after sulfasalazine injection was found ($F_{(3,27)} = 3.72$ $p < 0.05$) without an interaction ($F_{(15,135)} = 0.80$ $p > 0.05$). Given that the purpose of the experiment was to determine when and if sulfasalazine reduced glutamate, the main effect across each dose of sulfasalazine against time was analyzed. An ANOVA with time as a repeated measure revealed no significance in the control group (SSZ 0, $F_{(6,42)} =$

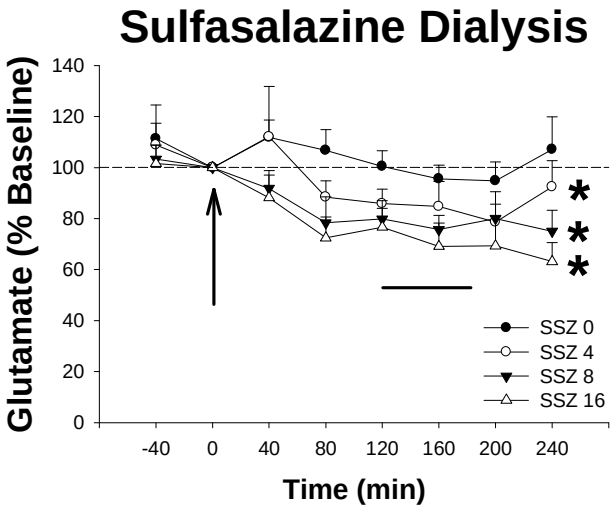


Figure 5.1 Sulfasalazine (0-16 mg/kg, IP) reduces glutamate concentrations in the medial prefrontal cortex. A) depicts % baseline glutamate levels across the experiment. Arrow is time of injection. Line from time 120-180 indicates when behavioral testing is conducted for later experiments. *N* of 7-8 per group. * indicates a significant main effect of time from baseline within treatments $p < 0.05$.

1.66 p > 0.05). Sulfasalazine did have a main effect of all doses (SSZ 4, $F_{(6,36)} = 2.46$ p < 0.05; SSZ 8, $F_{(6,42)} = 7.27$ p < 0.001; SSZ 16, $F_{(6,42)} = 8.81$ p < 0.001). Further deconstruction of the main effect revealed that during all of the following behavioral testing as shown by the solid line from 120-180 minutes SSZ 8 and SSZ 16 treatment significantly reduced glutamate in the prefrontal cortex.

Sensorimotor gating reflects the ability of the central nervous system to inhibit irrelevant sensory information and allow focus on salient information from the environment; deficits in sensorimotor gating occur in schizophrenic patients and prepulse inhibition is used to model this phenomenon (Braff et al., 2001b; Geyer et al., 2001; Swerdlow et al., 2006; Kumari et al., 2008). As shown in Figure 5.2, sulfasalazine was administered twice daily for five days (8 mg/kg, IP); testing was conducted 2 hours following the last injection to examine the effects of sub-chronic inhibition of cystine-glutamate exchange on sensorimotor gating deficits. An ANOVA with drug treatment as a between subjects measure and prepulse intensity as a repeated measure produced a main effect of treatment ($F_{(1,9)} = 5.26$ p < 0.05) in the absence of a significant interaction

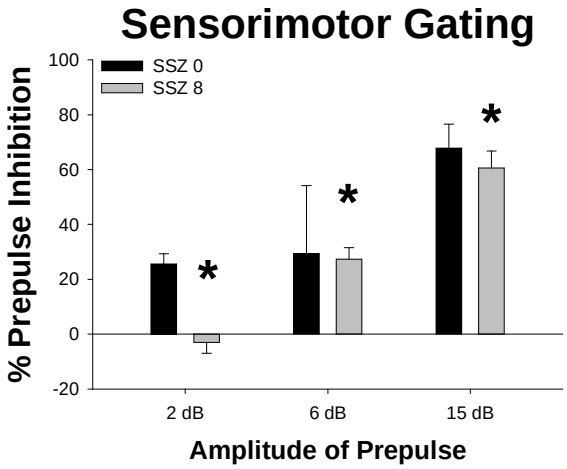


Figure 5.2 Effect of sub-chronic treatment of sulfasalazine on sensorimotor gating. The data is expressed as the mean (+SEM) prepulse inhibition displayed by rats receiving 5 daily injections produced a main effect of treatment on prepulse inhibition. (N = 4-7). * indicates a main effect of treatment and significance from SSZ 0.

($F_{(2,18)}$ = 1.10 p > 0.05) indicating that subchronic inhibition of system x_c^- is sufficient to produce deficits in sensorimotor gating.

Figure 5.3 Effect of acute or sub-chronic sulfasalazine treatment on elevated plus maze. The data are shown as the mean (+SEM) time in open arm following daily sulfasalazine treatments (0-8 mg/kg, IP, *N* = 4) on day 1 and 5. * indicates significance from day 1 SSZ 0 (p < 0.05) and + from day 5 SSZ 0 p = 0.07 using pairwise comparisons.

Anxiety, another symptom common in schizophrenia and related to patients overall satisfaction with life, can be measured with the amount of time spent in the open arms of the elevated plus maze as shown in Figure 5.3. As depicted, the amount of time spent in the open arms of the maze is significantly reduced with both an acute injection of sulfasalazine on day 1 (8mg/kg, IP) and sub-chronic following 5 daily injections with testing. An ANOVA with treatment as a between subjects measure and day of treatment as a repeated measure revealed a significant interaction ($F_{(1,6)}$ = 7.76 p < 0.05). Pairwise comparisons revealed a significant difference between SSZ 0 on day 1 and 2 and between SSZ 0 and SSZ 8 on day 1 (p < 0.05), with a trend towards significance between SSZ 0 and SSZ 8 on day 2 (p = 0.07) in the absence of differences with SSZ 8 between days (p = 0.83). This indicates that both acute and sub-chronic inhibition of system x_c^- is anxiogenic. All further sulfasalazine testing was conducted with acute injections.

An additional measure of anxiety is the open field paradigm which measures the amount of time the animal spends in the center of the maze with a decrease signifying anxiety-like behaviors. In Figure 5.4a animals received an acute injection of

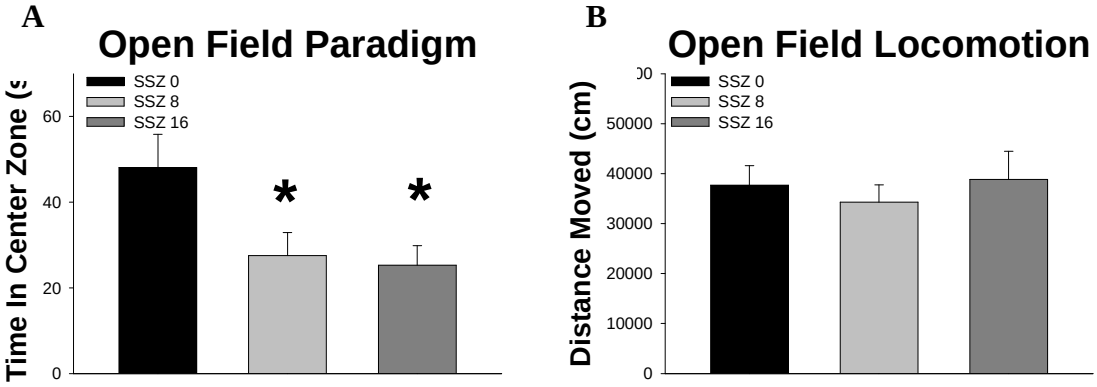


Figure 5.4 Sulfasalazine in the open field paradigm. Animals received an acute injection of sulfasalazine (0-16 mg/kg, IP, *N* = 10-13) and placed in an open field. A) depicts time spent in the center zone while B) represents total locomotion throughout testing. Data is expressed as the mean (+SEM). * indicates a significant difference from SSZ 0 using Tukey HSD post hoc.

sulfasalazine (0-16 mg/kg) and the amount of time the animals spent in the center of the maze was recorded. An ANOVA with treatment as a between subjects factor found significant differences in the amount of time spent in the center of the maze ($F_{(2,34)} = 4.38$ $p < 0.05$). Tukey post hoc analyses indicate that both sulfasalazine treatments (8-16 mg/kg) are significantly different than sulfasalazine 0 ($p < 0.05$). Decreased time in the center zone is not attributable to a change in locomotor activity since, as depicted in Figure 5.4b, there is no significant difference during testing ($F_{(2,34)} = 0.02$, $p > 0.05$).

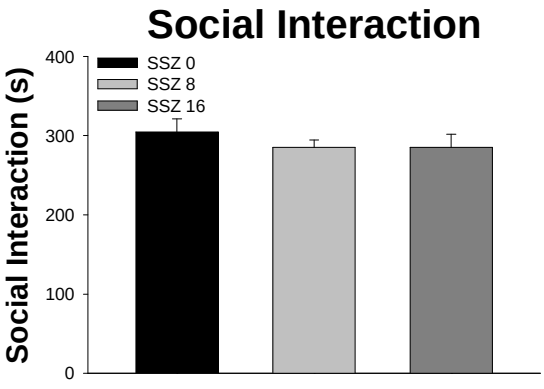


Figure 5.5 Sulfasalazine in social interaction. Acute sulfasalazine (0-16 mg/kg, IP, *N* = 5-7) does not produce a deficit in social interaction.

Social interaction is a prominent negative symptom of schizophrenia and measures the amount of time animals engage in social behaviors. Unexpectedly

since it is a prominent negative symptom of schizophrenia, as shown in Figure 5.5, there are no significant differences in social interaction activity following an acute injection of sulfasalazine (0-16 mg/kg, IP) ($F_{(2,16)} = 0.12$, $p > 0.05$).

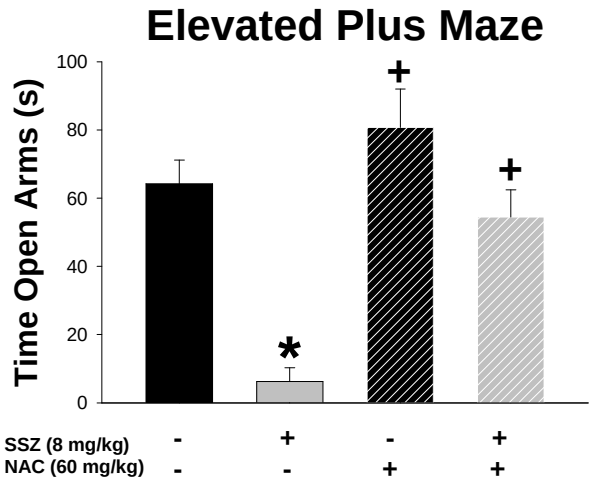


Figure 5.6 Sulfasalazine and N-acetylcysteine in elevated plus maze. Data is expressed as the mean (+SEM) of the time spent in the open arms. Sulfasalazine (0-8 mg/kg, IP) was given two hours and N-acetylcysteine (0-60 mg/kg, IP, *N* = 4-9) one hour before testing. * indicates significant difference from SSZ 0 / NAC 0 and + significant from SSZ 8 / NAC 0 using Tukey HSD.

Since sulfasalazine inhibits system x_c^- the next goal was to test whether the behavioral effects could be reversed by stimulation of system x_c^- with N-acetylcysteine. Using elevated plus maze, animals received an acute injection of sulfasalazine (0-8 mg/kg, IP) followed one hour later by N-acetylcysteine (0-100 mg/kg, IP). Figure 5.6 depicts time spent in open arms. An ANOVA with treatments as a between subjects factor revealed a significant difference ($F_{(3,24)} = 16.38$ $p < 0.01$). Tukey post hoc revealed a significant difference between SSZ 0 / NAC 0 and SSZ 0/ NAC 0. Furthermore, N-acetylcysteine reversed the effects of sulfasalazine as both SSZ 0 / NAC 100 and SSZ 8 / NAC 100 were significant from the sulfasalazine alone treated group.

Cognitive deficits including working memory, attention and behavioral flexibility are the best predictors of functional disability of schizophrenics. To elucidate the role of system x_c^- in cognitive deficits, acute sulfasalazine was administered prior to each testing phase in attentional set shifting which tests visual cue learning, an attentional set shift and reversal learning. An ANOVA was used to analyze cognition with sulfasalazine (8

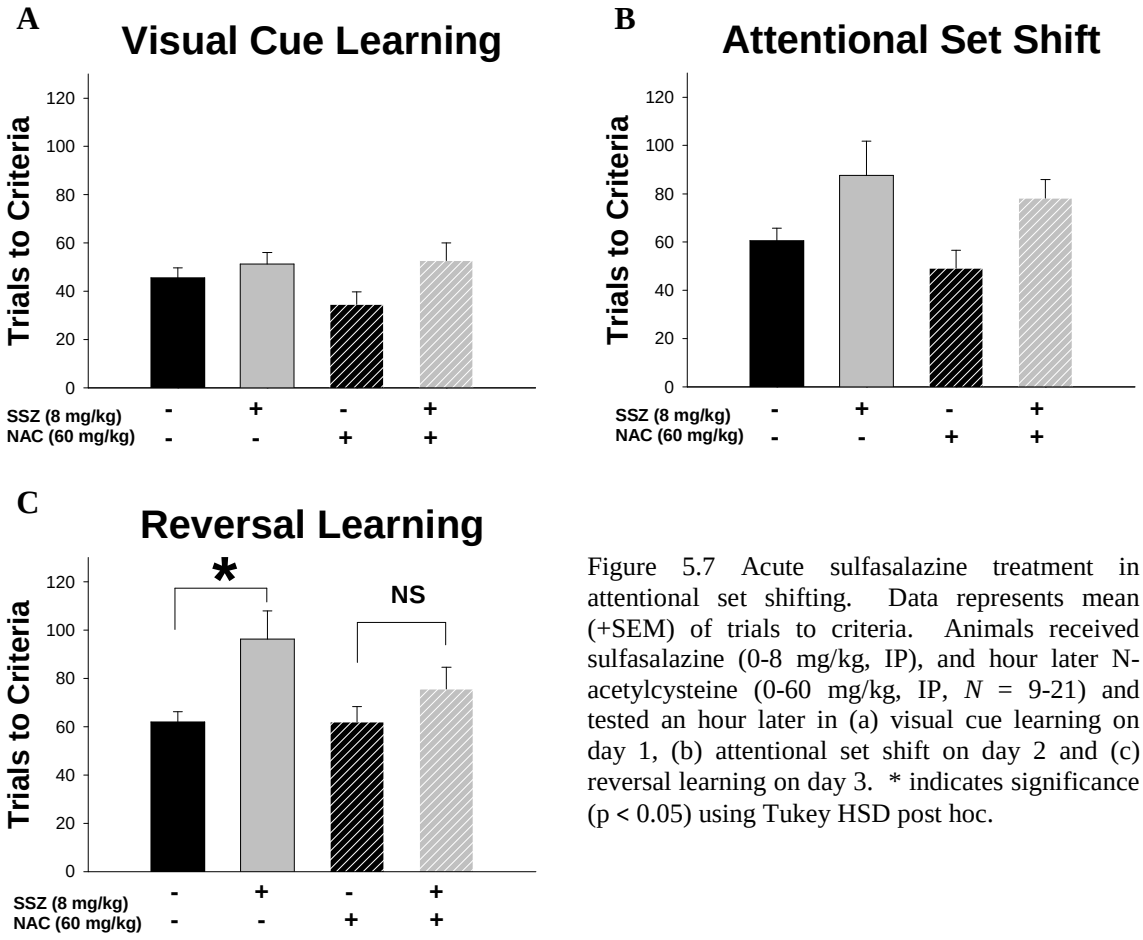


Figure 5.7 Acute sulfasalazine treatment in attentional set shifting. Data represents mean (+SEM) of trials to criteria. Animals received sulfasalazine (0-8 mg/kg, IP), and hour later N-acetylcysteine (0-60 mg/kg, IP, N = 9-21) and tested an hour later in (a) visual cue learning on day 1, (b) attentional set shift on day 2 and (c) reversal learning on day 3. * indicates significance ($p < 0.05$) using Tukey HSD post hoc.

mg/kg) as a between subjects factor on each day of testing. As shown in Figure 5.7, a main effect of treatment was found on day two ($F_{(3,50)} = 3.55$ $p < 0.05$) and day three ($F_{(3,50)} = 4.60$ $p < 0.05$) but not day one (Figure 5.7a, $F_{(3,50)} = 1.90$ $p > 0.05$). In Figure 5.7b, post hoc analysis indicate that on day two, there was a trend towards significance between SSZ 0 / NAC 0 and SSZ 8 / NAC 0 ($p = 0.08$). Post hoc analysis on day three reversal learning revealed sulfasalazine treatment significantly increased the number of trials to reach criteria from controls as shown in Figure 5.7c. Further, N-acetylcysteine treatment attenuated sulfasalazine-induced deficits on day three shown by no significant difference from N-acetylcysteine alone treated animals.

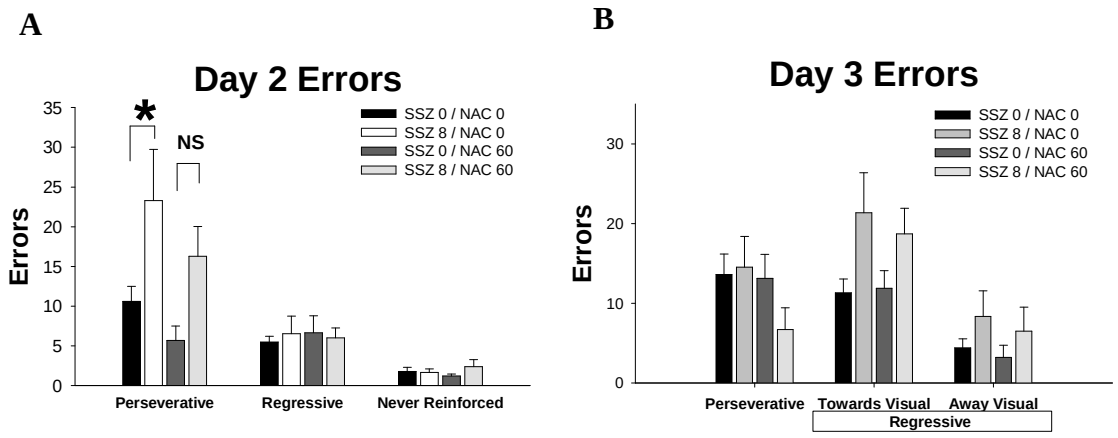


Figure 5.8 Types of errors committed by sulfasalazine treated animals in attentional set shift. (A) Depicts errors committed on day 2 on attentional set shift with a specific increase in perseverative errors while (B) shows errors on day 3 during reversal learning. * indicates significance ($p < 0.05$) using Tukey HSD post hoc analyses.

Attentional set shifting allows the analysis of the different types of impairments. Perseverative errors are an index of how quickly animals are able to inhibit a previously learned strategy which is now incorrect, regressive a measure of the animals ability to maintain the new strategy and never reinforced shows the ability of the animal to ignore an ineffective strategy. Figure 5.8a depicts the effect of acute sulfasalazine on set shifting specifically increasing the amount of perseverative errors using ANOVA with treatment as a between subjects factor ($F_{(3,50)} = 3.87$ $p < 0.05$) with no changes in regressive or never reinforced errors ($F_{(3,50)} = 0.17$ and $F_{(3,50)} = 0.50$, respectively, $p > 0.05$). Post hoc analysis indicates that sulfasalazine vehicle treated animals had increased perseverative errors from vehicle treated animals. Day three reversal learning errors are depicted in Figure 5.8b. An ANOVA with treatment as a between subjects factor reveals significance in the regressive towards the visual cue errors ($F_{(3,50)} = 2.82$ $p < 0.05$) in the absence of significance in perseverative or regressive away from the visual cue errors ($F_{(3,50)} = 1.13$ and $F_{(3,50)} = 1.02$ respectively, $p > 0.05$). Tukey HSD post hoc analysis

reveals a trend towards significance for sulfasalazine treated animals compared to vehicle.

Discussion

Altered glutamatergic neurotransmission and deficient glutathione may account for many of the pathological changes in schizophrenia (Olney et al., 1999; Coyle, 2006; Enomoto et al., 2007; Stone et al., 2007; Berk et al., 2008a) although few studies, if any, distinguish between synaptic or extrasynaptic glutamate pools. The primary findings in this chapter indicate that inhibition of system x_c^- is sufficient to produce behavioral deficits similar to those observed in schizophrenia. Importantly, acute sulfasalazine treatment reduces medial prefrontal extrasynaptic glutamate for a minimum of four hours. Further, inhibition of system x_c^- produces behavioral deficits in sensorimotor gating, measures of anxiety and cognition without effect on other measures of schizophrenia including spontaneous locomotion to novel environment or social interaction. Collectively, these data further characterize the potential involvement of system x_c^- in the neuropathology of schizophrenic symptoms.

Preclinical and clinical reports have shown that administration of N-acetylcysteine has therapeutic potential for schizophrenic deficits (see Chapters 2, 3, 4; Baker et al., 2008; Berk et al., 2008c; Lavoie et al., 2008; Bulut et al., 2009; Carmeli et al., 2012). Chapter 2 provides evidence than phencyclidine-induced deficits are reversed by N-acetylcysteine infusion into the prefrontal cortex and this effect was prevented with co-administration of the system x_c^- inhibitor (*S*)-4-carboxyphenylglycine (CPG) suggesting N-acetylcysteine stimulates system x_c^- to reverse deficits in sensorimotor gating. Chapter 3 and 4 show that system x_c^- stimulation reverses MAM-induced deficits in reversal learning. Clinically, N-acetylcysteine has been used as an adjunct therapy in

the treatment of schizophrenia and shown beneficial effects and improvement in overall symptom severity, mismatch negativity and improved prefrontal cortex synchronization using EEG recordings (Berk et al., 2008c; Lavoie et al., 2008; Carmeli et al., 2012). Authors of these trials suggest that N-acetylcysteine produces therapeutic effects by increasing glutathione levels; however, it is likely that N-acetylcysteine stimulates cystine-glutamate exchange thereby also exerting an effect on levels of extrasynaptic glutamate. This chapter examined whether system x_c^- inhibition is sufficient to produce behavioral deficits related to schizophrenia.

Sulfasalazine has been shown in astrocytic cultures to potently inhibit system x_c^- whereas its metabolites, sulfapyridine and 5-aminosalicylic acid fail to do so and in fact are anti-inflammatory agents used to treat inflammation in the gut (Gout et al., 2001; Chung and Sontheimer, 2009). These labs have also investigated the ability of sulfasalazine to reduce tumor cell growth since primary brain tumors exclusively rely cystine uptake by system x_c^- for glutathione synthesis. Additionally, elevated extrasynaptic glutamate is thought to lead to activated NMDA receptor induced excitotoxic neuronal cell death to allow space for tumor growth. Evidence that sulfasalazine penetrates the blood brain barrier includes the observation that chronic sulfasalazine treatment limited the growth rate of glial tumors *in vivo*, an effect attributable to central blockade of system x_c^- (Gout et al., 2001; Chung et al., 2005; Lyons et al., 2007). The microdialysis data presented in this chapter also suggests sulfasalazine crosses the blood brain barrier since an acute systemic injection of sulfasalazine dose dependently reduces extrasynaptic glutamate in the prefrontal cortex. This is the first report of systemic administration of a system x_c^- inhibitor leading to a reduction in

extrasynaptic glutamate in the prefrontal cortex. This result is not surprising since mRNA of xCT, the active subunit of system x_c^-, is present in the medial prefrontal cortex, the same region microdialysis was conducted in this study (see Chapter 2). However, another study reported no change in baseline extrasynaptic glutamate following system x_c^- inhibition with (*S*)-4-carboxyphenylglycine (CPG) (Melendez et al., 2005) suggesting the possibility that the observed reduction in medial prefrontal glutamate is secondary to the main site of sulfasalazine action in a structure which projects to the prefrontal cortex. Although this would have to be shown experimentally, sulfasalazine either directly or indirectly reduces extrasynaptic medial prefrontal glutamate. Given the critical role of glutamate signaling within the prefrontal cortex and the broad range of systems system x_c^- regulates particularly synaptic signaling and glutathione synthesis (Baker et al., 2002b; Conn and Pin, 1997; Dringen and Hirrlinger, 2003), it is of little surprise that inhibition of cystine-glutamate exchange produces behavioral deficits.

Interestingly, system x_c^- inhibition with sulfasalazine produces deficits in sensorimotor gating, models of anxiety and cognitive deficits including attentional set shift and reversal learning. As described in Chapter 2, sensorimotor gating reflects the ability of the central nervous system to inhibit irrelevant sensory information and allow focus on salient information from the environment; deficits in sensorimotor gating occur in schizophrenic patients and prepulse inhibition is used to model this phenomenon (Braff et al., 2001b; Geyer et al., 2001; Swerdlow et al., 2006; Kumari et al., 2008). Interestingly, sulfasalazine treated animals display sensorimotor gating deficits at lower prepulse intensities similar to glutathione deficient mice suggesting the prepulse inhibition mediated circuitry requires a more intense prepulse compared to control

animals (Kulak et al., 2011). Deficits in prepulse inhibition implicate the dorsolateral prefrontal cortex since this is a brain region critical for executive functioning and found to have altered cortical activation in schizophrenics (Smith and Jonides, 1999; Eisenberg and Berman, 2010).

Anxiety, while common in many neurological disorders, is present in individuals with schizophrenia; studies have shown that anxiety and even depression in patients are strongly associated with lower general life satisfaction (Huppert et al., 2001; Braga et al., 2005). Two measures of anxiety are employed in this chapter, elevated plus maze and open field which both measure the amount of time the animal spends in an open space away from the relative safety of walls or enclosed spaces. The amount of time spent in the open arms of the elevated plus maze has been validated as an indicator of anxiety-related behavior with less time in the open arms signifying increased anxiety (Pellow et al., 1985). Open field measures the amount of time spent in the center of a large open space; less time in the center indicates anxiety since traditional anxiolytic compounds such as benzodiazepines increase center exploratory behavior (Prut and Belzung, 2003; Choleris et al., 2001). Interestingly, acute inhibition of system x_c^- with sulfasalazine is anxiogenic in both the elevated plus maze and open field paradigm. Further, subchronic daily treatment of sulfasalazine remains anxiogenic in elevated plus maze even after repeated testing whereas vehicle treated animals increase the amount of time spent in the open arms over time. Additionally, the effects of sulfasalazine are completely reversed with administration of N-acetylcysteine after sulfasalazine treatment in elevated plus maze. This is not surprising since addition of cystine *in vitro* also reverses sulfasalazine effects on system x_c^- (Gout et al., 2001; Chung et al., 2005). These results suggest that

system x_c^- contributes to states of anxiety; however the specific brain region and neurotransmitter system mediating these behaviors will require further investigations.

Of surprise was the inability of sulfasalazine treatment to produce deficits in social interaction and to a lesser extent, on locomotion. Spontaneous locomotor activity was not altered with sulfasalazine treatment in a novel environment in the open field paradigm; reduced time in the center of the field was not attributable to reduced locomotion. Locomotor activity has been used as an index of the positive symptoms of schizophrenia since evidence suggests similar circuitry for psychosis in humans and hyperlocomotion in rodents. In support, compounds shown to enhance dopamine signaling such as amphetamine produce psychosis and paranoia in healthy individuals and hyperlocomotion in rodents (Robinson and Becker, 1986; Valvassori et al., 2008). However, the fact that sulfasalazine does not produce hyperlocomotion is consistent with findings that N-acetylcysteine treatment attenuates without complete reversal of methamphetamine-induced hyperlocomotion even with a high dose (300 mg/kg, IP) nor does it reverse acute cocaine-induced hyperlocomotion at 60 mg/kg, IP (Fukami et al., 2004; Madayag et al., 2007). Further, schizophrenia models, including MAM, have increased sensitivity to stimulant induced hyperlocomotion in the absence of spontaneous hyperlocomotion (Flagstad et al., 2004; Lodge and Grace, 2007; Phillips et al., 2012). Social interaction is a very prominent negative symptom of schizophrenia and N-acetylcysteine reverses phencyclidine-induced deficits thereby suggesting inhibition of system x_c^- would also lead to deficits (Baker et al., 2008). Surprisingly, acute administration of sulfasalazine was insufficient to produce behavioral deficits indicating a) social interaction is not directly modulated by system x_c^- or b) the administration

parameters employed here (0, 8 or 16 mg/kg, IP two hours before testing) were not sufficient to produce deficits in social interaction and perhaps a higher dose or more likely a sub or chronic treatment regimen of sulfasalazine could produce deficits in rodent social interaction. Further investigations need to be conducted to conclusively rule out the involvement of system x_c^- on social behaviors.

Behavioral flexibility, an additional measure of executive functioning deficient in schizophrenics, is thought to be mediated by human dorsolateral prefrontal cortex and rodent medial prefrontal cortex (Ravizza and Carter, 2008; Floresco et al., 2009). Multiple cortical and subcortical regions form a circuit mediating behavioral flexibility; abnormal activity in the different regions produces specific behavioral deficits that can be measured using a maze based attentional set shift model (Floresco et al., 2006a; Floresco et al., 2006b; Floresco et al., 2008; Ghods-Sharifi et al., 2008). Whereas MAM neurodevelopmental treatment produced deficits in reversal learning in the attentional set shifting paradigm, acute sulfasalazine treatment also increased trials to criteria on day three (reversal learning) with a strong trend on day two (attentional set shift). Prefrontal cortical activity has been shown to mediate the set shift on day 2 and the orbitofrontal cortex has been linked to reversal learning on day 3 (Floresco et al., 2008; Ghods-Sharifi et al., 2008). Interestingly, this suggests that system x_c^- contributes to cortical activity in both regions; decreased extrasynaptic glutamate has been shown in the prefrontal cortex while effects in the orbitofrontal cortex have yet to be examined. The types of errors committed by sulfasalazine treated animals further implicates system x_c^- in the modulation of cortical circuitry and activity. Specifically, a day 2 increase in perseverative errors measuring the rats ability to disengage from a previously learned

strategy, implies abnormal connections into or out of the prefrontal cortex (Block et al., 2007). An increase of day three regressive errors indicates sulfasalazine-induced inability to maintain a new strategy; regressive errors towards the visual cue indicate impaired ability to overcome a learned non-reward (Ghods-Sharifi et al., 2008). Measures in attentional set shifting and the types of errors committed correlate to behavioral deficits in schizophrenics that can be measured using the Wisconsin Card Sorting test and reversal learning paradigms (Gold et al., 1997; Waltz and Gold, 2007; Szoke et al., 2008). The ability of system x_c^- to acutely modulate these types of behaviors observed in schizophrenics implicates system x_c^- mediating executive functioning.

The results presented in this chapter implicate system x_c^- as a potential underlying factor contributing to the pathophysiology of symptoms of schizophrenia. The mechanism of sulfasalazine-induced deficits is likely due to inhibition of system x_c^- but other factors have not been ruled out. In support, acute sulfasalazine reduces prefrontal extrasynaptic glutamate and has been shown to block system x_c^- *in vitro* (Figure 5.1, (Gout et al., 2001; Chung et al., 2005; Chung and Sontheimer, 2009). This could a) lead to a reduction in glutathione in both astrocytes and neurons since system x_c^- transports cystine which is the rate limiting step in glutathione synthesis and astrocytes provide glutathione precursors for neurons (Bannai, 1984; Bridges, 2011; Dringen and Hirrlinger, 2003), and b) decreased extracellular glutamate reduces tone on metabotropic group two glutamate receptors thereby disinhibiting synaptic neurotransmitter release (Baker et al., 2002b; Mohan et al., 2011). Unregulated synaptic activity can lead to enhanced post-synaptic excitation and desynchronization of regional pyramidal cell firing which is necessary for cognitive processing (Gonzalez-Burgos and Lewis, 2008). Whether

inhibition of system x_c^- induces behavioral deficits through decreased glutathione synthesis or by depleted extrasynaptic glutamate will need to be further investigated to confirm or deny the pathway of acute sulfasalazine treatment on behavioral deficits similar to those observed in schizophrenia.

This chapter examined behavioral measures of adult inhibition of system x_c^- by sulfasalazine on schizophrenia-like symptoms. Sulfasalazine induces deficits in some measures (sensorimotor gating, anxiety and cognition) while having little effect on others (hyperlocomotion, social interaction). This is the first evidence of direct manipulation of system x_c^- leading to symptoms of schizophrenia, and while extremely interesting, will need to be further investigated in additional behaviors, potentially non-schizophrenic behaviors, as well as mechanistically to understand the potential impact of cystine-glutamate exchange in a wide range of behaviors and disorders of the nervous system.

VI. SULFASALAZINE IN UTERO MODIFIES CYSTINE-GLUTAMATE EXCHANGE AND BEHAVIOR

Introduction

Abnormal neurodevelopment is hypothesized to contribute to the pathophysiology of schizophrenia (Weinberger, 1987; Marenco and Weinberger, 2000; Rapoport et al., 2005). In support, prenatal (maternal malnutrition, infection or stress) or perinatal (obstetrical complications) insults increases the likelihood of developing schizophrenia. Further, motor, social or cognitive deficits are present during childhood before the diagnosis which typically occurs after psychosis emerges in late teens to early twenties (for review: (Lewis and Levitt, 2002)). Neurogenesis occurs in utero and is a highly regulated process. Glutamate and GABA are critical neurotransmitters which modulate immature neurons migratory pathways and facilitate final positioning and organization (Behar et al., 1998; Behar et al., 1999; Behar et al., 2000; Behar et al., 2001; Hirai et al., 1999; Kihara et al., 2002). Non-vesicular release of these neurotransmitters act on immature neurons NMDA and $GABA_A$ ionotropic receptors to facilitate migration (Nguyen et al., 2001; Lujan et al., 2005; Manent and Represa, 2007).

In the adult brain extrasynaptic glutamate is found in micromolar concentrations and are of nonvesicular origin since levels are insensitive to blockade of voltage dependent Na^+ and Ca^{2+} receptors (Timmerman and Westerink, 1997; Baker et al., 2002b). Further, system x_c^- has been shown to contribute a significant amount of nonvesicular extrasynaptic glutamate in the nucleus accumbens, striatum, hippocampus and prefrontal cortex (Baker et al., 2002b; Massie et al., 2011; De Bundel et al., 2011; Chapter 5). System x_c^- transports one cystine in for every glutamate out into the extrasynaptic space (for review see Bridges, 2011). The contribution of system x_c^- to in

utero nonvesicular glutamate during neurogenesis has not been directly studied however system x_c^- is expressed by immature neurons although it's role has not been elucidated (Murphy et al., 1990). Sulfasalazine is a potent inhibitor of system x_c^- (Chung et al., 2005; Chung and Sontheimer, 2009) and acute administration produces schizophrenic-like symptoms in rodents (Chapter 5). Sulfasalazine is capable of crossing the placental barrier (Peppercorn, 1984) and the blood-brain barrier (Lyons et al., 2007; de Groot and Sontheimer, 2011; Chapter 5). Using sulfasalazine to inhibit system x_c^- in utero has not been attempted so effective administration parameters and the long-term behavioral effects on offspring have not yet been established.

This chapter will examine the impact of varying concentrations of sulfasalazine on gestation day 17 (same time frame of MAM administration, Chapter 3 and 4) to assess whether decreased cystine-glutamate exchange in utero is capable of producing schizophrenic-like deficits in adult male offspring. Specifically, attentional set shifting and measures of system x_c^- will be examined. The importance of this stems from the lack of knowledge on the impact of altered in utero system x_c^- activity. Further, cognitive deficits including reversal learning are observed in schizophrenics (Waltz and Gold, 2007; Murray et al., 2008) and poorly treated with current antipsychotics.

The purpose of this chapter is to assess whether in utero manipulation of system x_c^- is sufficient to produce long lasting changes in cystine-glutamate exchange and cognitive measures.

Materials and Methods

Sulfasalazine Treatment: Timed pregnant Sprague-Dawley rats were given an acute injection of sulfasalazine (0, 8 or 16 mg/kg, IP; Sigma Chemical Co., St Louis, MO) dissolved in saline with NaOH on gestational day 17. Following vaginal birth all mothers and offspring were left undisturbed until weaning on postnatal day 22. Male offspring were single caged for testing as adults (PND 60+).

Attentional Set Shifting: The maze consisted of four-arms (60 x 20 x 12 cm) in the shape of an X connected by a center area (12 x 12 cm); constructed of black Plexiglas with a movable wall to block an arm to form a "T" configuration. Dividers at the end of each arm concealed the sugar pellet reward such that the rat was unable to see it from the center of the maze. The visual cue, when used, was laminated with black and white diagonal stripes and placed on the floor of an arm. Prior to habituation, rats were handled for five minutes each day for seven days and food deprived to 85% of their free-feeding weight. The day before habituation, each rat was given approximately 30 sugar pellets in their home cage to acquaint them with the pellets.

Habituation: Day one, 5 pellets were placed in each arm, 3 throughout the length of the arm and 2 behind the divider at the end. The rat was placed in the center of the maze and allowed to freely explore for up to 15 minutes. On the second day, 3 pellets were placed in each arm, one in the center, the other 2 behind the divider and the rats freely explored for up to 15 minutes. On the third day and all remaining days till criteria was achieved, one pellet was placed behind the divider in each arm. After the rat consumed a pellet behind the divider on any day, he was picked up and placed at the entrance to another

baited arm to habituate him to handling within the maze. If the rat consumed all sugar pellets on any day, the maze was rebaited with the following habituation day's configuration or with 4 pellets, one behind each divider after habituation day 2. After the maze was rebaited 3 times and all pellets consumed within 15 minutes, criteria was met and turn bias was run the following day. If the rat did not consume all sugar pellets within 15 minutes, he continued habituation daily until he met criteria.

Turn bias: During turn bias and testing, one arm was blocked with the movable wall to form a "T" configuration with the visual cue in one of the side arms. In turn bias, both side arms were baited with sugar pellets behind the dividers. The rat was placed in the stem arm at the bottom of the "T" and allowed to enter either arm; after consuming the pellet he was placed back in the stem arm and again allowed to enter either arm. If he entered the same arm again, he was returned to the stem arm until he turned into the opposite arm and ate the remaining sugar pellet. When he successfully ate both sugar pellets, he was placed back in his home cage signaling the end of the trial. The next trial, the rat began from a different arm such that over 7 trials, the rat started from each of 3 arms 2 times and the remaining arm once and the visual cue was placed in either the right or left arm as equally as possible. Inter-trial time was about 10 seconds to rebait the maze. The direction the rat turned first the most out of 7 trials was considered his turn bias for the rest of testing.

Testing: As in turn bias, one arm was blocked with the movable wall, either the right or left arm contained the visual cue and the rat began in the stem arm. Trials were grouped into a block of 12 with the stem arm in arms 1-3 as shown in Figure 3.3. In the probe trial, arm 4 was the stem arm and the visual cue was placed in the turn bias arm. Within

treatment groups, the probe arm switched such that at least one animal in each group used each arm as the stem arm for the probe trial. Each testing day required the following:

- 10 consecutive correct trials using the 12 trial block in stem arms 1-3
- 1 correct probe trial from stem arm 4
- If correct probe trial, testing was complete
    - If incorrect probe trial
        - 5 consecutive correct trials using the 12 trial blocks
        - 1 correct probe trial from stem arm 4
        - This continues till correct probe trial

Day 1: Visual-cue learning; see Figure 3.3a: On day one, the rat was trained in a simple discrimination task to attend to the visual cue such that the food reward was placed in each arm with the visual cue 50% of the time. Turing towards the visual cue and attaining the reward was a correct choice. In the probe trial the rat turned towards the visual cue.

Day 2: Attentional Set Shift; see Figure 3.3b: On day 2 the complex task of an attentional set shift required the rat to switch from a visual to a spatial cue with the new strategy for reward turning into the arm opposite of his turn bias. The probe trial required the rat to turn in the opposite direction of the visual cue.

Day 3: Reversal learning; see Figure 3.3c: Reversal learning required the rat to enter the arm opposite the strategy from day 2 such that the rats turn bias arm now contained the food reward and the rat turned towards the visual cue on the probe trial.

Tissue Glutathione Analysis: Animals were rapidly decapitated (PND 100+) and punches taken from the medial prefrontal cortex. The tissues were homogenized in a 4:1

dialysis buffer(5 mM glucose, 140 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.2 mM KCl, and 0.15% phosphate buffer saline, pH 7.4): mobile phase solution (75 mM $NaH_2PO_4 \cdot H_2O$, 1.8 mM $C_8H_{17}O_3Sna$, 25 μM EDTA, 0.1% TEA and CH_3CN pH to 3.0 with phosphoric acid). One fraction was analyzed in the BCA method to determine protein content. The other fraction analyzed thiol content using HPLC with EC detection (Decade II, MagicDiamond electrode set at +1.89V, Antec Leyden, Netherlands).

^{14}C Cystine Uptake: Animals were rapidly decapitated and brains extracted. All tissue punches of the prefrontal cortex were treated with TBOA (100 μM) and ^{14}C-L-cystine (approximately 0.3 μM). Experimental groups are SSZ or control rat's male offspring (PND 100) in the presence or absence of SSZ (500 μM). The punches were washed and solubilized with 1% SDS. One fraction (200 uL) of the solubilized tissue was used for determining [14C]-L-cystine uptake by a scintillation counter. The other fraction (25 uL) was used to determine protein content by the BCA method. Uptake was measured in counts/μg protein.

Statistics: Attentional set shifting was analyzed using analysis of variance (ANOVA) with treatment as a between subjects variable. System x_c^- measures are analyzed with *t* test.

Results

In order to determine whether inhibition of system x_c^- in utero has a long term effect on adult behavior, attentional set shifting measured cognitive functioning in sulfasalazine treated offspring as illustrated in Figure 6.1. To analyze the effect of sulfasalazine on attentional set shifting an ANVOA with treatment as a between subjects measure found no significant differences on any of the tasks (day one: $F_{(2,26)} = 0.61$; day two: $F_{(2,26)} = 0.188$ $p > 0.05$; day three: $F_{(2,26)} = 2.56$ $p = 0.10$). However, there was a trend towards an increase in trials to reach criteria with sulfasalazine 16 mg/kg in utero on the reversal learning task.

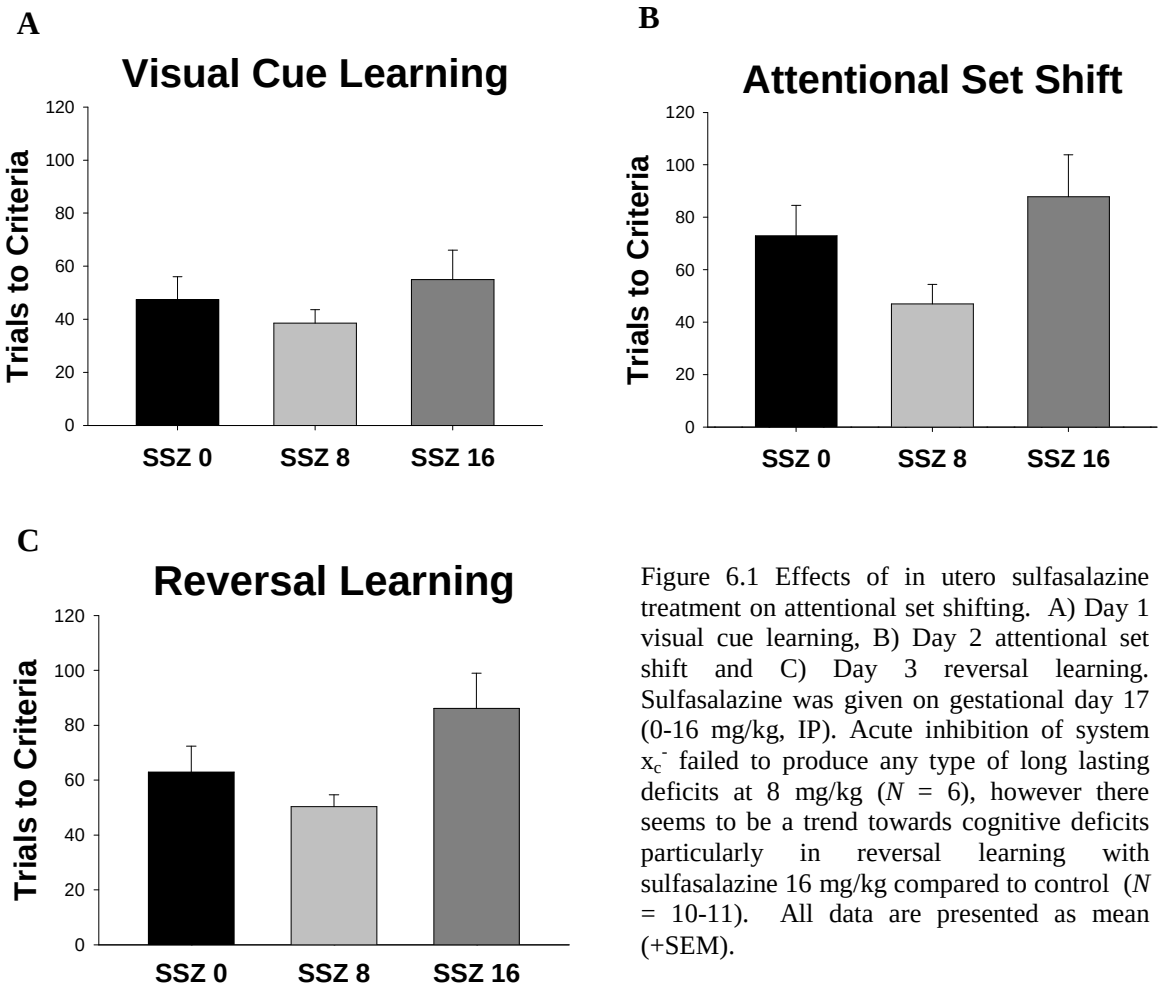


Figure 6.1 Effects of in utero sulfasalazine treatment on attentional set shifting. A) Day 1 visual cue learning, B) Day 2 attentional set shift and C) Day 3 reversal learning. Sulfasalazine was given on gestational day 17 (0-16 mg/kg, IP). Acute inhibition of system x_c^- failed to produce any type of long lasting deficits at 8 mg/kg (N = 6), however there seems to be a trend towards cognitive deficits particularly in reversal learning with sulfasalazine 16 mg/kg compared to control (N = 10-11). All data are presented as mean (+SEM).

Unlike behavioral effects from in utero sulfasalazine treatment, there are no apparent long term changes in system x_c^- measures in adult offspring. Only sulfasalazine 16 mg/kg in utero was tested in these paradigms since there was no behavioral effect in attentional set shifting at the 8 mg/kg dose. In Figure 6.2a there is no effect of in utero sulfasalazine treatment on glutathione tissue punches from the prefrontal cortex ($T = 1.11$ $p > 0.05$). There is also no significant difference in *ex vivo* system x_c^- dependent ^{14}C cystine uptake in the medial prefrontal cortex. Figure 6.2b illustrates this effect ($T = 1.52$ $p > 0.05$) using *t* test.

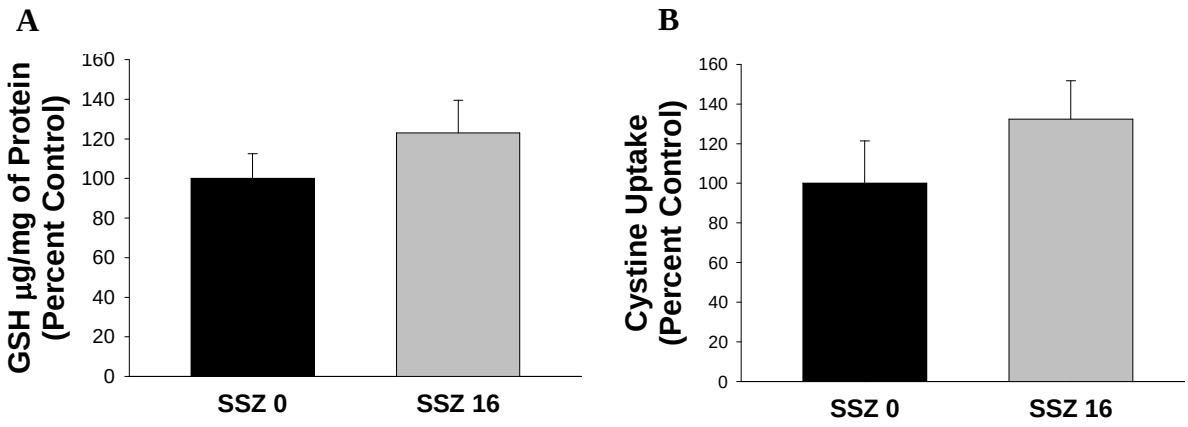


Figure 6.2 In utero sulfasalazine treatment on adult glutathione and system x_c- dependent ^{14}C cystine uptake in the medial prefrontal cortex. A) shows no significant difference in tissue punches taken from the medial prefrontal cortex on glutathione concentrations ($N = 9, 5$). B) indicates no changes in *ex vivo* cystine uptake in the same region ($N = 5$ per group) using *t* test.

Discussion

Nonvesicular glutamate release has a central role in cortical neuronal migration and organization during development (Behar et al., 1999; Hirai et al., 1999; Manent et al., 2005; Manent and Represa, 2007). This chapter examines the impact of in utero inhibition of system x_c^- on long term system x_c^- activity and behavior in offspring. Sulfasalazine administration on gestational day 17 had no long term behavioral effect at the lowest dose (8 mg/kg) however there seems to be a modest trend emerging for behavioral deficits in the reversal learning task in offspring from a higher dose (16 mg/kg). There does not appear to be any long lasting effects from an acute injection on gestation day 17 of sulfasalazine on system x_c^- activity measured through glutathione and *ex vivo* uptake in the prefrontal cortex. Together, these data indicate that there may be a modest long term behavioral deficit from in utero system x_c^- inhibition without persistent changes in system x_c^- activity.

Sulfasalazine treated offspring have normal levels of prefrontal glutathione with no apparent change in system x_c^- activity. These animals have no deficits in visual cue learning or attentional set shift with only a modest yet insignificant deficit in the reversal learning task. This supports the claim that long term changes in system x_c^- activity contributes to cognitive deficits as found in Chapter 3. MAM treated offspring have a long term significant increase in the potential for *ex vivo* cystine-glutamate exchange (present in juveniles and adults) potentially a compensatory response to reduced glutathione concentrations. Offspring also have deficits in reversal learning and adult N-acetylcysteine treatment is able to attenuate these behavioral deficits (see Chapter 3). If

altered system x_c^- activity contributes to cognition, why is in utero manipulation of system x_c^- insufficient to produce long term behavioral deficits?

Sulfasalazine treated offspring may not have long term behavioral deficits because a) altered system x_c^- activity is unrelated to cognitive deficits. This seems unlikely since N-acetylcysteine attenuates MAM-induced deficits likely by restoration of glutathione and system x_c^- activity and acute sulfasalazine treatment in adults produced deficits in cognitive processes (see Chapter 5) likely due to depleted extracellular glutamate resulting in unregulated synaptic neurotransmitter release. These results support the involvement of cystine-glutamate exchange to cognitive deficits in attentional set shifting. Or b) the administration parameters employed here (acute sulfasalazine injection on gestation day 17) is insufficient to produce long term changes in system x_c^- and behavioral deficits. This seems more likely since inducing behavioral deficits by in utero sulfasalazine treatment has not been attempted and finding the ideal dose, number of injections and timing of injections to produce long term changes in cystine-glutamate exchange could require a significant amount of work.

In utero manipulation of system x_c^- was hypothesized to produce long term changes in behavioral deficits given the critical role glutamate has in neurodevelopment. First, sulfasalazine is a potent inhibitor of system x_c^- activity whereas its metabolites sulfapyridine and 5-aminosalicylic are not (Gout et al., 2001; Chung and Sontheimer, 2009). Of importance for this chapter is the ability of sulfasalazine to cross the placental barrier (Peppercorn, 1984) for fetal penetration of the blood brain barrier. Inhibition of system x_c^- reduces extracellular glutamate *in vitro* (Chung and Sontheimer, 2009) and *in vivo* studies show systemic administration of system x_c^- reduces extracellular glutamate in

the prefrontal cortex of adults (see Chapter 5). It therefore seems likely that sulfasalazine is capable of reducing extracellular glutamate in the developing fetus.

As stated above, a potential factor limiting the ability of sulfasalazine to produce long term deficits is timing of insult. Cortical neurogenesis occurs between gestational days 14 to 20 (Bayer et al., 1993). All cortical principle cells originate from neuroepithelial cells lining the ventricles. Earlier born neurons populate the deepest cortical layers while the neurons born later in development form the more superficial layers with one exception, layer I neurons adjacent to the pial surface are generated first (Sidman and Rakic, 1973; McConnell and Kaznowski, 1991). Studies with the antimitotic agent MAM have shown that treatment on gestation day 17 induces specific changes in cortical layers II-IV while leaving the deeper layers V-VI, which are born earlier, undisturbed; treatment prior to day 17 leads to reductions in all cortical layers (Cattabeni and Di Luca, 1997; Moore et al., 2006) indicating the importance of injection timing in neurodevelopmental models of schizophrenia. Here, sulfasalazine was administered on gestation day 17 due to the effects of MAM treatment producing schizophrenia-like deficits when administered on day 17. However, as discussed in Chapters 3 and 4, it remains unclear whether MAM's effects are due to antimitotic properties or inhibition of neuronal migration by DNA methylation making in utero sulfasalazine timing optimization more difficult since it remains unclear whether inhibition of mitosis or migration is ideal to produce long term deficits

The ability of sulfasalazine to produce long term deficits was expected given the critical role non-vesicular glutamate has in neuronal migration during neurodevelopment. Neuroepithelial cells undergo mitosis in the germinal layers lining the ventricular system

and must migrate along radial glial cells to their final destination (Sidman and Rakic, 1973; Hatten, 1993). Non-vesicular glutamate and GABA have both been shown to promote neuronal migration and serve as stop signals when neurons reach their final destination (Behar et al., 1998; Behar et al., 1999; Behar et al., 2000; Behar et al., 2001; Hirai et al., 1999; Simonian and Herbison, 2001; Kihara et al., 2002). In support, immature neurons express NMDA and GABA receptors and glutamate and GABA are detected early in the microenvironment of migrating neurons (Nguyen et al., 2001; Lujan et al., 2005; Manent and Represa, 2007). Further, NMDA antagonists including MK801 and APV treatment but not other ionotropic glutamate receptor antagonists lead to strong impairments in neuronal motility *in vitro* and *in vivo* (Behar et al., 1999; Hirai et al., 1999; Manent et al., 2005). Work in cerebellar granule cells have shown that NMDA receptor activation leads to influx of Ca^{2+} frequencies necessary for migration and insufficient Ca^{2+} results in loss of movement (Komuro and Rakic, 1993; Kumada and Komuro, 2004). $GABA_A$ receptor activation, which has been shown to depolarize immature neurons promoting activation of voltage-gated calcium channels and NMDA receptors, promotes migration by increasing intracellular Ca^{2+} concentrations (Ben-Ari et al., 1997). Finally, mutants with genetic deletion of munc18-1 and munc13-1/2, proteins necessary for vesicular priming, are incapable of vesicular neurotransmitter release have normal cortical layering and synapse formation (Verhage et al., 2000; Varoqueaux et al., 2002) indicating synaptic neurotransmitter release does not contribute to neurodevelopment. Thus, reduced non-vesicular glutamate has the potential to impair neuronal migration and cellular organization.

Given the critical role of glutamate in neurodevelopment and the possible contributions of system x_c^- to extrasynaptic glutamate concentrations, it is interesting that xCT knockout mice have a normal appearance, normal lifespan and no changes in cortical or hippocampal width (Sato et al., 2005; De Bundel et al., 2011). As expected, there is a decrease in extrasynaptic glutamate but surprisingly, there is comparable glutathione concentrations in the hippocampus, striatum and other non-CNS organs such as the liver and pancreas (Sato et al., 2005; Massie et al., 2011; De Bundel et al., 2011). Behavior measures of xCT mutants include comparable to wild type spontaneous locomotion in an open field paradigm and a tendency towards less efficient learning with intact reference memory in Morris water maze (De Bundel et al., 2011). Interestingly, mutants have significant deficits in spatial working memory measured in delayed alternation Y-maze task (De Bundel et al., 2011); no other behavioral tasks have been measured. The absence of glutathione deficits suggests that behavioral deficits are induced by reduced extracellular glutamate or possibly due to abnormal cellular organization or circuitry, although this would have to be confirmed. It would also be interesting to evaluate neurodevelopmental processes in the mutants. As with any genetic mutation, there are likely compensatory measures to bring the environment in and around a cell to homeostasis making it impossible to assume that a lifetime depletion of cystine-glutamate exchange would have the same effects as an acute or chronic reduction in activity although further studies need to be conducted.

As stated above, in utero sulfasalazine treatment does not produce long term changes in behavior or system x_c^- activity. While it seems unlikely that system x_c^- activity has no effect on cognition (see Chapter 5), it is possible that these administration

parameters are insufficient to produce long lasting deficits. Further, it is possible that there is a compensatory upregulation of other nonvesicular glutamate transporters or migratory signals in response to insufficient cystine-glutamate exchange. There are many possible explanations for the minimal effect of in utero sulfasalazine; however, the ability of sulfasalazine to produce even a modest change in cognitive task is significant in itself, has never been shown and will require further characterization.

VII. DISCUSSION:

SCHIZOPHRENIA AND SYSTEM X_C^-

Schizophrenia is a lifelong disorder comprised of a number of symptoms including positive (hallucinations, delusions, paranoia), negative (anhedonia, blunted affect, social deficits) and cognitive deficits (executive function, behavioral flexibility, working memory, attention) (Andreasen, 1995; Elvevag and Goldberg, 2000; Blanchard and Cohen, 2006). The etiology of schizophrenia remains largely unknown however current research suggests a combination of multiple factors contributing to schizophrenia including a genetic predisposition and pre or postnatal environmental stressors leading to abnormal neurodevelopment (Weinberger, 1987; Chua and Murray, 1996; Lewis and Levitt, 2002; Lang et al., 2007; Brown, 2011). Many of the symptoms, particularly the cognitive deficits but also the positive and negative symptoms, have been linked to abnormal dorsolateral prefrontal cortical activity (Bunney and Bunney, 2000; Callicott et al., 2003; Jansma et al., 2004; Aalto et al., 2005; Eisenberg and Berman, 2010). In support, schizophrenics have abnormal pyramidal neuron activation leading to altered network oscillatory activity in the dorsolateral prefrontal cortex (Cho et al., 2006; Gonzalez-Burgos and Lewis, 2008; Gonzalez-Burgos et al., 2010).

In the dorsolateral prefrontal cortex schizophrenics have decreased oscillations in the gamma range (30-90 Hz) which have been shown to contribute to cognitive deficits (Lisman and Idiart, 1995; Lisman, 1999; Howard et al., 2003; Tallon-Baudry et al., 2004; Sejnowski and Paulsen, 2006). Desynchronization is a result of abnormal dorsolateral prefrontal cortical pyramidal cell activation. This is caused by changes in prefrontal glutamatergic activity including changes in NMDA receptor activation shown by non-competitive NMDA receptor antagonists producing all symptoms associated with schizophrenia in rodents and healthy individuals (Javitt and Zukin, 1991; Verma and

Moghaddam, 1996; Marino and Conn, 2002; Coyle et al., 2003; Vollenweider and Geyer, 2001; Rung et al., 2005). Evidence suggests that NMDA receptor antagonists preferentially inhibit receptors located on GABAergic interneurons due to their fast spiking properties (Jones and Buhl, 1993; Lei and McBain, 2002; Maccaferri and Dingledine, 2002; Homayoun and Moghaddam, 2007; Lewis et al., 2012). Further, schizophrenics have shown abnormalities in prefrontal GABAergic interneurons (Hashimoto et al., 2003b; Mellios et al., 2009; Fung et al., 2010) suggesting altered NMDA receptor mediated GABAergic activity in the dorsolateral prefrontal cortex contributing to pyramidal neuron desynchronization and symptoms of schizophrenia.

Astrocytes have also been shown capable of regulating synaptic plasticity and pyramidal cell activity (Barker and Ullian, 2010; Poskanzer and Yuste, 2011). They are uniquely positioned to regulate network synchronization based on the ability of one astrocyte to contact approximately 100,000 synapses, pyramidal cell bodies and other astrocytes (Bushong et al., 2003; Oberheim et al., 2006). Communication between astrocytes and neurons are critical for normal synaptic functions. Particularly astrocytic glutamate release has been shown to contribute to neuronal oscillatory activity (Fellin et al., 2004; Fellin et al., 2006; Carmignoto and Fellin, 2006) Glutamate is released by astrocytes at a concentration sufficient to activate extrasynaptic glutamate receptors (Baker et al., 2002b; Fellin et al., 2006; Makani and Zagha, 2007). System x_c^- may be a critical mechanism whereby astrocytes release glutamate to regulate network activity. First, system x_c^-exchanges one intracellular glutamate for an extracellular cystine (Baker et al., 2002b); cystine uptake is the key step in glutathione synthesis (Dringen and Hirrlinger, 2003) and externalized nonvesicular glutamate is capable of activating

extrasynaptic glutamate receptors particularly mGluR2/3 and NMDA receptors (Baker et al., 2002a; Mohan et al., 2011). Glutathione has been shown to be important in NMDA receptor activation by reducing the extracellular redox sites on the NMDA receptor potentiating its activity (Kohr et al., 1994; Lipton et al., 2002). Thus system x_c^- may represent an additional mechanism to regulate network activity based on the capacity to release glutamate and modulate synaptic neurotransmitter release through activation of mGluR2/3 and NMDA receptors.

Given the critical role of system x_c^- for synaptic functioning, altered or diminished activity could result in abnormal network synchronization through either 1) a decrease in extrasynaptic glutamate or 2) a decrease in glutathione concentrations. A decrease in extrasynaptic glutamate would lead to diminished extrasynaptic glutamate receptor activation. Diminished NMDA receptor activation would alter neuronal intracellular functioning (Makani and Zagha, 2007; Hardingham and Bading, 2010) and potentially contribute to diminished oscillatory activity (Fellin et al., 2004). Additionally, reduced extrasynaptic glutamate would also decrease activation of metabotropic glutamate receptors. Group II mGluR activation has been shown to negatively modulate presynaptic vesicular neurotransmitter release (Conn and Pin, 1997; Xi et al., 2002; Melendez et al., 2005). Therefore, extrasynaptic glutamate released from system x_c^- may regulate synaptic activity and neuronal activation; abnormal exchange could potentially lead to abnormal pyramidal neuron activation and synchronization contributing to symptoms of schizophrenia.

System x_c^- may also contribute to network synchronization via cystine uptake. Glutathione synthesis is limited by the availability of astrocytic cysteine (Dringen and

Hirrlinger, 2003) and regulates oxidative stress and redox balance (Dringen, 2000). Of particular importance is the ability of glutathione to reduce the extracellular redox site of the NMDA receptor to potentiate its activity (Kohr et al., 1994; Sies, 1999; Lipton et al., 2002). Given the contribution of NMDA receptor activation on GABAergic interneurons to regulate pyramidal network oscillations it is not surprising that deficient glutathione concentrations alters short and long term plasticity and induces NMDA receptor hypoactivation (Steullet et al., 2006). Therefore, deficits in glutathione concentrations may contribute to abnormal pyramidal neuron activation and desynchronization through reduced NMDA receptor activation through abnormal GABAergic interneuron activity.

Given the critical role of glutamate and glutathione in neuronal activation, altered astrocytic system x_c^- has the potential to lead to pyramidal neuron desynchronization. This likely occurs through both reduced extrasynaptic glutamate release and cystine uptake resulting in deficits in glutathione synthesis. Altered cystine-glutamate exchange in schizophrenics is supported by evidence of reduced glutathione concentrations (Do et al., 2000; Raffa et al., 2009) and increased expression of xCT, the active subunit of system x_c^- (Baker et al., 2008) suggesting abnormal cystine-glutamate exchange in schizophrenics and that this may contribute to the pathophysiology of this disorder. This is of great importance and will require more research since targeting system x_c^- may normalize gamma oscillations in the dorsolateral prefrontal cortex and lead to therapeutic treatments for the cognitive symptoms of schizophrenia.

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