ASSESSMENT OF THE FACE VALIDITY, CONSTRUCT VALIDITY, AND ETIOLOGICAL VALIDITY OF A NOVEL ANIMAL MODEL OF SCHIZOPHRENIA

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Abstract

Schizophrenia is a devastating neurological disorder that affects approximately 1% of the population and is characterized by positive, negative, and/or cognitive symptoms. Positive symptoms are associated with increased glutamate (Glu) and/or dopamine (DA) signaling in the nucleus accumbens (NAcc) while negative and cognitive symptoms are related to decreased Glu and/or DA signaling in the medial prefrontal cortex (mPFC) and hippocampus. The etiology of the disorder remains unknown, yet it is believed to be the result of a combination of genetic and neurodevelopmental factors. In order to improve our understanding of schizophrenia it is important to develop and further characterize animal models with relevance to the human condition.

Prior research has demonstrated that rodents exposed to neonatal treatment with domoic acid (DOM) (20 μg/kg), a kainate (KA) receptor (KAR) agonist, during a critical period of brain development (i.e. post-natal day (PND) 8-14) produces animals that exhibit several behavioural, neurochemical, and neuroanatomical abnormalities that are characteristic of schizophrenia.

The purpose of this thesis is to further examine the potential of DOM treatment in producing an animal model of schizophrenia by assessing cognitive functioning (i.e. face validity) using the attentional set-shifting and puzzle box paradigms, employing immunohistochemistry to examine dopamine transporter (DAT), D1 receptor, and D2 receptor as markers of the DA system in the mPFC (i.e. construct validity), and by

assessing active caspase-3, an executioner caspase in cellular apoptosis, in the mPFC (i.e. etiological validity).

Behavioural results indicate improved cognitive flexibility among DOM-treated males in the attentional set-shifting paradigm and improved short-term memory and problem solving ability among DOM-treated females in the puzzle box. Neurochemical results indicate increased DAT staining in the right PRL of DOM-treated females, decreased caspase-3 staining, as evidenced by optical density measurement, in the right PRL among DOM-treated males, and decreased caspase-3 staining, as evidenced by cell count measurement, in the right PRL among DOM-treated females. These results serve to strengthen the face validity, construct validity, and etiological validity of the model as they demonstrate cognitive alterations, neurochemical alterations in adulthood which are associated with behavioural symptomatology, and neurochemical alterations during development.

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List of Abbreviations

Abbreviation	Term
6-OHDA	6-hydroxydopamine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CA	cornu ammonis
cAMP	cyclic adenosine monophosphate
CamKII	calcium/calmodulin-dependent protein kinase II
CD	compound discrimination
CNS	central nervous system
COMT	catechol-O-methyltransferase
CPP	3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid
DA	dopamine
DAB	diaminobenzidine
DAG	diacylglycerol
DAT	dopamine transporter
DISC-1	disrupted in schizophrenia-1
DOM	domoic acid
DOPA	L-3,4-dihydroxyphenylalanine
ED	embryonic day
EDS	extra-dimensional shift
EtOH	ethanol
G-protein	guanine nucleotide-binding proteins
GABA	γ-aminobutyric acid

GAD67	glutamic acid decarboxylase 67
Glu	glutamate
IDS	intra-dimensional shift
IgG	immunoglobulin G
iGluR	ionotropic glutamate receptor
ILC	infralimbic cortex
IP3	inositol 1,4,5-triphosphate
KA	kainate
KAR	kainate receptor
M	medium
MAO	monoamine oxidase
mPFC	medial prefrontal cortex
mGluR	metabotropic glutamate receptors
NAcc	nucleus accumbens
NET	norepinephrine transporter
NHS	normal horse serum
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartic acid receptor
O	odour
PBS	phosphate-buffered saline
PCP	phencyclidine
PET	position emission tomography
PFC	prefrontal cortex
PIP-2	phosphatidylinositol 4,5-bisphosphate
PND	post-natal day
PPI	pre-pulse inhibition

PRL	prelimbic cortex
REV	reversal
SAL	saline
s.c.	subcutaneous
SD	simple discrimination
SEM	standard error of the mean
T	trial
TBS	tris-buffered saline
TNF-α	tumor necrosis factor α
TrkB	type 2 neurotrophic tyrosine kinase receptors
VH	ventral hippocampus
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area

Chapter 1

General Introduction

1.1 Schizophrenia

Schizophrenia is a debilitating psychiatric disorder that afflicts approximately 1% of the population (Goeree et al., 2005) and is associated with significant emotional and economic impact for patients, families, and society as a whole. For example, issues related to health-care costs and unemployment in patients with schizophrenia have been shown to cost the Canadian government approximately $6.85 billion annually (Goeree et al., 2005). While schizophrenia is certainly a prevalent and significant disorder, it was only identified relatively recently by Emil Kraepelin and Eugen Bleuler (as reviewed in Brockington and Leff, 1979; as reviewed in Tamminga and Holcomb, 2005). Schizophrenia was originally identified by Emil Kraepelin when it was referred to as "dementia praecox", meaning premature dementia (as reviewed in Brockington and Leff, 1979), before being subsequently classified as schizophrenia by Eugen Bleuler in 1911 (as reviewed in Tamminga and Holcomb, 2005).

The symptomatology of schizophrenia is extremely diverse and is characterized by (i) positive symptoms; marked by the presence of abnormal behaviours (e.g. delusions), (ii) negative symptoms; marked by absence of normal behaviours (e.g. social withdrawal) or (iii) cognitive symptoms; marked mainly by learning and/or memory deficits (e.g. working memory dysfunction) (DSM-IV-TR, 2000). The core diagnosis of schizophrenia requires various criteria to be met, including (i) the presence of two of the following: delusions, hallucinations, disorganized speech, disorganized behaviour, negative symptoms, (ii) social or occupational dysfunction, (iii) symptom duration of at least six

months, (iv) the absence of a diagnosis of schizoaffective disorder or a mood disorder, and (v) the absence of substance abuse or a general medical condition which would explain behavioural findings (DSM-IV-TR, 2000). Based on the constellation of symptoms that are manifested, schizophrenia can then be sub-categorized into various subtypes including residual, disorganized, catatonic, paranoid, and undifferentiated schizophrenia (DSM-IV-TR, 2000). Each subtype of schizophrenia has certain features, in addition to core features of schizophrenia, which predominate. For example, the diagnosis of disorganized schizophrenia requires the presence of both disorganized behaviour and flattened affect (DSM-IV-TR, 2000). Also, slow and abnormal movements typify catatonic schizophrenia, while preoccupation with delusions or hallucinations characterize paranoid schizophrenia (DSM-IV-TR, 2000). Residual schizophrenia can be considered a somewhat less severe version of paranoid schizophrenia and, finally, undifferentiated schizophrenia is typified by symptoms of schizophrenia which do not meet the criteria of any of the other subtypes (DSM-IV-TR, 2000).

The heterogeneity of schizophrenia, as seen through the various behavioural manifestations and subcategories, is further demonstrated in terms of developmental time course and gender differences. In terms of developmental time course, while patients are typically diagnosed with schizophrenia at some point between the late teenage years or early adulthood, the diagnosis has also been seen to occur in children and in the elderly (Rabins et al., 1984). Additionally, patients often exhibit a prodromal period prior to

diagnosis where, while the diagnostic criteria for schizophrenia are not met, behaviour is often described as atypical and may include introversion, peculiar interests, suspiciousness, or sudden changes in personality (Gourzis et al., 2002). The prodromal period has also been shown to include deficits in both academic and social functioning in individuals who are subsequently diagnosed with schizophrenia (Allen et al., 2005). Gender differences also seem to exist during the prodromal period, with males more frequently exhibiting negative and cognitive symptoms (Choi et al., 2009). While there is inherent variability between patients in terms of disease progression, following the prodromal period, there is a standard progression of schizophrenia which involves negative symptoms emerging first, often during the late teenage years or during the early twenties, cognitive symptoms following shortly after, and positive symptoms generally not appearing until years later (DeLisi, 1992). Gender difference also exist during the clinical period, as men exhibit an earlier onset and more pronounced negative and cognitive symptoms (Goldstein and Lewine, 2000).

The existence of an early prodromal period points to a neurodevelopmental etiology of the disorder, which is further supported by epidemiological studies demonstrating that various prenatal and perinatal factors are associated with the subsequent development of schizophrenia. These factors include prenatal exposure to influenza (Mednick et al., 1994), other types of maternal infection (Brown, 2006), maternal stress (Malaspina et al., 2008), and prenatal exposure to toxins (Opley et al., 2008). Notably, these factors are all associated with alterations in glutamate (Glu) tone, a primary neurotransmitter system

that is implicated in schizophrenia (Parsons et al., 1998; Deutsch et al., 2001; Dracheva et al., 2001; Gluck et al., 2002) which is also capable of altering dopamine (DA) functioning, another of the key neurotransmitter systems implicated in schizophrenia, by modulating DA release (Kretschmer, 1999).

Evidence of the genetic contribution to schizophrenia comes from various sources including adoption studies (Kety et al., 1994) and twin studies (Gottesman and Shields, 1982; Tsuang et al., 1991). In fact, the concordance rate in dizygotic twins has been shown to be 10-15% in dizygotic twins and roughly 50% in monozygotic twins (Onstad et al., 1991). Also, several potential susceptibility genes for schizophrenia have been recently identified and include genes for dysbindin, neuregulin 1, disrupted in schizophrenia 1 (DISC-1), catechol-O-methyltransferase (COMT), and D-amino-acid oxidase (as reviewed in Owen et al., 2005).

As can be seen from the variability in developmental time course, gender characteristics, subtypes, and genetic contributions, schizophrenia is a complex neurodevelopmental disorder that results from an interaction of environmental and genetic factors. However, the etiology of schizophrenia remains unclear. In order to increase our understanding of the etiology of schizophrenia and to improve upon the current treatment regimes, it is necessary to develop novel animal models of the disorder that more closely resemble the human condition.

1.1.1 Behavioural alterations

Schizophrenia is a debilitating neurological disorder that is characterized by (i) positive symptoms; marked by the presence of abnormal behaviours (e.g. delusions and hallucinations), (ii) negative symptoms; marked by absence of normal behaviours (e.g. social withdrawal, avolition) or (iii) cognitive symptoms; marked mainly by learning and/or memory deficits (e.g. temporal memory dysfunction) (DSM-IV-TR, 2000).

1.1.1.1 Positive symptoms

Positive symptoms of schizophrenia refer to the presence of abnormal behaviours such as hallucinations, delusions, disordered thought, and psychomotor agitation, and are believed to be due in part to DA hyperactivity in the mesolimbic pathway (Stone et al., 2007). Hallucinations involve perception of stimuli which, in fact, are not present, and can involve any of the sensory modalities, although auditory hallucinations are most common. Not surprisingly, the interpretation of the patient with respect to the source of these voices, as well as the content of the hallucinations, varies substantially between patients. Delusions, which are defined as persistent, obviously false beliefs, are another common positive symptom of schizophrenia. The most common forms of delusion involve delusions of grandeur, which often involve an inflated sense of self-importance, delusions of persecution where one often feels as though they are being conspired against, and delusions of reference where random, meaningless events are given some greater personal meaning (Kimhy et al., 2005). Disordered thought is perhaps the most common positive symptom and is typified by irrational thought that does not involve delusions or

hallucinations (Kerns and Berenbaum, 2002). The manifestations of hallucinations, delusions, and disordered thought appear quite similar and have thus been hypothesized to be the result of a common underlying pathology in the ability to filter out irrelevant information (Braff et al., 1977; Adler et al., 1982; Light and Braff, 2000). Finally, a lesser known and perhaps more discrete positive symptom is psychomotor agitation, where hyperactivity and/or stereotyped movements are seen. While the general public tends to associate positive symptoms most strongly with schizophrenia, it is in fact the negative and cognitive symptoms which generally emerge first and have a more significant impact on daily functioning (Rabinowitz et al., 2012).

1.1.1.2 Negative symptoms

Negative symptoms of schizophrenia refer to the absence of normal behaviours and include blunted affect, anhedonia, alogia, and social withdrawal (DSM-IV-TR, 2000). The neurobiological basis of negative symptomatology is believed to be due, in part, to DA hypoactivity in the mesocortical pathway (Stone et al., 2007). Blunted affect, or flattened affect in a more severe form, involves an apparent lack of facial emotion on the part of a patient and may or may not be associated with anhedonia, which involves a subjective lack of emotion (DSM-IV-TR, 2000). Alogia involves poverty in speech or thought and is often manifested as short, meaningless responses to questioning (DSM-IV-TR, 2000). Avolition involves a lack of motivation to participate in normal daily activities and is closely related to social withdrawal (DSM-IV-TR, 2000). Blunted affect, alogia, and avolition may all culminate in social withdrawal, the most common negative

symptom of schizophrenia, which manifests as either an inability or disinterest in maintaining social relationships (DSM-IV-TR, 2000).

1.1.1.3 Cognitive symptoms

Although many of the positive (e.g. delusions, hallucinations, and disordered thought) and negative symptoms (e.g. alogia) bear resemblance to cognitive symptoms, the cognitive group of symptoms are more restricted to attentional deficits, learning and memory impairments, and abnormalities in executive functioning (Gold and Harvey, 1993). Specific findings include impairments in processing speed (Schatz, 1998), reasoning (Corcoran, 2003), working memory (Conklin et al., 2000), temporal memory (Schwartz et al., 1991), vigilance (Mar et al., 1996), and social cognition (Hall et al., 2004). However, the inter-related aspects of the positive, negative, and cognitive categories of symptoms relates in part to similarities in the underlying neurobiological abnormalities that are responsible for the behavioural manifestations of schizophrenia (as reviewed in Fallon et al., 2003). The significance of neurocognitive symptoms in schizophrenia is supported by the findings that cognitive impairments are present, to some degree, in almost all patients (Green et al., 2004), and that the cognitive symptom category is most strongly correlated with functional ability (Green, 1996).

1.1.2 Neuroanatomical alterations

The significant heterogeneity of schizophrenia in terms of potential contributing etiological agents and symptom categorization is reflected in the diversity of associated

neuroanatomical and neurochemical abnormalities in the clinical population. These include both gross structural abnormalities such as increased lateral ventricular size and decreased cortical volume (DeLisi et al., 1991), cortical surface abnormalities (Crespo-Facorro et al., 2000), and fine structural abnormalities of the basal ganglia (Heckers et al., 1991), thalamus (Pakkenberg, 1992), corpus callosum (Bachmann et al., 2003), prefrontal cortex (PFC) (Breier et al., 1992), and hippocampus (Takahashi et al., 2000), to name just a few structures. Of these areas, two of the prime research targets are the PFC and the hippocampus. This is likely due to the clear connection between these areas and the negative and cognitive symptoms of schizophrenia which, while not being considered by the general public to be the symptoms most associated with schizophrenia, have been shown to have the strongest effect on daily functioning and are thought to be linked to core changes in the ability to filter and process information (Rabinowitz et al., 2012).

1.1.2.1 Hippocampus

The hippocampus is a limbic structure, located in the medial temporal lobe, which consists of the dentate gyrus and the hippocampus proper, which is sub-divided into cornu ammonis (CA) 1, CA2, and CA3 (El Falougy and Benuska, 2006). The CA1, CA2 and CA3 form a well-defined feed-forward Glu pathway known as the trisynaptic circuit (El Falougy and Benuska, 2006). First, the perforant pathway projects from the entorhinal cortex to the molecular layer of the dentate gyrus, which then projects to pyramidal cells of the *stratum radiatum* of CA3 via mossy fibers (El Falougy and

Benuska, 2006). Finally, pyramidal cells of CA3 project to pyramidal cells of CA1 via Schaffer collaterals (El Falougy and Benuska, 2006). The trisynaptic circuit plays a role in induction of long-term potentiation, which is believed to be a cellular mechanism for learning and memory, one of the key functions of the hippocampus (Teyler and Discenna, 1984; Matynia et al., 2002). The hippocampus also has a role in various pathologies, including Alzheimer's disease (Phillips et al., 1991; Du et al., 2001), temporal lobe epilepsy (McDonald et al., 1991; Cendes et al., 1993), and schizophrenia (as reviewed in Harrison, 2004). In terms of schizophrenia, various structural and neurochemical hippocampal abnormalities have been identified in the clinical population, including decreased cell counts and cellular disorganization (Jönsson et al., 1999), reduced overall volume (Narr er al., 2005), and elevated levels of brain-derived neurotrophic factor (BDNF) and type 2 neurotrophic tyrosine kinase (TrkB) receptor expression (Takahashi et al., 2000).

1.1.2.2 Prefrontal cortex

The PFC is defined as the cortical areas which receive input from the mediodorsal nucleus of the thalamus, and can be divided into the lateral PFC, the orbital PFC, and the medial PFC (mPFC) (Fuster, 2001). The PFC is specialized to aid in the control of affect, emotion, social behaviour, and executive function (Fuster, 2001). The diverse behavioural roles of the PFC have led to its implication in various conditions such as attention deficit hyperactivity disorder (Castellanos and Tannock, 2002; Durston et al., 2006), mood disorders (Drevets et al., 1998; Uranova et al., 2004), and schizophrenia. In

terms of schizophrenia, various prefrontal abnormalities have been identified in the clinical population, including reduced gray matter volumes (Thompson et al., 2001), altered TrkB receptors (Takahashi et al., 2000), altered tyrosine hydroxylase levels (Akil et al., 1999), and reductions in mean neuronal size in layer III (Rajkowska et al., 1998).

1.1.3 Neurochemical alterations

The neurochemistry of schizophrenia parallels the behaviour manifestations of the disorder in terms of complexity and heterogeneity as several neurotransmitter systems have been implicated in the pathogenesis and pathophysiology of schizophrenia. Such systems include the serotonin system (Joyce et al., 1994; Roth et al., 2004), the acetylcholine system (Mukherjee et al., 1994; Borda et al., 2002), the γ-aminobutyric acid (GABA) system (Beasley et al., 2002; Hashimoto et al., 2003), the DA system (Laruelle et al., 1996; Lindströma et al., 1999), and the Glu system (Dracheva et al., 2001; Gluck et al., 2002; Bauer et al., 2008). While the DA system received much of the early attention with respect to research, the Glu system has been the source of significant research recently and attempts have been made to merge the DA and Glu hypotheses of schizophrenia into a single, coherent model (Seeman, 2009).

1.1.3.1 Glutamate

Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS) (Ozawa et al., 1998). Proper levels of Glu signaling in the developing brain are critical as Glu has been shown to play an important role in regulating neuronal

survival, differentiation, and synaptogenesis (McDonald et al., 1990). Additionally, abnormal Glu transmission is related to variety of neuropsychiatric diseases, including schizophrenia (Parsons et al., 1998; Deutsch et al., 2001).

1.1.3.1.1 Biosynthesis of glutamate

While Glu can be synthesized via several biosynthetic pathways, transamination and the glutamine cycle predominate in the production of Glu (as reviewed in Feldman, 1997). Transamination involves transaminase catalysis of α-ketoglutarate, a constituent of the citric acid cycle, with an α-amino acid to produce both an α-keto acid and Glu (Platt, 2007). The glutamine cycle is catalyzed by glutaminase and involves the conversion of glutamine and water to Glu and ammonia (Platt, 2007). However, transamination differs from the glutamine cycle in that the original driving force behind the former pathway is glucose, while Glu itself drives the latter pathway through the conversion of Glu to glutamine via glutamine synthase. Thus, there is no net production in the glutamine cycle, since Glu serves as both the original reactant and the final product. However, it has been found that there is differential expression of glutamine synthase, which is predominantly expressed in astrocytes, and glutaminase, which is predominantly expressed in neurons, thereby favouring the net production of Glu in neurons (as reviewed in Feldman, 1997). Following Glu synthesis by transamination or the glutamine cycle, Glu is released by presynaptic neurons and diffuses across synapses to exert an effect on postsynaptic Glu receptors.

1.1.3.1.2 Glutamate receptor system

The large variety in Glu receptors can be broadly divided in either ionotropic Glu receptors (iGluRs) or metabotropic Glu receptors (mGluRs) (Ozawa et al., 1998). Ionotropic Glu receptors can be further categorized as either α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs), kainate receptors (KARs), and N-methyl-D-aspartic acid (NMDA) receptors (NMDARs) (Ozawa et al., 1998). When activated by ligand binding, iGluRs serve as cation-specific channels, thereby allowing the influx of sodium, the efflux of potassium, and, in certain cases, the influx of calcium, with a net excitatory effect due to electrochemical gradients favouring sodium influx to potassium efflux (Platt, 2007). However, AMPARs that contain a GluR2 subunit that has not undergone post-transcriptional RNA glutamine-to-arginine editing, KARs that contain either GluR5 or GluR6 subunits that have not undergone post-transcriptional RNA glutamine-to-arginine editing, and NMDARs can also allow the influx of calcium ions, a finding that has significance based on the various intracellular effects of calcium (as reviewed in Vissel et al., 2001). Increased intracellular calcium levels can lead to the activation of proteins including protein kinase C, which can in turn stimulate AMPARs and KARs (Wang et al., 2005). Increase intracellular calcium can also activate calcium/calmodulin-dependent protein kinase II (CamKII), which stimulates the insertion of additional AMPARs and KARs in the neuronal membrane and which enhances Glu release via synapsin phosphorylation (Fink and Meyer, 2002). Finally, calcium-induced CamKII activation can activate nitric oxide synthase, also resulting in increased Glu release (Fink and Meyer, 2002). Metabotropic Glu receptors can be further

classified into mainly postsynaptic Group I receptors (e.g. $mGluR_1$, $mGluR_5$), mainly presynaptic Group II receptors (e.g. $mGluR_2$, $mGluR_3$), or mainly presynaptic Group III receptors (e.g. $mGluR_4$, $mGluR_6$, $mGluR_7$, $mGluR_8$) (Ozawa et al., 1998). When activated by a ligand, mGluRs activate guanine nucleotide-binding protein (G-protein) linked cascades that ultimately increase intracellular concentrations of inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG) and cyclic adenosine monophosphate (cAMP) (Lambert, 1993). Presynaptic mGluRs serve to decrease NMDAR activity via G_i protein while postsynaptic mGluRs serve to increase NMDAR activity via G_q proteins (Lambert, 1993).

1.1.3.1.3 Glutamate degradation

Following presynaptic release and activation of postsynaptic Glu receptors, Glu is catabolized by several pathways which mirror the anabolic pathways. Firstly, Glu can be converted to glutamine via glutamine synthase, a component of the glutamine cycle (as reviewed in Feldman, 1997). Secondly, transaminase can function bi-directionally and thereby convert an α-keto acid and Glu to α-ketoglutarate and an α-amino acid (as reviewed in Feldman, 1997). Finally, a novel mechanism exists which involves the conversion of Glu to α-ketoglutarate via Glu dehydrogenase (as reviewed in Feldman, 1997). In addition to the three catabolic pathways of Glu degradation, excitatory amino-acid transporters are also able to serve as reuptake mechanisms to transport Glu from the synapse into presynaptic neurons and glial cells (as reviewed in Feldman, 1997).

1.1.3.1.4 Glutamate in CNS development

Glutamate is the primary excitatory neurotransmitter in the mammalian CNS and approximately half of all neurons in the brain are classified as Glu-releasing (Ozawa et al., 1998). Proper levels of Glu signaling in the developing brain are critical as Glu has been shown to play an important role in regulating neuronal survival, differentiation and synaptogenesis (McDonald and Johnston, 1990). Altered Glu signaling in the developing brain, specifically through treatment with NMDAR antagonists, has been shown to disrupt proper development by triggering apoptosis (Ikonomidou et al., 2001) and by causing subjects to display various behavioural abnormalities, including prepulse inhibition (PPI) deficits and increased locomotor activity, well into adulthood (Harris et al., 2003; Kawabe et al., 2007). In addition to NMDAR-mediated effects on development, KARs have also been shown to significantly affect the developing brain, as several persistent behaviours changes including alterations in social behaviour (Ryan et al., 2011), reward seeking behaviour (Burt et al., 2008a), and latent inhibition (Adams, 2009), among others, have been observed following neonatal treatment with low doses of domoic acid (DOM), a KAR agonist, from post-natal day (PND) 8-14. The previous findings have contributed to the development of a novel animal model of schizophrenia that is produced by the administration of a Glu agonist, a neurotransmitter that has been extensively implicated in the pathogenesis of schizophrenia (Buka et al., 2001; Dracheva et al., 2001; Meador-Woodruff et al., 2001; Gluck et al., 2002; Bauer et al., 2008; Malaspina et al., 2008).

1.1.3.1.5 Role of glutamate in schizophrenia

The findings that Glu is the primary excitatory neurotransmitter in the CNS (Ozawa et al., 1998) and that alterations in Glu tone during development can have significant effects on neuroanatomy (McDonald and Johnston, 1990) and behaviour (Harris et al., 2003; Kawabe et al., 2007) are significant in that they demonstrate the impacts of developmental insults to the Glu system in terms of significant, persistent behavioural abnormalities. Such observations have led to early Glu dysfunction being implicated in disorders such as schizophrenia. The potential role of Glu dysfunction in schizophrenia has been demonstrated by various studies showing that factors including maternal stress and prenatal viral infections, both of which are associated with altered Glu signaling, are also associated with schizophrenia (Buka et al., 2001; Malaspina et al., 2008). Also, various studies have demonstrated Glu system alterations in the post-mortem brains of aged individuals diagnosed with schizophrenia. These include decreased PFC KAR binding and alterations in KAR subunit expression (Meador-Woodruff et al., 2001), increased expression in the PFC of various NMDAR subunits (Dracheva et al., 2001), Glu transporters (Bauer et al., 2008) and phosphate-activated glutaminase, an enzyme that converts glutamine to Glu (Gluck et al., 2002). However, some of the first and most significant support for the role of Glu in schizophrenia came from the observation that phencyclidine (PCP) and ketamine, both of which are NMDAR antagonists, can elicit positive, negative, and cognitive symptoms in both humans and animal models (Geyer et al., 1984; Sams-Dodd, 1997; Adams and Moghaddam, 1998; Adler et al., 1999a; Rasmussen et al., 2007). Other studies revealed that treatment with PCP decreases DA

activity in the prefrontal lobes and that this hypoactivity was responsible for the manifestation of negative and cognitive symptoms (Jentsch et al., 1999). While the DA hypothesis of schizophrenia, which proposes that DA hypoactivity at D1 receptors in the PFC underlies both cognitive and negative symptoms while DA hyperactivity at D2 receptors in subcortical structures underlies positive symptoms, has been the most influential theory of schizophrenia, this discovery helped pave the way for the Glu hypothesis of schizophrenia (Olney et al., 1999). The Glu hypothesis proposes that NMDAR hypofunction is responsible for various aspects of the symptomatology observed in schizophrenia (Olney et al., 1999). However, as the Glu system, which acts through NMDARs, AMPARs, and KARs, can serve to modulate the release of DA (Kretschmer et al., 1999) these two hypotheses are not mutually exclusive and can compliment each other, as will be discussed in subsequent sections.

1.1.3.2 Dopamine

Dopamine is a catecholamine neurotransmitter that is important in various processes including cognition (as reviewed in Nieoullon, 2002), motivation (as reviewed in Wise, 2004), reward (as reviewed in Ikemoto, 2007), and motor functioning (Brück et al., 2001). Dopaminergic abnormalities have been implicated in various disorders including Parkinson's disease (Leenders et al., 1986; Frost et al., 1993), attention-deficit hyperactivity disorder (LaHoste et al., 1996; Dougherty et al., 1999), and schizophrenia (Seeman et al., 1993; Laruelle et al., 1996; Lieberman, 2004). The association between aberrant DA pathways and schizophrenia has been investigated extensively following

early findings that drugs which function as dopamine antagonists can treat schizophrenia symptoms, leading to both the DA hypothesis of schizophrenia and to the development of novel various treatment regimes, including improved antipsychotic drugs, which modulate the DA system (Kapur et al., 2000; Potkin et al., 2003).

1.1.3.2.1 Biosynthesis of dopamine

The synthesis of DA is dependent on phenylalanine crossing the blood-brain barrier via L-type amino acid transporters, a process that occurs in competition with the uptake of tryptophan (as reviewed in Elsworth and Roth, 1997). Following the uptake of phenylalanine, phenylalanine hydroxylase converts phenylalanine to tyrosine, which is then converted to L-3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (as reviewed in Elsworth and Roth, 1997). DOPA is subsequently transported into presynaptic vesicles via vesicular monoamine transporter (VMAT), where aromatic-L-amino acid decarboxylase functions to convert DOPA to DA (as reviewed in Elsworth and Roth, 1997). While this marks the end of the biosynthetic pathway in DA neurons, other adrenergic and noradrenergic neuronal populations contain additional enzymes to further modify DA into norepinephrine and, in turn, epinephrine (as reviewed in Elsworth and Roth, 1997).

1.1.3.2.2 Dopamine pathways

The DA system can be understood as four main pathways, with these pathways originating at the site of DA cell body localization, namely the substantia nigra, the

ventral tegmental area (VTA), and the arcuate nucleus of the mediobasal hypothalamus (as reviewed in Feldman, 1997). Dopaminergic cells which are located in the substantia nigra project to the striatum of the basal ganglia, forming the nigrostriatal system (as reviewed in Feldman, 1997). The nigrostriatal system functions in motor control and damage to this system is associated with Parkinson's disease (Graybiel et al., 1990). Dopaminergic cells which are located in the VTA can project to either the limbic system (e.g. hippocampus, amygdala, nucleus accumbens (NAcc), etc.), forming the mesolimbic system, or to the cortex, forming the mesocortical pathway (as reviewed in Björklund and Dunnett, 2007). The mesolimbic system functions in motivation and reward-based behaviour (Wightman and Robinson, 2002) while the mesocortical pathway involves higher cognitive functions (Moghaddam et al., 1997). Finally, DA cells in the arcuate nucleus of the mediobasal hypothalamus project to the median eminence of the hypothalamus, thereby forming the tuberoinfundibular pathway which controls the release of prolactin from the anterior pituitary gland (as reviewed in Vallone et al., 2000).

1.1.3.2.3 Dopamine receptor system

Dopaminergic receptors are classified into two main categories: D1-like receptors and D2-like receptors (as reviewed in Feldman, 1997). The D1-like receptor family, which contains both the D1 and D5 receptors, is linked to G_s G-proteins which function to increase adenylate cyclase-based formation of cAMP (as reviewed in Sidhu and Niznik, 2000). The D2-like receptor family, which contains the D2, D3, and D4 receptors, is linked to G_i G-protein which serves a role which is antagonistic to the D1-like receptor

family by decreasing adenylate cyclase-based formation of cAMP (as reviewed in Sidhu and Niznik, 2000). Additionally, D2 is unique among the DA receptors in that it serves as the autoreceptor, thereby regulating presynaptic DA release (as reviewed in Sidhu and Niznik, 2000).

1.1.3.2.4 Dopamine degradation

The two main mechanisms for DA degradation include reuptake, the primary mechanism, and enzymatic degradation. Reuptake is accomplished by dopamine transporter (DAT) and norepinephrine transporter (NET), both of which are presynaptic transporters which allow the recycling of pre-synthesized DA (as reviewed in Feldman, 1997). Enzymatic degradation involves the actions of both monoamine oxidase (MAO) and COMT (as reviewed in Elsworth and Roth, 1997). While MAO is effective in reducing DA levels, its localization on mitochondrial membranes prevents direct synaptic degradation of DA, although there is a net decrease in DA as a result of the actions of MAO (as reviewed in Feldman, 1997). However, COMT exists in both cytosolic and membrane-bound forms, thereby allowing for both the intracellular and extracellular destruction of DA (as reviewed in Feldman, 1997).

1.1.3.2.5 Dopamine in CNS development

The DA system has an extended developmental period which, in the rat, extends from embryonic day (ED) 12, which is approximately equivalent to week five of gestation in human development, through to PND 14, which is approximately equivalent to the third

trimester in human development (Ugrumov, 1997). However, both post-mortem studies and *in vivo* position emission tomography (PET) studies have shown that peak cortical and subcortical DA tone is not reached until early adulthood (McGeer and McGeer, 1981;Wong et al., 1984). Thus, if early alterations to the DA system occur when the system is developing between E12 and PND14, and if such alterations persist, behavioural abnormalities may not become apparent until later periods, such as early adulthood, when DA signalling is more prominent. In fact, studies have shown that early, selective, 6-hydroxydopamine (6-OHDA)-induced degeneration of DA terminals produces abnormalities in anxiety levels and memory ability with treatment on PND4 or PND10 producing stronger effects than treatment on ED13 or ED17 (Shabanov et al., 2005). Additionally, postnatal stress from PND1-13 has been shown to produce animals who manifest increased DA metabolites when exposed to restraint stress in adulthood (Cabib et al., 1993), thereby confirming that various early manipulations, including 6-OHDA-induced DA terminal degeneration and stress exposure, can produce long-term DA abnormalities that persist into adulthood. These findings are significant as they demonstrate that early neurodevelopmental factors, which are believed to be involved in the pathogenesis of schizophrenia, may affect DA development and contribute to the later manifestations of the disease in early adulthood.

1.1.3.2.6 Role of dopamine in schizophrenia

The first evidence that schizophrenia is associated with alterations in the DA system came from the observation that chlorpromazine, a DA antagonist used for its anxiolytic

properties, was effective in treating the positive symptoms of schizophrenia (as reviewed in Carlson, 2007). This discovery led to the development of various other DA antagonist drugs that became classified as antipsychotic drugs. It was also observed that DA agonists such as amphetamine and cocaine are effective in producing positive symptoms of schizophrenia and that these symptoms are counteracted by treatment with antipsychotics (as reviewed in Carlson, 2007). These observations support the argument that antipsychotic drugs exert their effects by blocking DA transmission, specifically at D2 receptors. Subsequent studies revealed that the mesolimbic DA system, with projections from the VTA to the NAcc and amygdala, is likely to be the site of DA hyperactivity underlying the positive symptoms of schizophrenia (Laruelle et al., 1996). However, cognitive symptoms appeared to differ from positive symptoms in that cognitive symptoms were unaffected by treatment with antipsychotics and thus unlikely to be due to DA hyperactivity at D2 receptors (as reviewed in Feldman, 1997). In fact, research showed that cognitive symptoms were associated with DA hypoactivity at D1 receptors in the PFC (as reviewed in Abi-Dargham and Moore, 2003). Evidence of both DA hyperactivity and hypoactivity led to the development of a new class of drugs, the atypical antipsychotics, which function either through more complex binding profiles or as partial DA agonists which are able to both reduce DA activity in the overstimulated NAcc and also increase DA activity in the understimulated PFC (Lieberman, 2004). Aripiprazole is one such partial agonist which has a higher affinity than DA for DA receptors but elicits less of a response than the natural ligand (Lieberman, 2004). Thus, aripiprazole acts as an antagonist in the overstimulated NAcc and as an agonist in the

understimulated PFC.

1.1.3.2.7 Relationship between glutamate and dopamine

The two most prominent neurotransmitters that have been implicated in schizophrenia are Glu and DA, leading to both the Glu hypothesis of schizophrenia and the DA hypothesis of schizophrenia. However, these two explanations are not mutually exclusive. In fact, the idea of hypofrontality has emerged with integrates both Glu and DA alterations and proposes that prefrontal Glu hypoactivity, due to NMDAR hypofunction, leads to both DA hyperactivity in the mesolimbic pathway and hypoactivity in the mesocortical pathway (as reviewed in Carlson, 2007). Such alterations in the DA system are of relevance to schizophrenia as DA hyperactivity in the mesolimbic system is speculated to underlie the manifestation of positive symptoms while DA hypoactivity in the mesocortical system is speculated to underlie the manifestation of negative and cognitive symptoms (Stone et al., 2007). It is hypothesized that prefrontal NMDAR hypofunction is the result of genetic factors and/or some environmental insult and that reduced Glu tone from neurons in the PFC can result in downstream alterations in the DA system (Olney et al., 1999). Glutamatergic neurons in the PFC project to the VTA where they innervate both DA neurons and GABA-containing neurons. In turn, the DA neurons in the VTA project back to the PFC and the GABAergic neurons either project directly to the NAcc or innervate other DA neurons in the VTA that project to the NAcc. Such an arrangement of pathways would cause any decreases in prefrontal Glu activity to result in an underactivition of GABAergic neurons in the VTA which, in turn, would result in

disinhibition of DA neurons that project to the NAcc, thereby resulting in DA hyperactivity in the NAcc. Also, since the glutamatergic neurons in the PFC synapse on DA neurons in the VTA, which project back to the PFC, decreased prefrontal Glu activity would be a self-reinforcing process (as reviewed in Carlson, 2007).

In addition to the interaction between the Glu and DA systems in terms of hypofrontality, alterations in Glu signaling can have a direct effect on DA neurons as many DA cell bodies throughout the brain possess Glu receptors (Ozawa et al., 1998). Therefore, increased Glu activity during brain development may lead to over-activation of such DA neurons and, if NMDAR and AMPAR containing unedited GluR2 subunits are activated, may result in calcium influx into DA neurons (Swanson et al., 1997). In addition to the role of NMDARs and AMPARs in the flux of calcium, KARs, which are targeted by DOM, are also able to transport calcium into the intracellular space in cases where either GluR5 or GluR6 subunits that have not undergone post-transcriptional RNA glutamine-to-arginine editing are present (as reviewed in Vissel et al., 2001). Once present intracellularly, calcium is able to activate a number of signal transduction cascades that can ultimately lead to changes in gene expression (Purves et al., 2008). Thus, it is possible for alterations in the Glu system to lead to alterations in gene expression in DA cells.

The idea of hypofrontality and the potential effects of NMDAR activation on gene expression in DA cells are examples of interactions between the Glu and DA systems and

highlight potential ways in which treatment with Glu agonists such as DOM may affect DA transmission. Additionally, alterations in these neurotransmitter systems have served, and continue to serve, to guide research on schizophrenia in terms of animal models.

1.2 Animal models

Animal models involve simulating a human condition in a non-human species and are therefore useful for testing novel treatments and for improving our understanding of various disorders (Geyer and Markou, 2000). There are currently a number of accepted animal models of schizophrenia, most notably (i) the neonatal hippocampal lesion model (Lipska et al., 1995), (ii) pre-natal immune challenge models (Zuckerman and Weiner, 2003), (iii) the PCP model (Rasmussen et al., 2007) and (iv) various genetic models (Hikida et al., 2007; Pletnikov et al., 2008).

1.2.1 Measures of reliability and validity in animal models

In order for an animal model to be beneficial it must be both reliable and valid. Reliability refers to the consistency with which the results of a given study are obtained, while validity, of which there are various types, refers to how well the model maps onto the specific clinical question. While there are various types of validity that define the usefulness of animal models, the significance of a model does not necessitate that all validity criteria are met, since the relevant criteria for a model are largely dependent on the purpose of the model. However, while acknowledging that different categories of validity are relevant for different studies, the four most commonly accepted categories of

validity for animal models of human conditions include (i) face validity, (ii) construct validity, (iii) etiological validity, and (iv) predictive validity (Geyer and Markou, 2000). Face validity refers to the similarity between the behavioural manifestations in a model and behavioural manifestations of a given disease in the clinical population (Geyer and Markou, 2000). However, face validity must often be supplemented with construct validity, which refers to the accuracy with which a test measures that which it is intended to measure (Geyer and Markou, 2000). For example, construct validity involves the identification and elimination of alternative explanations for a given experimental finding. This type of validity is often difficult to establish since the concept of what a test should be attempting to measure changes as advancements improve our understanding of the human condition. An animal model with etiological validity involves recapitulating the clinical etiology (Geyer and Markou, 2000). Again, etiological validity is often difficult to establish since the etiology of the human condition is often poorly understood. Predictive validity, which is considered by some to be the most important form of validity, is often used to refer to the ability of a model to identify novel therapeutic compounds (Geyer and Markou, 2000). Two lesser known forms of validity include convergent validity and discriminant validity. Convergent validity refers to similarities between a given model and other models while discriminant validity refers to unique features of a given model (Geyer and Markou, 2000).

1.2.2 Modelling schizophrenia symptoms in rodents

The symptomatology of schizophrenia is extremely diverse and is characterized by (i)

positive symptoms; marked by the presence of abnormal behaviours (e.g. delusions), (ii) negative symptoms; marked by absence of normal behaviours (e.g. social withdrawal) or (iii) cognitive symptoms; marked mainly by learning and/or memory deficits (e.g. working memory dysfunction) (DSM-IV-TR, 2000).

1.2.2.1 Positive symptoms

Positive symptoms of schizophrenia refer to the presence of abnormal behaviours such as hallucinations, delusions, disordered thought, and psychomotor agitation, and are believed to be due in part to DA hyperactivity in the mesolimbic pathway (Stone et al., 2007). While animal models are clearly unable to mimic certain positive symptoms, such as hallucinations and delusions, psychomotor agitation and disordered thought are more reproducible (Rueter et al., 2004; Wedzony et al., 2008). Additionally, it is thought that impaired sensorimotor gating and an inability to filter out irrelevant information, which may be the core components of disordered thought, may also underlie hallucinations and delusions (Braff et al., 1977; Adler et al., 1982). Therefore, since impaired sensorimotor gaiting and deficits in filtering out irrelevant information can be easily assessed in rodents through tests of PPI and latent inhibition, respectively, these tests have become gold-standard behavioural tests in animals as they appear to assess the core functional disturbances of the disorder, while eliminating the need to assess clinical symptoms such as hallucinations and delusions.

1.2.2.2 Negative symptoms

Negative symptoms of schizophrenia refer to the absence of normal behaviours and include blunted affect, anhedonia, alogia, and social withdrawal (DSM-IV-TR, 2000). The neurobiological basis of negative symptoms is believed to be due, in part, to DA hypoactivity in the mesocortical pathway (Stone et al., 2007). The main negative symptoms which can be demonstrated in animal models include social withdrawal (Audet et al., 2009; Ryan et al., 2009), the gold-standard in terms of negative symptomatology, anhedonia (Daenen et al., 2003), and emotionality (Koike et al., 2009). Social withdrawal is often assessed either using a dynamic interaction whereby two rodents are placed in an open field chamber with the amount of interaction time spent by the subjects being measured (Audet et al., 2009). Alternatively, a more passive interaction can be performed by placing a test subject in a two-chamber arena which has a divider separating the test subject from a stimulus rat. This allows for the assessment of the amount of time spent by the test subject in proximity to the stimulus rat as a measure of social behaviour (Ryan et al., 2009). Other methods for assessing negative symptoms include the use of an elevated plus maze which has two arms with walls and two arms without walls (Koike et al., 2009). The amount of time spent by the subjects in the walls without arms provides a measure of the emotionality of the rat. Subjects who are less emotionally driven may spent more time in the unprotected arms than do those who are more fearful. Also, anhedonia can be assessed by placing an animal in a pool without an escape platform and measuring the length of time before the animal aborts attempts to escape (Daenen et al., 2003).

1.2.2.3 Cognitive symptoms

The cognitive symptoms of schizophrenia are considered to include attentional deficits, learning and memory impairments, and abnormalities in executive functioning (DSM-IV-TR, 2000). The significance of neurocognitive symptoms in schizophrenia is supported by the findings that cognitive impairments are present, to some degree, in almost all patients (Green et al., 2004), and that cognitive symptom category is most strongly correlated with functional ability (Green, 1996). Cognitive symptoms are perhaps most easily replicated in animal models, and include tests such as (i) PPI (Braff and Geyer, 1990; Adams et al., 2008), (ii) latent inhibition (Alves and Silva, 2001; Zuckerman and Weiner, 2003), (iii) the radial 8-arm maze (Enomoto and Floresco, 2009), (iv) the puzzle box (Abdallah et al., 2011), and (v) digging tasks (Eggerton et al., 2005).

Pre-pulse inhibition is one of the more basic assessments of cognitive functioning as it involves reflexive behaviour. Pre-pulse inhibition, which, along with latent inhibition, are two gold-standard behavioural tests of schizophrenia, is an assessment of the sensorimotor gating reflex, which shows great consistency across species (Swerdlow et al., 1999). Pre-pulse inhibition involves the normal suppression of the acoustic startle reflex when pre-exposed to a lower, non-startling pre-pulse tone. Normally, exposure to a pre-pulse, which is not consciously discernible from the pulse, decreases the response to a subsequent startle pulse. However, schizophrenia is associated with impaired PPI, such that the pre-pulse does not decreased the response to the subsequent startle pulse (Grillon et al., 1992).

Assessment of latent inhibition, which is a phenomenon of basic classical conditioning, is another method of assessing lower-level cognitive ability. Latent inhibition involves the ability to filter out irrelevant information and occurs when pre-exposure to a stimulus, which is not associated with a noteworthy outcome (e.g. aversive event), prevents the later conditioning of the original stimulus with a subsequent event (e.g. aversive event). For example, a subject may be pre-exposed to a sucrose solution, with no adverse outcome (e.g. shock exposure). Subsequently, if the subject, while consuming sucrose, was to receive a mild shock, the association between the sucrose solution and the shock would be weak compared to the association that would be established if their first exposure to the sucrose solution had been paired with a shock. Those with schizophrenia demonstrate impaired latent inhibition, with strong associations being formed even with numerous pre-exposures without an adverse outcome (Gray et al., 1995).

The radial 8-arm maze, a more complex task than PPI or latent inhibition, involves spatial working memory. Impaired working memory is now recognized as a possible core cognitive symptom in schizophrenia and can be assessed in the radial 8-arm maze by baiting all eight arms with food and determining the number of arm choices that are made before the food reward is received from all arms, with lower total number of arm choices indicating superior spatial working memory.

The puzzle box and digging task involve the assessment of more high-level cognitive processes. As of yet, these paradigms have not been frequently used to evaluate higher-

order cognitive processes in animal models of schizophrenia. One reason for this may be that, when compared to PPI and latent inhibition, both the puzzle box and digging task are quite time-intensive for the experimenter. However, since higher-order cognition is known to be affected in schizophrenia (Bustini et al., 1999; Pantelis et al., 2009), assessing models with these two tasks may better inform researchers about the nature of cognitive deficits that are present in various animal models of schizophrenia. In turn, should there prove to be cognitive changes in these tasks, they are ideal targets for assessments of predictive validity, given that cognitive symptoms are known to have the most significant effect on daily functioning in those with schizophrenia and are thought to be core in the manifestation of the disorder (Rabinowitz et al., 2012).

The puzzle box is able to assess short-term memory ability, long-term memory ability, and problem solving ability. This is accomplished by placing a subject in an aversive, brightly lit chamber which is separated from a more comforting, dark chamber. In order to enter the less aversive chamber, the subject must discover how to dislodge the barrier that separates the chambers. Additionally, subjects are tested a number of times per day and the barrier changes each day over the three days of testing, thereby allowing for the assessment of problem-solving, short-term memory, and long-term memory.

Finally, cognitive flexibility can be assessed using a digging procedure known as the attentional set-shifting paradigm. Cognitive flexibility involves shifting attention from one focus to another (Owen et al., 1993; Konishi et al., 1998), involves inhibitory control

and working memory components (Pantelis et al., 2009), and has been shown to be disrupted in various populations (Dias et al., 1996; Birrell and Brown, 2000; Rodefer et al., 2005). In this test, subjects are provided with two bowls, one of which is baited with food, with bowels varying in terms of the digging medium (M) that they contain and their odour (O). In order to solve various discrimination problems and obtain a food reward, subjects must learn to attend to a relevant cue (i.e. digging M in a bowl) while ignoring an irrelevant cue (i.e. O of a bowl). The task is to use the cues of digging M or O to select the "correct" (i.e. baited) bowl. Subsequently, the relevant and irrelevant cues are then shifted and cognitive flexibility is assessed. This is conceptually equivalent to a human neuropsychology test known as the Wisconsin Card Sorting Task, which often identifies abnormalities in cognitive flexibility and attentional set-shifting in the clinical population (Everett et al., 2001).

1.2.3 Rodent models of schizophrenia

Animal models of human conditions are intrinsically difficult to develop, with schizophrenia being a particularly strong example due, in part, due to the diagnostic symptoms of schizophrenia (e.g. hallucinations and delusions). However, the heterogeneity of schizophrenia provides researchers with a variety of behaviours that can be exhibited in rodents while also serving to focus researchers on core neurobehavioural symptoms, including positive symptom behaviours (e.g. psychomotor agitation and disordered thought), negative symptom behaviours (e.g. social withdrawal, anhedonia, and abnormal emotionality), and cognitive symptoms behaviours (e.g. working memory

deficits, PPI deficits, impaired latent inhibition, altered problem solving, and decreased cognitive flexibility). These models can take various forms, but the most prominent categories are genetic models, lesion-based models, drug-induced models, and developmental models).

1.2.3.1 Genetic based models

While several potential susceptibility genes for schizophrenia have been recently identified (e.g. genes for dysbindin, neuregulin 1, DISC-1, and D-amino-acid oxidase) (as reviewed in Owen et al., 2005), the DISC-1 animal model seems to hold the most promise. Functionally, DISC-1 is believed to be involved in regulation of neuronal proliferation, neuronal process growth, and neuronal adhesion (Miyoshi et al., 2003; Sasaki et al., 2005). Disrupted in schizophrenia-1 animal models have shown face validity through studies demonstrated increased locomotor activity, enlarged ventricles, and altered PPI (Hikida et al., 2007). However, while the DISC-1 animal model seems to have a degree of etiological validity due to the genetic association between DISC-1 and schizophrenia, it is recognized that schizophrenia is the result of a complex interplay of many genetic and environmental factors, with no single etiological factor being responsible. Therefore, it is important to both recognize the importance of the DISC-1 genetic model and to attempt to develop additional models with improved etiological validity.

1.2.3.2 Lesion based models

In terms of neonatal lesion-based models, the neonatal ventral hippocampal (VH) lesion model has received the most attention. The VH lesion model appears to satisfy construct and face validity criteria as it has been shown to produce increased locomotor behaviour in response to various stimuli (e.g. novelty, forced swimming, and amphetamine exposure) (Lipska et al., 1993), alterations in PPI (Lipska et al., 1995), impaired latent inhibition (Greksch et al., 1999), and social withdrawal (Becker et al., 1999). Additionally, the VH lesion model has shown stress-induced alterations in levels of both DA and its metabolites in the NAcc (Lillrank et al., 1999). Predictive validity has also been shown in the VH lesion model as haloperidol has been observed to reverse abnormalities in locomotor behaviour (Lipska et al, 1993) and clozapine has been observed to reverse PPI abnormalities (Le Pen and Moreau, 2002). This model is also attractive due to its developmental nature, thereby supporting an argument for etiological validity. However, while the VH lesion model appears to exhibit etiological, face, construct, and predictive validity, the extent of damage inflicted in this model is not representative of hippocampal abnormalities in the clinical population, which mainly involve more subtle alterations to neuronal number, structure, connectivity, and neurochemical alterations (Gao et al., 1997; Zaidel et al., 1997; Webster et al., 2002; Zhang and Reynolds, 2002) rather than gross structural lesions.

1.2.3.3 Drug-induced models

Drug-induced models, particularly those involving DA agonists, have had significant appeal in schizophrenia research, largely based on similarities between their mechanism of action and the DA model of schizophrenia. Face validity and predictive validity have been shown with amphetamine administration, which stimulates DA release, as studies have demonstrated that amphetamine-induced hyperlocomotion and stereotypy are both reversible with antipsychotic treatment (Megens et al., 1992). Additionally, antipsychotic-reversible abnormalities have been shown in terms of PPI (Abekawa et al., 2008). Other DA-based drug-induced models involve the direct injection of DA (Swerdlow et al., 1990) or DA agonists (Swerdlow et al., 1994), which can also produce PPI deficits which are reversible with antipsychotic treatment. While the various pharmacological methods for increasing DA tone in the brain seem to satisfy the face and predictive validity criteria, this relates predominantly to the positive symptoms of schizophrenia. In fact, the negative and cognitive symptoms of schizophrenia are more associated with hypodopaminergia than hyperdopaminergia, thereby limiting the face and predictive validity of DA drug-induced models to positive symptoms.

Consequently, acute Glu-based models, which are able to mimic positive, negative, and cognitive symptoms, have supplanted DA-based pharmacologic models. The most widely accepted Glu-based model involves acute injections of PCP, a non-competitive NMDAR antagonist. This treatment has been shown to produce behavioural symptoms of schizophrenia such as hyperlocomotion (Adams and Moghaddam, 1998), social

isolation (Sams-Dodd, 1997), impaired PPI (Geyer et al., 1984), and impaired attentional set-shifting (Egerton et al., 2005). Additionally, predictive validity is confirmed in the PCP-model as PCP-induced hyperlocomotion, social isolation, and PPI deficits are reversed with clozapine (Freed et al., 1980; Bakshi et al., 1994; Steinpries et al., 1994). Neurochemical abnormalities consistent with schizophrenia have also been observed following acute PCP administration, including increased levels of DA and Glu in the PFC and NAcc (Adams and Moghaddam, 1998). However, despite the various advantages of the PCP-model of schizophrenia, it is not able to satisfy the criteria of etiological validity, thereby further limiting their usefulness in terms of identifying and characterizing the underlying etiology and neurodevelopmental nature of schizophrenia.

1.2.3.4 Developmental models

Developmental models are somewhat unique in that they can include drug-induced models, lesion models, or genetic models, so long as the experimental manipulation occurs during animal development to produce symptoms at a later point. Therefore, the VH lesion model is not only a lesion-based model, but also a developmental model. Another well-studied developmental model involves neonatal non-competitive NMDA receptor antagonism through the administration of dizocilpine, although the administration dosage and time-period can vary somewhat between studies. Regardless, the neonatal dizocilpine model has been shown result in reduced hippocampal volume and cell counts (Harris et al., 2003), PPI deficits (Harris et al., 2003), impaired spatial working memory (Kawabe et al., 2007), and increased locomotor activity (Hoffman,

1992), thereby demonstrating face validity.

In addition to the ability of developmental models to produce behavioural alterations that are characteristic of schizophrenia, the NMDAR antagonism model and neonatal VH lesion model appear to reflect the neurodevelopmental nature of schizophrenia and therefore appear to satisfy the criteria of etiological validity. However, further examination of these models leads to questions of the relevance of large lesions to the hippocampus and of relatively high dose exposure to NMDAR antagonists with respect to the types of insults that are likely to affect the clinical population. Therefore, additional animal models which involve more subtle brain insults must be developed. Also, due to the heterogeneous nature of the disorder, it is unlikely that any animal model will effectively capture all relevant characteristics of schizophrenia (Powell and Miyakawa, 2006). Therefore, in addition to the importance of novel animal models for improving our understanding of the etiology of schizophrenia and to screen potential therapeutants, novel models must be developed to more effectively mimic such a complex disorder. The current study focuses on further characterizing a novel animal model of schizophrenia that involves subtle alterations in Glu signaling in the developing brain (see Section 1.3).

The current model (i.e. subcutaneous (s.c.) injections of 20 μg/kg of DOM from PND 8-14) has been shown to produce subjects that exhibit various behavioural, neurochemical, and neuroanatomical alterations that are characteristic of schizophrenia without inducing

gross structural lesions (Doucette et al., 2004; Bernard et al., 2007; Doucette et al., 2007; Adams et al., 2008; Burt et al., 2008). In addition to the face validity shown in the DOM model, the model is advantageous as, based on our current understanding of schizophrenia, it appears to satisfy the criteria of etiological validity as it is developmental in origin and, unlike some alternative models, involves exposure to very low doses of a neurotoxin that targets the Glu system, a system that has been shown to be affected in the clinical population (Dracheva et al., 2001; Gluck et al., 2002; Bauer et al., 2008).

1.3 Domoic acid model of schizophrenia

1.3.1 Domoic acid

Domoic acid, an amnestic shellfish toxin that is produced by *Pseudo-nitzschia* species, gained prominence in 1987 following the consumption of contaminated *Mytilus edulis* harvested around Prince Edward Island (Perl et al., 1987). Effects of varying severity were seen in those exposed to DOM, ranging from gastrointestinal symptoms to short-term memory loss, seizures, and death (Perl et al., 1990). Domoic acid is structurally similar to KA and acts in a dose-dependent fashion, exerting its effects at lower doses via KA receptors and, at higher doses, via a variety of Glu receptors (Tasker et al., 1996).

1.3.2 Domoic acid exposure during development

Domoic acid has a short half-life and poorly penetrates the blood-brain barrier, thereby reducing its toxicity in older individuals (Preston and Hynie, 1991). However, the ability

of DOM to enter the brain during development prior to blood-brain barrier development, combined with its ability to cross the placenta, make DOM a more potent neurotoxin during development (Jeffery et al., 2004). *In vivo*, rodents exposed to DOM during development have shown various persistent behavioural and neurobiological abnormalities. Behavioural alterations include seizure-like symptoms in response to a novel spatial environment (Doucette et al., 2004), altered PPI (Adams et al., 2008), altered latent inhibition (Adams, 2009a), and social withdrawal (Ryan et al., 2009). Neurobiological alterations involving increased mossy fiber sprouting (Bernard et al., 2007) and decreased hippocampal glutamic acid decarboxylase 67 (GAD 67) and parvalbumin (Gill et al., 2010) have also been seen. Neonatal exposure to DOM has generated interest as a novel animal model of schizophrenia due to its effects on the Glu system, which is extensively implicated in schizophrenia, its developmental nature, and the various behavioural and neurochemical abnormalities that result from early exposure.

1.3.3 Neonatal low-dose domoic acid exposure as an animal model of schizophrenia

Treatment with low doses of DOM (20 μg/kg) from PND 8-14 has been shown to have potential as a novel animal model of schizophrenia. Support comes from studies demonstrating that treatment with DOM produces statistically significant neurochemical alterations in the hippocampus that are characteristic of schizophrenia, including changes in BDNF (Doucette et al., 2004), TrkB receptors (Bernard et al., 2007), and GAD 67 (Adams, 2009). Also, it has been demonstrated that treatment with DOM produces statistically significant alterations in PPI (Adams et al., 2008) and latent inhibition

(Adams, 2009), two "gold standard" behavioural tests for animal models of schizophrenia, as well as in social withdrawal (McInnis, 2009; Ryan et al., 2009) and emotionality (Doucette et al., 2007), both of which are negative symptoms of schizophrenia. Additionally, alterations have been seen in reward seeking behaviour (Burt et al., 2008a; Burt et al., 2008b), which is considered to reflect a positive symptom of schizophrenia, and in temporal memory ability, which is considered to reflect cognitive symptomatology (Robbins, 2010).

These behavioural manifestations of schizophrenia are associated with DA hyperactivity in the mesolimbic DA system and hypoactivity in the mesocortical system, thus suggesting that neonatal treatment with the KAR agonist DOM may produce persistent alterations in the DA system. The mechanisms through which DOM-induced neurodevelopmental alterations could occur are numerous. Ostensibly, an increase in KAR activity during a critical period of development could alter the maturation and expression of not only KARs but also other neurotransmitter systems, including the GABA system and DA system, which depend on KAR activity (Rodríguez-Moreno and Lerma, 1998). For example, DOM-induced alterations in the DA system are plausible as KARs, a subtype of iGluR on which DOM acts, appear to regulate the mesocorticolimbic DA system by modulating DA release (Kalivas et al., 1989). Thus, treatment with DOM may cause excessive DA release which could, in turn, result in compensatory changes in the mesocorticolimbic DA system. Additionally, evidence suggests NMDAR involvement in DOM-mediated effects on conditioned O preference (Tasker et al., 2005).

This finding is significant in that NMDAR can flux calcium ions following the displacement of a channel-blocking magnesium ion, a process which may be aided by KAR activity (Purves et al., 2008). The intracellular presence of calcium can then activate various cascades that result in alterations in gene expression which, in turn, can have persistent effects on protein production. In addition to the indirect role of KARs in activating NMDARs, which can then flux calcium, KARs can also directly flux calcium, specifically KARs that contain either GluR5 or GluR6 subunits that have not undergone post-transcriptional RNA glutamine-to-arginine editing (as reviewed in Vissel et al., 2001).

Thus, while the exact mechanism of action is undetermined, the regulatory role of KARs in the mesocorticolimbic DA pathway and the involvement of both non-edited KARs and NMDARs in calcium flux suggests that neonatal treatment with DOM can produce persistent alterations in the DA system. Such alterations in the DA system may also manifest as cognitive dysfunctions such as impaired attentional set-shifting, a cognitive symptom of schizophrenia.

The current study was conducted to further examine the potential of DOM treatment in producing an animal model of schizophrenia by assessing the face validity, construct validity, and etiological validity of the model. Face validity was further characterized using the attentional set-shifting and puzzle box paradigms to assess mPFC-dependent higher-order cognitive functions which have been shown to be altered in the clinical

population. These mPFC-dependent behaviours have been associated with altered DA tone, therefore the behavioural assessment was followed with immunohistochemical analyses of D1 receptor, D2 receptor, and DAT in the mPFC in an attempt to understand previous behavioural findings and any current behavioural findings (i.e. construct validity). Finally, after assessing behavioural alterations and any neurobiological contributions to these behaviours, potential mechanisms of both the neurobiological and behavioural alterations were examined using immunohistochemistry to assess caspase-3, which is an executioner caspase in the apoptotic pathway, in the mPFC (i.e. etiological validity). Therefore, the current study allowed for further examination of the potential of DOM treatment in producing an animal model of schizophrenia by assessing mPFC-dependent higher-order cognitive functioning (i.e. face validity), assessing DA markers related to higher-order cognitive behaviours in the mPFC (i.e. construct validity), and assessing caspase-3, a potential contributor to any behavioural and/or neurobiological alterations following neonatal DOM treatment (i.e. etiological validity).

These studies were performed with the objective of characterizing neurochemical abnormalities in the mPFC and possible cognitive abnormalities with the hypothesis that DOM treatment would produce alterations in caspase-3 and DA marker staining in the mPFC with consequent cognitive deficits in the attentional set-shifting and puzzle box paradigms.

Chapter 2

Assessment of cognitive functioning in adult rats treated neonatally with domoic acid

Summary

Cognitive symptoms of schizophrenia involve learning and/or memory impairments and abnormalities in executive functioning (Gold and Harvey, 1993). More specifically, findings include impaired processing speed (Schatz, 1998), reasoning (Corcoran, 2003), working memory (Conklin et al., 2000), temporal memory (Schwartz et al., 1991), vigilance (Mar et al., 1996), and social cognition (Hall et al., 2004). Cognitive symptoms have been often replicated in animal models using tests such as the radial 8-arm maze (Enomoto and Floresco, 2009), PPI (Braff and Geyer, 1990; Adams et al., 2008), latent inhibition (Alves and Silva, 2001; Zuckerman and Weiner, 2003), digging tasks (Egerton et al., 2005), and the puzzle box (Abdallah et al., 2011). The significance of neurocognitive symptoms in schizophrenia is supported by the findings that cognitive impairments are present, to some degree, in almost all patients (Green et al., 2004), and that cognitive symptom category is most strongly correlated with functional ability in the clinical population (Green, 1996).

The extent to which cognitive symptoms affect the clinical population, combined with the previous findings of behavioural abnormalities in animal models of schizophrenia using both the puzzle box procedure and digging tasks, provides rationale for the current study. The current study was conducted in order to further examine the face validity of the neonatal DOM model by determining the effects of neonatal DOM treatment on attentional set-shifting ability using a digging task and on problem solving ability, short-term memory, and long-term memory using the puzzle box paradigm. Results showed

statistically significant treatment effects in the attentional set-shifting task with DOM-treated males completing the IDS task with fewer errors than SAL-treated male counterparts. Additionally, significant treatment effects were seen in the puzzle box during trials assessing short-term memory and problem solving ability, as evidenced through DOM-treated females completing trial (T) 2, T3, T4, and T10 more quickly than DOM-treated males.

2.1 Introduction

Glutamate is the primary excitatory neurotransmitter in the mammalian CNS (Ozawa et al., 1998).. Proper levels of Glu signaling in the developing brain are critical as Glu has been shown to play an important role in regulating neuronal survival, differentiation, and synaptogenesis (McDonald et al., 1990). Additionally, abnormal Glu transmission is related to a variety of neurodegenerative diseases, including schizophrenia (Parsons et al., 1998; Deutsch et al., 2001).

Past research in our laboratory has shown that alterations in Glu signaling by early treatment with DOM, a KAR agonist, produces adult animals that manifest several behavioural abnormalities and neurochemical alterations that are characteristic of schizophrenia, including altered reward seeking (Burt et al., 2008), social withdrawal (Ryan et al., 2011), and PPI deficits (Adams et al., 2008), which reflect each of the major categories of symptoms of schizophrenia. However, due to the heterogeneity of schizophrenia and variability both between patients and between animal models in terms

of symptomatology, it is important to fully characterize each model in order to better understand the relationship between the mechanism by which the model was produced and the resulting behavioural and neurochemical effects. Additionally, complete evaluation of the wide array of cognitive domains in a model may provide insight into the diversity of cognitive patterns that may categorize various subcategories of schizophrenia.

Higher-end cognitive symptomatology can be assessed by examining attentional-set shifting ability. Attentional-set shifting is a cognitive ability which involves shifting attention from one focus to another (Owen et al., 1993; Konishi et al., 1998), involves inhibitory control and working memory components (Pantelis et al., 2009), and has been shown to be disrupted in various populations (Dias et al., 1996; Birrell and Brown, 2000; Rodefer et al., 2005). These include the clinical schizophrenic population (Jazbec et al., 2007; Pantelis et al., 2009), rodents with damage to the mPFC, a key area involved in schizophrenia (Birrell and Brown, 2000), macaques with damage to the lateral PFC (Dias et al., 1996), and in NMDAR antagonist-induced animal models of schizophrenia (Rodefer et al., 2005; Broberg et al., 2008).

The digging task paradigm has proven useful for examining attentional set-shifting abnormalities. The digging task assesses the ability of subjects to solve various discrimination problems in order to obtain a food reward by attending to a relevant cue while ignoring an irrelevant cue (see Table 1). The task is to use the cues of digging M or O to select the “correct” (baited) bowl.

Table 1. Examples of the stimulus pairs that were used during testing in the digging task.

Odour Pairs	Medium Pairs
Lemon and vanilla	Mop and letters
Orange and banana	Sponge and sand
Maple and coconut	Imitation moss and white bedding
Almond and cinnamon	Packing cardboard and brown bedding

Subsequently, the relevant and irrelevant cues are then shifted and cognitive flexibility is assessed (see Table 2). This is conceptually identical to the human neuropsychological test known as the Wisconsin Card Sorting task (Lysaker et al., 1995; Haut et al., 1996).

Cognitive symptomatology can also be assessed by examining puzzle box performance, which provides measures of short-term memory, long-term memory, and problem solving ability. In the clinical population, abnormalities have been observed in terms of short-term memory (Goldberg et al., 1998), long-term memory (Hill et al., 2004), and problem solving ability (Bustini et al., 1999). Previous findings using the puzzle box are rather limited due to its relatively recent development and have focused mainly on characterizing strain differences (Lucurto et al., 2003; Lad et al., 2010). However, a study by Abdallah et al. (2011) has recently assessed the performance of mice from five animal models of schizophrenia, including those treated sub-chronically with dizocilpine, those lacking the GluR1 subunit of AMPARs, those over-expressing D2 receptors in the striatum, and those with lesions to the mPFC or hippocampus. Results showed impaired performance in each animal models, with the effects being strongest in animals subjected to hippocampal lesioning.

The puzzle box procedure involves placing a subject in an aversive, brightly lit chamber and measuring the latency for the subject to enter a less aversive, dark chamber. Passage from one chamber to another is made progressively more difficult by decreasing the size of the door separating the two chambers and by obstructing the door with various objects.

Table 2. Example of a possible combination of stimulus pairs for a rat shifting from digging M to O as the relevant dimension in the digging task. The correct elements are shown in bold with the S+ column representing the correct stimulus pairs and the S- column representing the incorrect stimulus pairs.

	Dimensions		Element combinations	
Discrimination	Relevant	Irrelevant	S +	S -
Single Discrimination	M		**sand**	marbles
Compound Discrimination	M	O	**sand**/banana	marbles/orange
			sand/orange	marbles/banana
Intradimensional Shift	M	O	**paper**/maple	gravel/peppermint
			paper/peppermint	gravel/maple
Reversal	M	O	**gravel**/maple	paper/peppermint
			gravel/peppermint	paper/maple
Extradimensional Shift	O	M	**lemon**/cotton	rum/sponge
			lemon/sponge	rum/cotton

Testing consists of four Ts per day over a three day period, with the latency to enter the goal box being measured and used as an indicator of problem solving ability, short-term memory, and long-term memory. Trials 1, 3, 6, and 10 are tests of problem-solving as they involve the first exposure of subjects to obstacles on the way to the goal box (see Table 3). Trials 2, 4, 7, 8, 11, and 12 assess short-term memory as they involved re-exposure to challenges that were recently faced (see Table 3). Trials 5 and 9 assess long-term memory as they involved re-exposure to challenges that were faced on the previous day (see Table 3).

The current study employed both the digging task and the puzzle box procedure in order to assess a variety of higher-order cognitive abilities such as attentional set-shifting ability, short-term memory, long-term memory, and problem solving ability in order to further characterizing the effects of neonatal administration of DOM, a Glu agonist, on cognitive functioning. This was done with the objective of assessing higher order cognitive functions in the neonatal DOM model of schizophrenia with the hypothesis that DOM-treated subjects would demonstrated cognitive deficits that are characteristic of schizophrenia.

2.2 Materials and methods

2.2.1 Experimental animals and injection procedure

All experiments were conducted on the offspring of untimed pregnant Sprague-Dawley rats (Charles River Laboratories, St Constant, QC). Dams were left undisturbed until the

Table 3. Puzzle box testing procedure.

Day	Trial	Obstacle	Measure
1	1	Large door	Problem solving
1	2	Large door	Short-term memory
1	3	Small door	Problem solving
1	4	Small door	Short-term memory
2	5	Small door	Long-term memory
2	6	Bedding material	Problem solving
2	7	Bedding material	Short-term memory
2	8	Bedding material	Short-term memory
3	9	Bedding material	Long-term memory
3	10	Cardboard plug	Problem solving
3	11	Cardboard plug	Short-term memory
3	12	Cardboard plug	Short-term memory

day of birth which was defined as PND 0. Within 24 hours of birth, each litter was culled to up to 14 pups (7 male and 7 female, when possible). Pups from each litter (n=10) were pseudo-randomly assigned to either saline (n=20) or DOM (n=20) treatment groups with an equal number of males and females in each treatment group.

Domoic acid was obtained from Diagnostic Chemicals Ltd. (Charlottetown, PE) and the drug was dissolved in sterile saline with injections being administered s.c. in a volume of 10 ml/kg. From PND 8-14, pups were weighed, marked for identification (i.e. ear-notched), and given a single daily s.c. injection of either 20 μg/kg DOM or equal volume of saline. Previous work in our laboratory has shown that at this dose, no overt signs of behavioural toxicity are apparent (Doucette et al., 2003). Pups were weaned on PND 21 and singly-housed in a colony room maintained at approximately 22°C on a 12 hour reverse light cycle with lights turning on at 07:00 h. All animals received water and food (Purina Lab Chow) *ad libitum*. All testing was conducted during the dark phase of the light/dark cycle with the experimenter blind to treatment. All procedures were conducted according to the guidelines established by the Canadian Council on Animal Care and in accordance with the Animal Care Committee at the University of Prince Edward Island.

2.2.2 Attentional set-shifting procedure

Subjects (n = 39; 10 male DOM, 10 male SAL, 10 female DOM, 9 female SAL) from nine litters were assessed for attentional set-shifting ability using a digging task from

PND 132-177. The attentional set-shifting paradigm assesses the ability of subjects to solve various discrimination problems in order to obtain a food reward by attending to a relevant cue (i.e. digging M in a bowl) while ignoring an irrelevant cue (i.e. O of a bowl). The task is to use the cues of digging M or O to select the "correct" (baited) bowl. Subsequently, the relevant and irrelevant cues are then shifted and cognitive flexibility is assessed. This is conceptually equivalent to a human neuropsychology test known as the Wisconsin Card Sorting task.

Prior to testing, rats were food restricted for at least 7 days to ensure motivation to perform the food-based task. Subjects were weighed prior to food restriction, prior to testing, and post-testing in order to monitor the effects of food restriction. During the first two days of the food restriction period, subjects were exposed to bowls with several pieces of Sugar Crisp Cereal. On subsequent food restriction days, the food reward was buried in bedding material in order to expose subjects to the digging action that would be required for successful completion of the task.

The attentional set-shifting paradigm consisted of a one-hour habituation trial on the day prior to the commencement of testing. The first day of testing consisted of two single discrimination tasks in order to familiarize subjects with attending to either digging M or O in order to obtain a food reward. The subsequent day, subjects faced a series of tasks including a simple discrimination (SD) test, a compound discrimination test (CD), an intra-dimensional shift (IDS) test, a reversal (REV) test, and an extra-dimensional shift

(EDS) test. Subjects were tested with each type of discrimination until a criterion value of six consecutive correct choices were made, at which point they would progress to the next type of discrimination. Choices were defined as vigorous exploration of the digging M. This allowed subjects to approach each bowl and explore the O and texture of each M without making a choice until the material became vigorously displaced. Performing an upper limit of 30 responses or failing to complete a given discrimination in two hours resulted in the rat being removed from the chamber for 24 hours, at which point they were returned to the chamber in order to complete the series of discriminations, starting with the last discrimination problem that was successfully completed. After reaching the maximum allotted time on three separate occasions, a subject was no longer tested.

In the test of attention set-shifting ability, subjects were tested in a black plexiglass chamber measuring 50 cm x 25 cm x 50 cm (length x width x height). Subjects were given a one-hour habituation trial on day one, two single discriminations (i.e. both digging material and O) on day two, and a series of discrimination problems on day three. Testing involved access being given to two food bowls which differed from each other with respect to two sensory domains, namely in terms of O and the digging M which they contain. One bowl contained a food reward, which was consistently associated with one element of a sensory domain (i.e. reward in cup with M1, not M2), while the other sensory domain was irrelevant (i.e. O1 and O2 do not predict food location). Subjects underwent a series of discrimination tests including a SD where one element was to be chosen over another, a CD in which an irrelevant dimension was added, an IDS where the

same dimension was linked to reward but two new elements are presented, a reversal in which the previously non-rewarded element was now the correct option, and finally an EDS where the correct sensory dimension was changed. For example, in a single test session a subject could be faced with the following discriminations (see Table 2): First, choose the bowl with M1 over the bowl with M2 (SD). Subsequently, choose M1 over M2 when an irrelevant O is linked to each food bowl (CD). Then choose M3 over M4 while, again, an irrelevant O is linked to each food bowl (IDS). The reversal trial then occurs in which M4 should be chosen over M3, still ignoring the O. Finally, attention should be directed to O instead of digging M and O5 should be chosen over O6, with M5 and M6 being irrelevant. Subjects were tested with each type of discrimination until reaching a criterion level of 6 consecutive correct choices, at which point they progressed to the next type of discrimination. Nine cohorts of four subjects were constructed with one member of each sex and treatment condition in each of the nine cohorts, and a tenth cohort of three subjects was also constructed. Each member of a particular cohort received the same order of digging M and O throughout the set-shifting procedure while no two cohorts received the same sequence. An example of the arrangement of discriminations is provided in Table 2.

2.2.3 Puzzle box procedure

Subjects (n=40) were assessed in the puzzle box following completion of the attentional set-shifting procedure, from PND 153-189, with at least a week separating the two behavioural testing paradigms for each subject. The testing arena was constructed of

plastic corrugated board and consisted of a brightly light start chamber measuring 70 cm x 35 cm x 45 cm (length x width x height) which was separated from the darker goal chamber measuring 40 cm x 35 cm x 45 cm (length x width x height) by a divider that either had a large doorway measuring 22 cm x 11 cm (length x height) or a small doorway measuring 10 cm x 11 cm (length x height) (see Figure 1). The doorway could be unobstructed, filled with bedding material that must be dug through, or filled with a cardboard plug that must be pulled out of the opening. Subjects were placed in the light chamber and the latency to pass into the dark chamber was measured. The negative reinforcement of avoiding a large, brightly lit area served as motivation for subjects to solve the various problems posed by the obstructions in the doorway in order to pass into the goal box.

Testing consisted of four trials per day over a three day period (see Table 3). On day 1, T1 and T2 involved a large open doorway for easy entry into the goal chamber while T3 and T4 involved a small open doorway. The subsequent day, T5 mirrored T3 and T4 while T6-8 involved the doorway being blocked by bedding material. On the final day of testing, T9 mirrored T6-8 and T10-12 involved the doorway being blocked by a cardboard plug. Subjects were given two minutes to enter the goal box on trials involving an open doorway or bedding material and three minutes on trials involving a cardboard plug. Following entry into the goal box, subjects left for five minutes on T1 and one minute on all other trials before being returned to the light chamber for the subsequent trial.

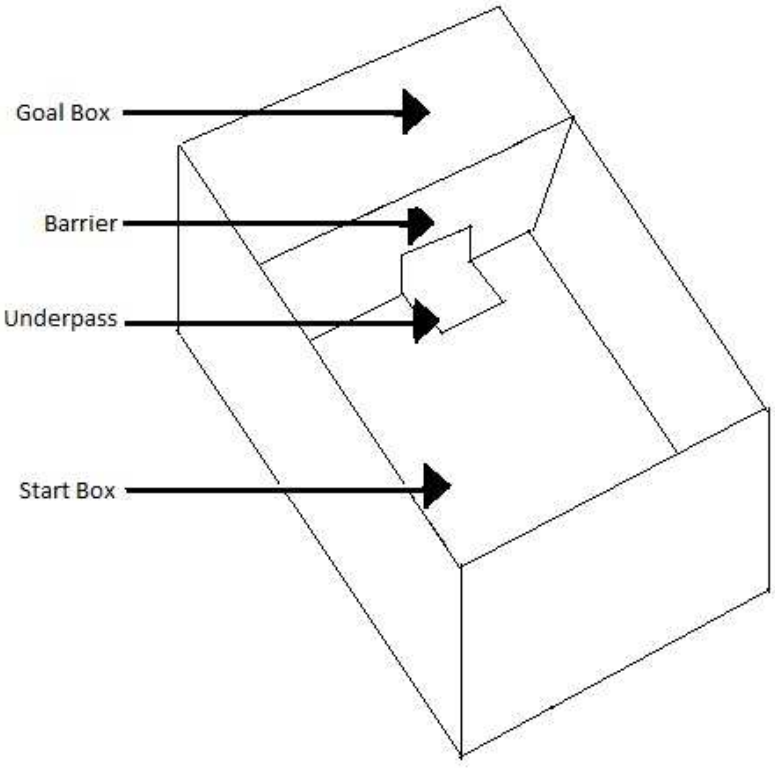


Figure 1. Diagram of the puzzle box used during testing.

2.2.4 Quantification of behaviour

All trials were video-recorded but scoring was performed concurrently with trial performance. Attentional set-shifting behaviour in the digging task was assessed by measuring the number of trials required to reach the criterion level of 6 consecutive correct choices for each type of discrimination. This provided a measure of the ability of subjects to shift attention from a previously learned rule to a new rule (i.e. cognitive flexibility) in order to receive food rewards with impaired attentional set-shifting ability being manifested as an increase in the number of trials to reach criterion.

Short-term memory, long-term memory, and problem solving ability in the puzzle box procedure was assessed by measuring the latency to enter the goal box on each trial. All trials were video-recorded but scoring was performed manually at the time of testing. Trials 1, 3, 6, and 10 were tests of problem-solving as they involved the first exposure of subjects to obstacles on the way to the goal box, thus increased latency to enter the goal box on these trials would indicate problem-solving deficits. Trials 2, 4, 7, 8, 11, and 12 assessed short-term memory as they involved re-exposure to challenges that were recently faced, thus increased latency to enter the goal box on these trials would indicate short-term memory deficits. Trials 5 and 9 assessed long-term memory as they involved re-exposure to challenges that were faced on the previous day, thus increased latency to enter the goal box on these trials would indicate long-term memory deficits.

2.2.5 Data analyses

The attentional set-shifting task data was separated for sex based on the extent of sex-specific findings in previous studies of the DOM model (Doucette et al., 2007; Adams et al., 2008; Adams 2009b; Burt et al., 2008a; Robbins, 2010; Ryan et al., 2011), and was analyzed using Student's *t*-tests for each trial type (PASW Statistics 18). A result of $p \leq 0.05$ indicated statistical significance.

The puzzle box data was also separated for sex and analyzed using Student's *t*-tests for each trial type (PASW Statistics 18). A result of $p \leq 0.05$ indicated statistical significance.

2.3 Results

For the attentional set-shifting task, results of Student's *t*-tests revealed a statistically significant treatment effect [$t(11) = -2.021$, $p = 0.039$] with DOM-treated males completing the IDS task with fewer errors than SAL-treated males (see Figure 2). No other statistically significant effects were observed in this task. See Table 4 for mean and standard error of the mean (SEM) values for the attentional set-shifting paradigm.

For the puzzle box task, results of Student's *t*-tests revealed statistically significant treatment effects in female subjects for T2 [$t(9.701) = -2.759$, $p = 0.021$], T3 [$t(9.198) = -1.946$, $p = 0.042$] , T4 [$t(18) = -2.585$, $p = 0.019$], and T10 [$t(11.744) = -1.947$, $p = 0.038$] (see Figures 3-6). In each of T2, T3, T4, and T10, DOM-treated females completed trials

Table 4. Mean ± SEM number of trials to criterion for subjects treated neonatally with DOM and SAL. Asterisk indicates a statistically significant difference between treatment groups ($p \leq 0.05$). Superscript "y" indicates groups in which a single subject performed, thereby preventing a calculation of SEM. Superscript "x" indicates a group in which no subjects performed.

	Trials to Criterion			
	Male		Female	
Trial Type	SAL	DOM	SAL	DOM
Single Discrimination	6.33 ± 0.33	6.60 ± 0.60	7.25 ± 0.95	6.00[y]
Compound Discrimination	11.00 ± 3.21	9.80 ± 1.32	7.00 ± 0.71	8.00[y]
Intradimensional Shift	7.89 ± 0.93	6.00 ± 0.00*	7.75 ± 0.85	6.00[y]
Reversal	10.00 ± 0.91	10.75 ± 2.25	8.00 ± 0.48	6.00[y]
Extradimensional Shift	9.00 ± 0.71	12.20 ± 2.94	8.25 ± 1.44	[x]

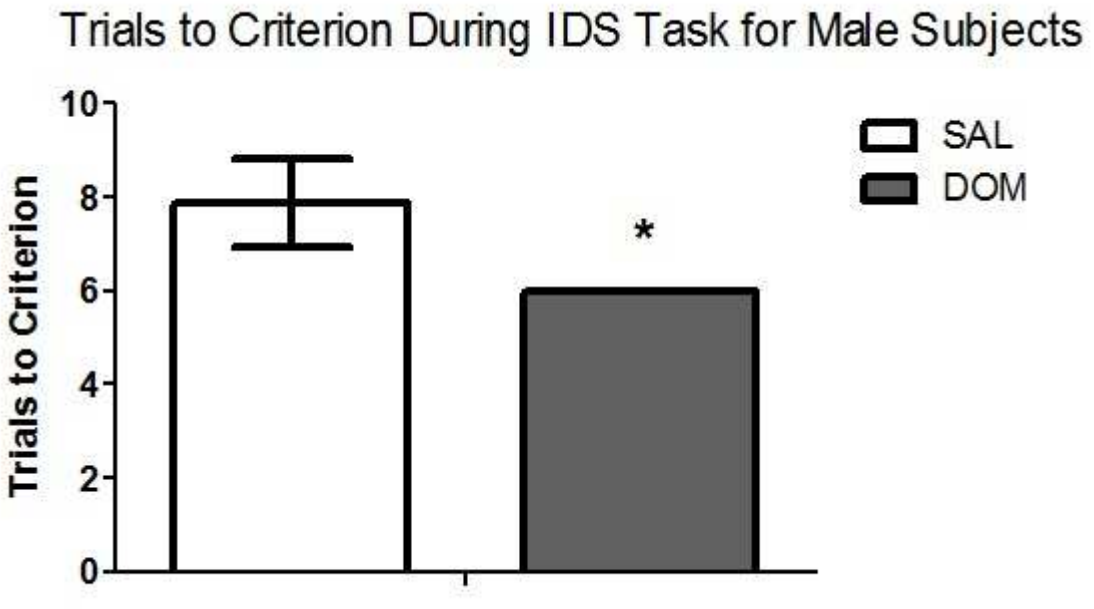


Figure 2. Mean ± SEM number of trials to criterion for DOM- and SAL-treated male subjects to complete the IDS portion of the attentional set-shifting task. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

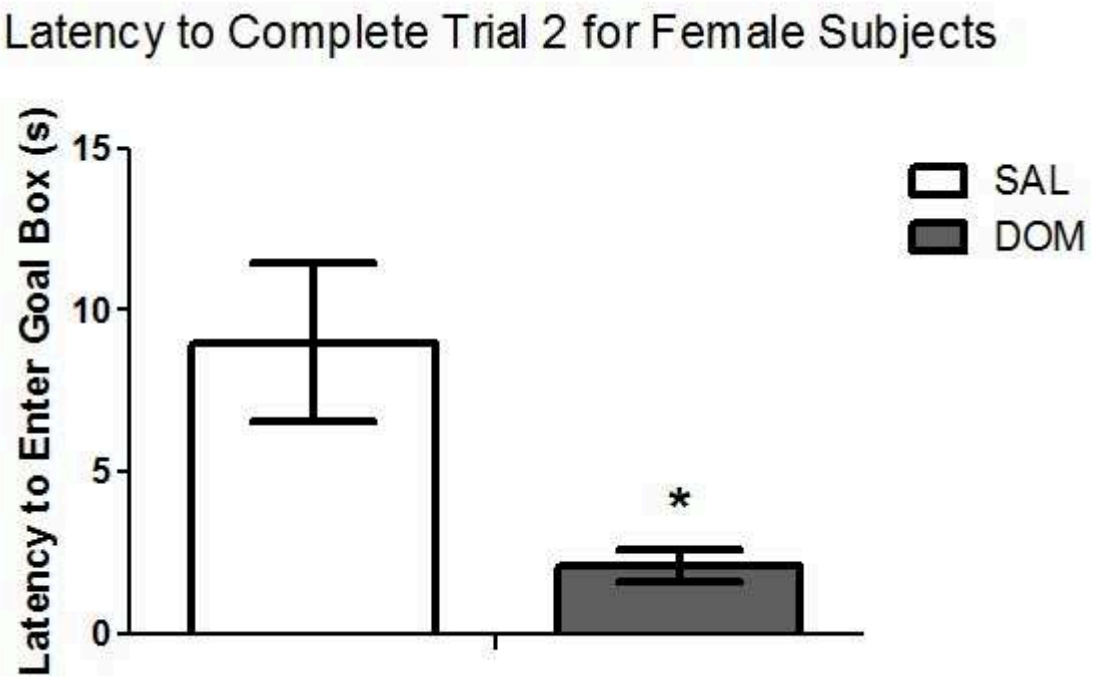


Figure 3. Mean ± SEM latency of DOM- and SAL-treated female subjects to enter the goal box during T2 of the puzzle box procedure. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

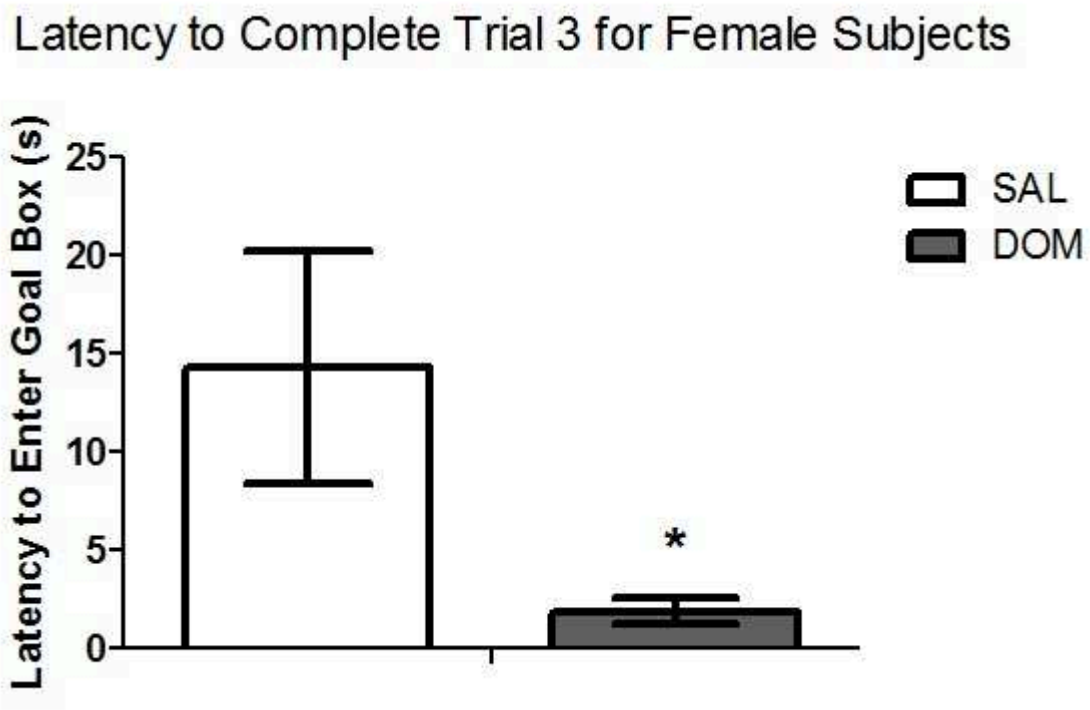


Figure 4. Mean ± SEM latency of DOM- and SAL-treated female subjects to enter the goal box during T3 of the puzzle box procedure. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

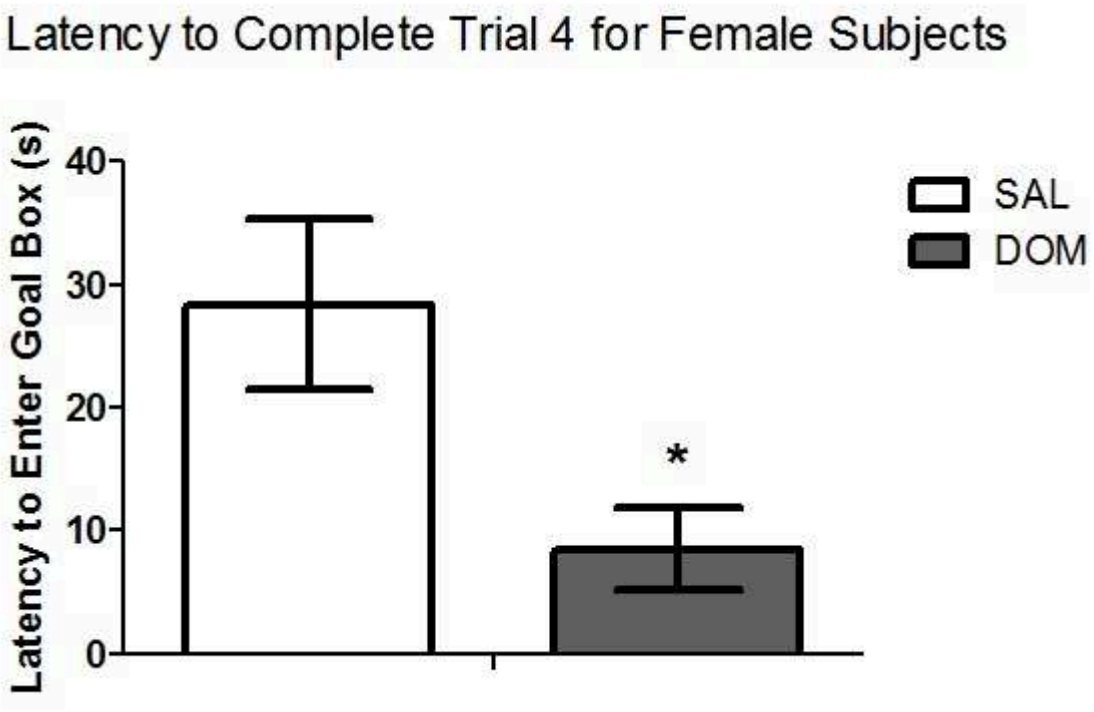


Figure 5. Mean ± SEM latency of DOM- and SAL-treated female subjects to enter the goal box during T4 of the puzzle box procedure. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

Figure 6. Mean ± SEM latency of DOM- and SAL-treated female subjects to enter the goal box during T10 of the puzzle box procedure. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

more quickly than SAL-treated female counterparts. No other statistically significant effects were observed in the puzzle box paradigm. See Table 5 for mean and SEM values for the puzzle box paradigm.

2.4 Discussion

The current study revealed minimal statistically significant DOM-induced effects in terms of trials to criterion for the attentional set-shifting paradigm, with the exception of the finding that DOM-treated males completing the IDS task with fewer errors than SAL-treated male counterparts.

The IDS task is one of the more basic types of trials in the attentional set-shifting paradigm as it merely involves applying a previously learned rule to a new set of stimulus. Therefore, deficits in more challenging tasks such as the REV and EDS task are generally expected to be seen more than are deficits in the IDS task. Also, while studies of marmoset monkeys has shown that dorsolateral PFC damage affects EDS task performance and orbitofrontal cortex damage affects REV task performance (Dias et al., 1996; Dias et al., 1997), the neurobiology of the IDS is less studied thus far, thereby preventing the extrapolation of the current result in the identification of cortical areas for further analyses. Thus, statistically significant effects on IDS performance are infrequently the target of the attentional set-shifting paradigm and are more poorly understood, in terms of neurobiology, as compared to the REV and EDS tasks. For

Table 5. Mean ± SEM latency to enter the goal box in subjects treated neonatally with DOM and SAL. Asterisk indicates a statistically significant difference between treatment groups ($p \leq 0.05$).

	Male		Female	
Trial	SAL	DOM	SAL	DOM
1	18.20 ± 3.43	20.80 ± 4.67	23.70 ± 6.21	20.40 ± 4.87
2	7.33 ± 2.94	10.70 ± 3.30	9.00 ± 2.45	2.11 ± 0.48*
3	9.11 ± 2.87	15.56 ± 9.12	14.30 ± 5.94	2.67 ± 0.62*
4	18.50 ± 6.04	38.00 ± 16.30	28.40 ± 6.91	8.50 ± 3.39*
5	8.30 ± 2.97	7.50 ± 1.93	10.50 ± 2.13	6.90 ± 1.65
6	22.60 ± 5.11	61.30 ± 16.26	61.00 ± 22.38	30.14 ± 4.51
7	29.14 ± 17.29	18.20 ± 4.56	26.13 ± 10.43	14.75 ± 6.68
8	29.78 ± 12.37	16.90 ± 8.38	19.86 ± 4.60	20.30 ± 7.84
9	17.67 ± 5.52	25.00 ± 14.61	29.25 ± 12.15	7.80 ± 1.57
10	17.78 ± 4.47	20.90 ± 4.18	38.20 ± 10.13	17.00 ± 4.00*
11	21.44 ± 9.40	25.80 ± 5.42	22.70 ± 6.03	23.20 ± 10.00
12	18.13 ± 11.46	24.00 ± 8.28	43.89 ± 12.82	54.10 ± 14.99

these reasons, the current finding of improved performance of the IDS task among DOM-treated male rats may be a spurious result. However, previous studies of the model have reported heightened exploratory behaviour in simple tasks, especially in male subjects (Gallant, 2009). Thus, enhanced digging in the IDS appears consistent with this finding as increased exploration may facilitate faster and more consistent responding.

The relative lack of statistically significant effects may be due to the low subject participation that was observed, as only 19 of the 39 subjects participated to some degree (see Table 6). However, this observation leads to some interesting findings related to treatment-based and sex-based differences in participation. One of these interesting findings was that 14 of 20 males participated to some degree while only 5 of 19 females participated to some degree. This observation also lead to further analysis using a chi-square analysis, which revealed that there was a statistically significant difference between males and females, when collapsed for treatment, in terms of participation, with male rats participating more often than females [$\chi^2(1, N = 39) = 7.74, p < 0.01$]. While this, combined with the observation that many studies use only male subjects (Fox et al., 2003; Barense et al., 2004; Cain et al., 2011), may indicate an innate difference between sexes in predisposition to perform the digging task, other studies have successfully included female rats (Lovic and Fleming, 2004; McLean et al., 2008). Thus, it does not appear as though an innate difference exists between males and females in ability or likelihood to participate in the digging task.

Table 6. Number of subjects participating in the digging task.

	Male		Female	
Trial Type	SAL	DOM	SAL	DOM
SD	9	5	4	1
CD	9	5	4	1
IDS	9	4	4	1
REV	8	4	4	1
EDS	8	4	4	0

Another interesting finding was that, while trials to criterion did not reveal any significant differences between DOM and SAL animals, 13 SAL-treated rats participated, at least to some degree, in the experiment while only 6 DOM subjects participated to some degree. Based on the previous chi-square finding, which indicated that there was a sex effect in terms of response likelihood with females having a very low response rate, attention was directed solely at male data. In terms of male data, 5 of 10 DOM-treated subjects participated to some degree while 9 of 10 SAL-treated subjects participated to some degree. This observation also lead to further analysis using a chi-square analysis of the likelihood of male subjects of each treatment condition to participate in the digging task. This analysis revealed that while a higher proportion of SAL-treated males participated to some degree, there was no statistically significant treatment effect in the male groups, although the statistical value approached significance [$\chi^2(1, N = 39) = 3.807, p = 0.051$].

Almost all of the subjects who did not participate in the digging task failed to dig even a single time in the experimental chamber, an observation that informs possible explanations for the low DOM group participation. One possible explanation is that increased sensitivity to novelty in the DOM-treated group could have contributed to their generalized exploratory behaviour and a lack of specific digging behaviour. This is supported by previous studies that have shown by changing a single element in the environment of DOM-treated male animals, they respond by treating the entire environment as if it was novel and, in turn, engage in generalized exploratory behaviour (Burt et al., 2008). Thus, by replacing the cups between trials, introducing a new digging

M and O, and experiencing lingering O from previous trials may have abolished any habituation to the chamber and reduced the amount of specific digging behaviour. Another possibility is that differences in stress levels between treatment groups contributed to low performance levels. It has previously been shown that in high-stress environments, such as the Morris water maze, DOM-treated subjects display novelty-induced seizure-like behaviour (Doucette et al., 2004). While it seems unlikely that the current study produced an equivalent level of anxiety as the Morris water maze, which is further supported by the lack of observable seizure-like behaviours, the act of an experimenter being positioned above a rat and repeatedly reaching into the maze may have evoked more stress than expected. Alternatively, it has been suggested that DOM-treated subjects demonstrate less stress and fear responses in environments such as the elevated plus maze which are less stressful than the Morris water maze (Doucette et al., 2007). Perhaps low levels of stress in a fairly bland testing chamber resulted in subjects becoming under-stimulated and losing interest in the testing chamber and its contents. This certainly seems possible based on the observation that the majority of the subjects who did not participate in the study merely sat in a single position until being removed from the maze.

However, despite the findings of sex-based and treatment-based differences in likelihood to participate in the study, the fact remains that a lower number of subjects than expected participated in the digging task. Aside from the previously mentioned explanations for low sex-specific and treatment-specific responding, potential explanations for the low

success rate in general include (i) inadequate training, (ii) inadequate food restriction, (iii) low motivation due to insufficient rewards for performance, (iv) intrinsic flaws, and/or (v) external effects.

Inadequate training does not seem likely based on the observation that when subjects were pre-exposed to buried Sugar Crisp rewards in their home-cage, digging behaviour was observed to occur almost immediately upon exposure to digging bowls. However, there was no pre-exposure to the O stimuli that were used, allowing for the possibility that inadequate training was, in fact, a contributing factor to poor performance. This also seems unlikely since subjects who did not participate in digging did not seem to dig a number of times and then, based on poor performance, grow frustrated and cease responding. Instead, the subjects that did not participate in digging almost universally did not dig a single time, thus suggesting that inadequate training with the O stimulus was not responsible for poor performance.

Additionally, inadequate food restriction does not seem likely to explain the poor performance. Although rat weight was not assessed daily, the 15 g and 12 g of rat pellets that were given to male and female subjects, respectively, for a period of at least 7 days was in line with the procedures set out by Lapiz et al. (2006). Also, both male and female subjects lost an average of 12% of their original body weight by the end of the experiment, which is in keeping with moderate food restriction.

The possibility that low motivation was responsible for low performance also seems unlikely. This is due to the observation that, on the final day of testing, all subjects were given, in their home cage, a bowl of 30 Sugar Crisp pieces, all of which were consumed by each subject within 3 minutes. However, these 30 pieces of Sugar Crisp were obtainable with minimal effort, so it remains possible that the effort required to learn the rules of the digging task and the effort of digging may have been greater than the reward of the Sugar Crisp. Also, the sheer number of rewards that could have been received during a testing session seems enough to abolish any motivation. However, this problem, which would only have applied to rats who progressed far in the series of trials and, therefore, received many rewards, was not apparent.

Intrinsic flaws in the experiment may have also negatively impacted the study. These include the O and digging M used, and the bowls used. For example, in the current study the scents were sprayed directly onto the digging M while, in previous studies, scents were administered to the rim of the bowl, which was made of absorbent terracotta (Fox et al., 2003). It is possible that the mixing of the O into the digging M, where the food reward was placed, resulted in a change in the flavour of the Sugar Crisp reward. However, again, the observation that the majority of the rats who did not participate in the digging task never dug a single time reduces the likelihood of this possibility. Alternatively, it is possible that the choice of digging M negatively impacted the study. For example, some of the digging materials (i.e. paper and sponge) were often removed from the digging cups, taken to a corner of the maze, and explored by subjects. Perhaps

choosing more small, fine digging M such as sand and gravel could have reduced this behaviour.

Finally, external effects may have negatively impacted the study. These include experimenter positioning, which involved sitting immediately behind the digging bowls, and experimenter action, which involved re-filling bowls between trials and reaching into the maze to replace bowls while the subject remained in the maze. It certainly seems possible that these behaviours served to increase the stress of the subjects and may have reduced the likelihood of their responding, despite the fact that they had one hour of pre-exposure to this type of experimenter behaviour during a habituation trial the day prior to testing. This is supported by a recent study which has shown both behavioural and neurochemical signs of an increased stress-response, as evidenced by increased closed-arm entries in the elevated plus-maze in DOM-treated subjects, increased expression of α2a adrenergic receptors, α2c adrenergic receptors, and mineralocorticoid receptors in the hippocampus of DOM-treated males, and a decreased ratio of glucocorticoid to mineralocorticoid receptors in the hippocampus of DOM-treated males (Gill et al., 2012). Regardless, the experimental design could be altered to allow for the digging bowls to be moved to and from the maze through an opening in the base of a wall, thereby reducing the need for the experimenter to reach into the maze. This would also allow a cover the be placed on the top of the maze to reduce the visibility of the experimenter. While these alterations do not appear to be used in the procedures outlined in the literature (Fox et al.,

2003; Barense et al., 2004; Cain et al., 2011), they may be helpful in reducing animal stress and improving performance.

In terms of alterations that could be made to improve the current study, aside from the previously mentioned alterations in maze structure and experimenter behaviour, there are also variations of the current digging paradigm which may be more effective. One of these variations has been described by Stefani et al., (2003) and involves the use of an elevated plus maze which has two arms, one of which is baited, which differ along the dimensions of brightness and texture. The same sequence of SD, CD, IDS, REV, and EDS trials as was used in the current study is employed in this variation, and the number of trials to reach a criterion level of correct responses is assessed. Advantages of this variation include the replacement of the O and M stimuli that were used in the current study with brightness and texture stimuli may be beneficial as O intensity of the two stimuli is different to match equally and any O from previous trials are likely to linger and potentially lead to confounding stimuli effects. Additionally, our laboratory has previously employed elevated plus maze paradigms to assess emotionality (Doucette et al., 2007), thereby supporting the assertion that DOM-treated animals are likely to participate in a procedure that involves an elevated plus maze.

The puzzle box paradigm revealed female-specific statistically significant differences in terms of latency to enter the goal box T2 and T4, which assess short-term memory, and for T3 and T10, which assess problem solving ability, with DOM-treated females

completing the above-mentioned trials more quickly than SAL-treated counterparts. These results are in agreement with previous studies in the laboratory which showed that animals treated neonatally with DOM show working memory deficits in the radial arm maze (McQuaid, 2009), both working and long-term memory deficits in temporal memory ability (Robbins, 2010) and abnormalities in other behaviours involving the mPFC such as PPI (Adams et al., 2008) and latent inhibition (Adams, 2009). Additionally, abnormalities in puzzle box performance have been demonstrated in various mice models of schizophrenia including those with mPFC lesions or hippocampal lesions, those treated sub-chronically with dizocilpine, those constitutively lacking the Glu1 subunit of AMPARs, and those over-expression D2 receptors in the striatum (Abdallah et al., 2011).

However, closer assessment reveals that, of the trials showing a statistically significant treatment effect, T2, T3, and T4 all involved either a small or a large open door through which subjects entered the goal box. Thus, the task was of minimal difficulty and perhaps required very little cognitive ability in terms of working memory capacity. A potential explanation for this observation is that the current study may involve confounding effects of treatment and stress level. Since the stress of being placed in a large, bright, uncovered chamber served as the motivation for animals to enter the dark chamber, differences in stress levels between groups may have interacted with and masked any short-term memory, long-term memory, or problem solving abnormalities. Thus, the shorter latency to enter the goal box that was observed among DOM-treated

females may have been due to an exaggerated stress-response to the aversive bright lights. This, again, is supported by recent studies of behavioural signs of the stress response and neurochemical markers of the stress system which have suggested an exaggerated stress-response in DOM-treated subjects (Gill et al., 2012). The relative role of stress in the current study could be assessed in the future by modifying the procedure to replace the aversive bright light stimulus for promoting entry into the goal box with a more appetitive stimulus, such as a food reward. An alternative explanation is that while these tasks required working memory ability, the cognitive load, in terms of the amount of information that guided behaviour, was minimal compared to other studies of working memory such as the radial arm maze. Thus, the current lack of working memory impairment may be due to the nature of the task as the vast majority of working memory tasks that have demonstrated impairments in animal models and in the clinical population involve a larger cognitive load (Holzman and Park, 1992; Goldberg et al., 1997; Enomoto et al., 2008).

In summary, statistically significant effects were observed in both of the employed paradigms, with DOM-treated males completing the IDS task with fewer errors than their SAL-treated male counterparts. Additionally, differences were seen in the puzzle box during trials assessing short-term memory and problem solving ability, as evidenced through DOM-treated females completing T2, T3, T4, T10 more quickly than SAL-treated females.

Chapter 3

Alterations in dopaminergic markers in rats treated neonatally with domoic acid

Summary

The dopaminergic system has long been implicated in the pathophysiology of schizophrenia. Links between DA and schizophrenia were first established by the finding that chlorpromazine, a DA antagonist used for its anxiolytic properties, is effective in treating the positive symptoms of schizophrenia (as reviewed in Ban, 2007), and that DA agonists such as amphetamine and cocaine are effective in producing positive symptoms of schizophrenia (Serper et al., 1999; Ban, 2007). These findings contributed to the current understanding of the DA contribution to schizophrenia, which suggests that prefrontal DA hypoactivity, mainly at D1 receptors, contributes to negative and cognitive symptomatology while mesolimbic DA hyperactivity, mainly at D2 receptors, contributes to positive symptomatology (as reviewed in Howes and Kapur, 2009). Thus, the current study was conducted in order to determine the effects of neonatal treatment with DOM on the integrity of the DA system in adults animals, as evidenced through potential alterations in D1 receptor, D2 receptor, and DAT in the mPFC. Results using cell counting methods revealed a statistically significant increase in DAT staining in the right PRL of DOM-treated females. No other significant differences were observed between DOM-treated and SAL-treated animals in terms of D1, D2, or DAT staining in the PRL or ILC.

3.1 Introduction

Dopamine is a catecholamine neurotransmitter that is important is various processes including cognition (as reviewed in Nieoullon, 2002), motivation (as reviewed in Wise,

2004), reward (as reviewed in Ikemoto, 2007), and motor functioning (Brück et al., 2001). Dopaminergic abnormalities have been implicated in various disorders including Parkinson's disease (Leenders et al., 1986; Frost et al., 1993), attention-deficit hyperactivity disorder (LaHoste et al., 1996; Dougherty et al., 1999), and schizophrenia (Seeman et al., 1993; Laruelle et al., 1996; Lieberman, 2004). The association between aberrant dopaminergic systems and schizophrenia has been investigated extensively, leading to both the DA hypothesis of schizophrenia and to the development of various treatment regimes, including antipsychotic drugs, which modulate the DA system (Kapur et al., 2000; Potkin et al., 2003).

While there are four main dopaminergic pathways, the mesolimbic pathway, which projects from the VTA to the limbic system (e.g. hippocampus, amygdala, NAcc, etc.) and mesocortical pathway, which projects from the VTA to the cerebral cortex, are most commonly implicated in schizophrenia. The mesolimbic system functions in motivation and reward-based behaviour (Wightman and Robinson, 2002) and increased activity is associated with positive symptomatology while the mesocortical pathway involves higher cognitive functions (Moghaddam et al., 1997) and hypoactivity is associated with negative and cognitive symptoms.

The mesolimbic and mesocortical DA pathways also differ from one another in terms of postsynaptic receptor distribution with D1-like receptors (e.g. D1 and D5 receptors) predominating in the mesocortical pathway and D2-like receptors (e.g. D2, D3, and D4

receptors) predominating in the mesolimbic pathway (as reviewed in Howes and Kapur, 2009). However, while there is variability between pathways in terms of postsynaptic receptors, the D2 receptor serves as an autoreceptor to regulate presynaptic DA release in both pathways (Gainetdinov et al., 1994).

Following the release of DA from mesocortical dopaminergic neurons and the subsequent action of DA on postsynaptic and presynaptic receptors, DA is removed from the synaptic cleft primarily through reuptake via DAT and NET but also, to a lesser degree, through enzymatic degradation via COMT and MAO (as reviewed in Feldman, 1997).

Thus, D1 receptors, D2 receptors, and DAT are key components of the DA system and immunohistochemical analyses of these markers can provide information on the structural integrity of the system, and various abnormalities in these markers have been observed in the clinical population. These findings include the effectiveness of first-line atypical antipsychotics, which act as D2 antagonists (Horacek et al., 2006). However, complications arise as the mechanism of action of these drugs remains somewhat unclear, with the relative roles of both presynaptic and postsynaptic D2 antagonism, serotonin receptor antagonism, and various combinations of receptor antagonism being the subject of some debate. Also, there is some degree of variability between atypical antipsychotics in terms of receptor affinity (Horacek et al., 2006). However, a more clear picture is offered by more recent findings of decreased cognitive symptoms in animals treated with SKF-38,393, a selective D1-like receptor partial agonist (McLean et al., 2009; Barnes et

al., 2012) and decreased positive and negative symptoms in animals treated with JNJ-37822681, a selective D2 receptor antagonist (Schmidt et al., 2012). Additional findings of dopaminergic receptor abnormalities in schizophrenia include increased prefrontal D1 receptor availability in a cohort consisting of both drug-naive and treated individuals (Abi-Dargham et al., 2002), decreased prefrontal D1 receptors via PET imaging in drug-naive patients (Okubo et al., 1997), and increased prevalence of the −141C D2 receptor allele in patients with schizophrenia (Arinami et al., 1997). In terms of DAT, treatment with modafinil, which functions through a mechanism that is not yet completely understood, has been shown to inhibit DAT functioning (Zokowska et al., 2009) and, also, to improve attentional set-shifting ability (Turner et al., 2004). These findings linking D1 receptors, D2 receptors, and DAT to the pathophysiology and treatment of schizophrenia can thereby serve as a guide in the assessment of the structural integrity of the dopaminergic system in the further characterization of the novel DOM-induced animal model of schizophrenia.

The previous findings of altered tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, in the mPFC of animals treated with DOM (Robbins, 2010) and findings relating to D1 receptors, D2 receptors, and DAT in the clinical population, combined with the association between dopaminergic hypoactivity in the mesocortical pathway and the expression of cognitive symptoms, has served as the theoretical rationale for the examination of D1 receptors, D2 receptors, and DAT in the mPFC in an attempt to further characterize a novel animal model of schizophrenia. Mechanistically, neonatal

treatment with DOM may alter DA markers as a result of the modulatory role of KARs on DA release causing compensatory alterations in the DA system. Alternatively, KAR activity could increase intracellular calcium either directly, through the action of KARs that contain either GluR5 or GluR6 subunits that have not undergone post-transcriptional RNA glutamine-to-arginine editing, or indirectly, through the secondary activation of NMDARs which can flux calcium, thereby affecting gene transcription.

The current study was performed with the objective of characterizing possible alterations in the DA system following neonatal exposure with DOM with the hypothesis that DOM-treated animals would demonstrate alterations in the DA system as evidenced through changes in D1 receptor, D2 receptor, and DAT.

3.2 Materials and methods

3.2.1 Experimental animals and injection procedure

Refer to section 2.2.1

3.2.2 Tissue collection and sectioning

Following behavioural testing, at approximately PND 196, subjects (n = 24; 6 of each sex x treatment group) were deeply anesthetized with sodium pentobarbitol (s.c. injection), transcardially perfused with 100 mL of phosphate buffered saline (PBS) and 100 mL of 4% paraformaldehyde in PBS, and brains were dissected from the skull and post-fixed for 24 hours in 4% paraformaldehyde in PBS. Tissue was then transferred to 0.08% sodium

azide in PBS until sectioning. Prior to sectioning on a vibratome, a small notch was placed in the right hemisphere of each brain to allow the identification of each hemisphere. Coronal sections (50 μm) were then obtained from areas of the mPFC (4.7 to 1.7 mm relative to bregma) (Paxinos and Watson, 1998). Tissue was sectioned in groups of six sections with the first three being kept for DAT, D1, and D2 immunohistochemistry, respectively, and the next three being kept as control sections for DAT, D1, and D2 immunohistochemistry. Tissue was stored in 24-well plates containing 0.08% sodium azide in PBS until the implementation of immunohistochemical procedures.

3.2.3 Immunohistochemical analyses

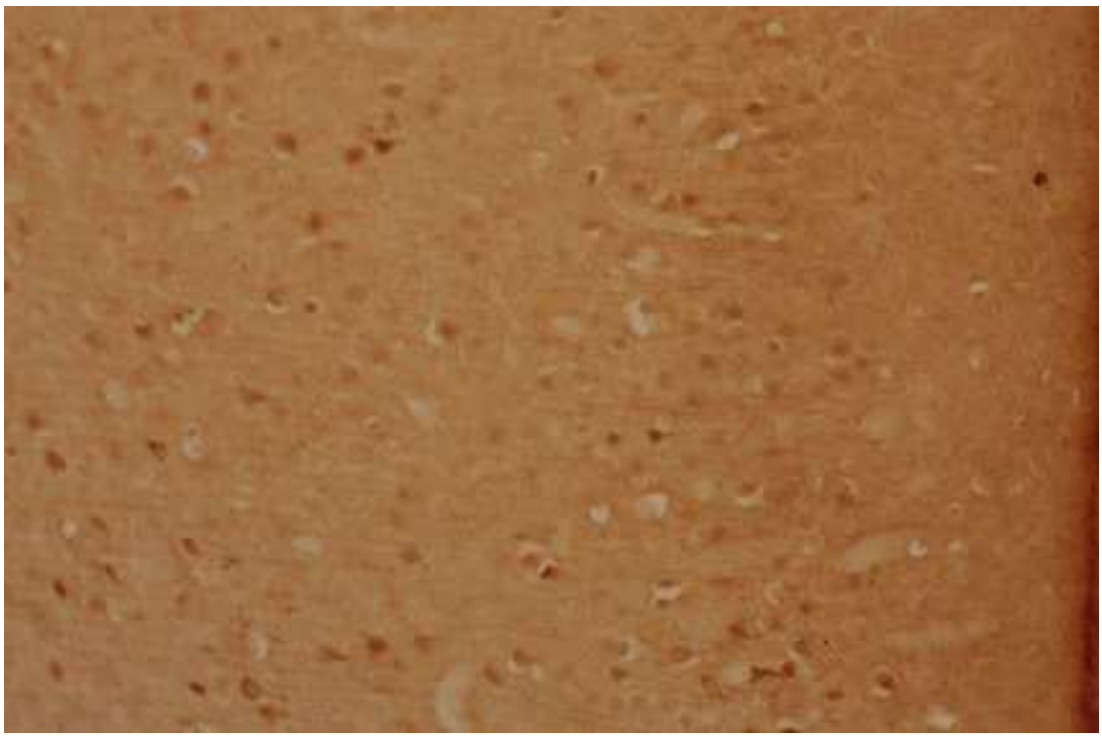
The immunohistochemical procedures used to assess DAT, D1, and D2 were adapted from Sesack et al. (1998), Huang et al. (1992), and Yung et al. (1995), respectively, and began with a series of rinses (3 x 5 min unless otherwise stated) in PBS followed by a 30 minute incubation in 1% hydrogen peroxide in PBS. Sections were then re-rinsed in PBS prior to being incubated for 30 minutes in 1% sodium borohydride in PBS. Another series of rinses in PBS was then conducted prior to a 30 minute incubation in blocking solution (5% normal horse serum (NHS), 2% bovine serum albumin (BSA), 0.2% milk, in Tris buffered saline (TBS)). Following incubation, blocking solution was removed and sections were treated with the primary antibody to either DAT, D1, or D2 (rabbit anti-DAT, polyclonal; rabbit anti-D1R, polyclonal; rabbit anti-D2R, polyclonal) (Millipore, Billerica MA, USA) at a working dilution of 1:1000 in PBS for approximately 60 hours at

4° C. Rinses (5 x 5 min) in TBS were then performed prior to incubation for 2 hours in biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vector Laboratories, Burlingame CA, USA) in TBS at a dilution of 1:100. Tissue was then rinsed in TBS followed by a 2 hour incubation period in avidin-biotin complex (Vector Laboratories, Burlingame CA, USA), after which the final set of rinses in TBS was performed. Sections were then floated in PBS onto slides and the peroxidase reaction was developed using 0.2% hydrogen peroxide in diaminobenzidine (DAB) for 6 minutes. Following treatment with DAB, sections were rinsed with approximately 2 mL of both PBS and distilled water and were left to dry overnight. The following day, sections were dehydrated by undergoing a series of 3 minute rinses in 70% ethanol (EtOH), 95% EtOH, 100% EtOH, 100% EtOH, xylene, and xylene again before a final 45 minute rinse in xylene. Sections were then cover-slipped using Permount.

3.2.4 Quantification of staining

Equivalent sections from separate subjects were identified using the appearance of the forceps minor corpus callosum as a landmark to identify sections approximately equivalent to a location 2.7 mm relative to bregma (Paxinos and Watson, 1998). Photographs (at 250 x magnification) of the mPFC were taken using a digital Cannon Rebel EOS xSi camera (Canon Canada Inc., ON, Canada) mounted on a MEIJI trinocular ML5000 series microscope (MEIJI Techno America, CA, USA). Instances of immunoreactivity were operationally defined as dark-brown or black punctated dots which represented sites of DAT, D1, or D2 localization (see Figures 7-13).

A



B

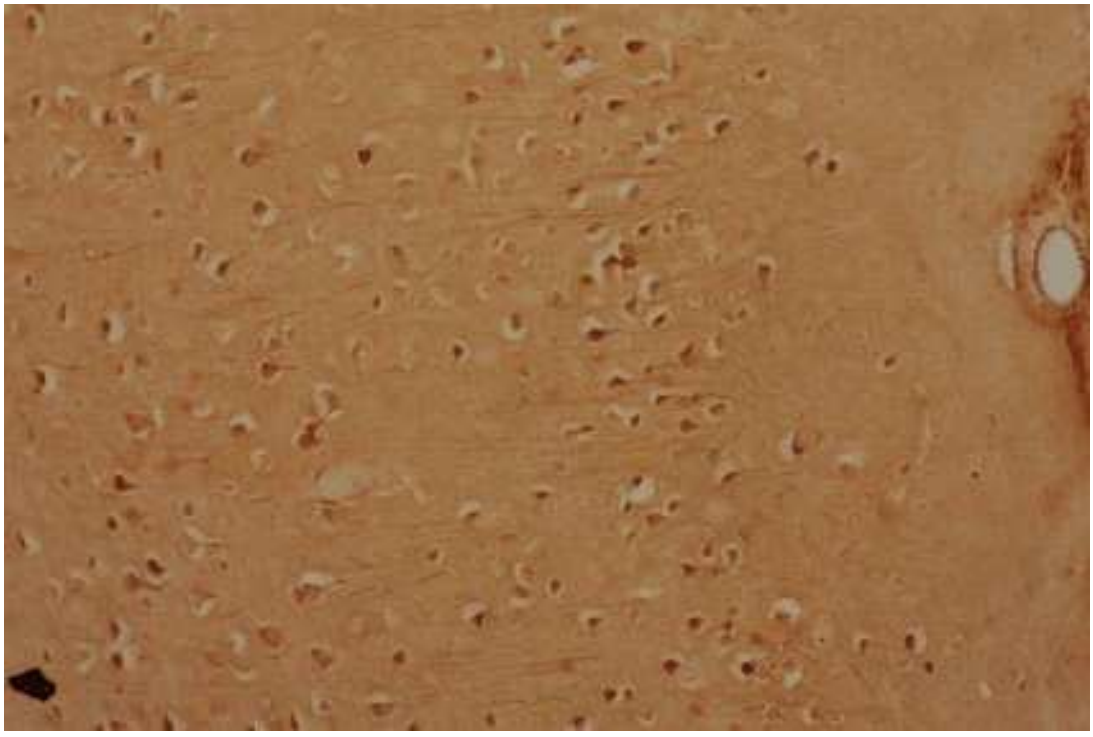
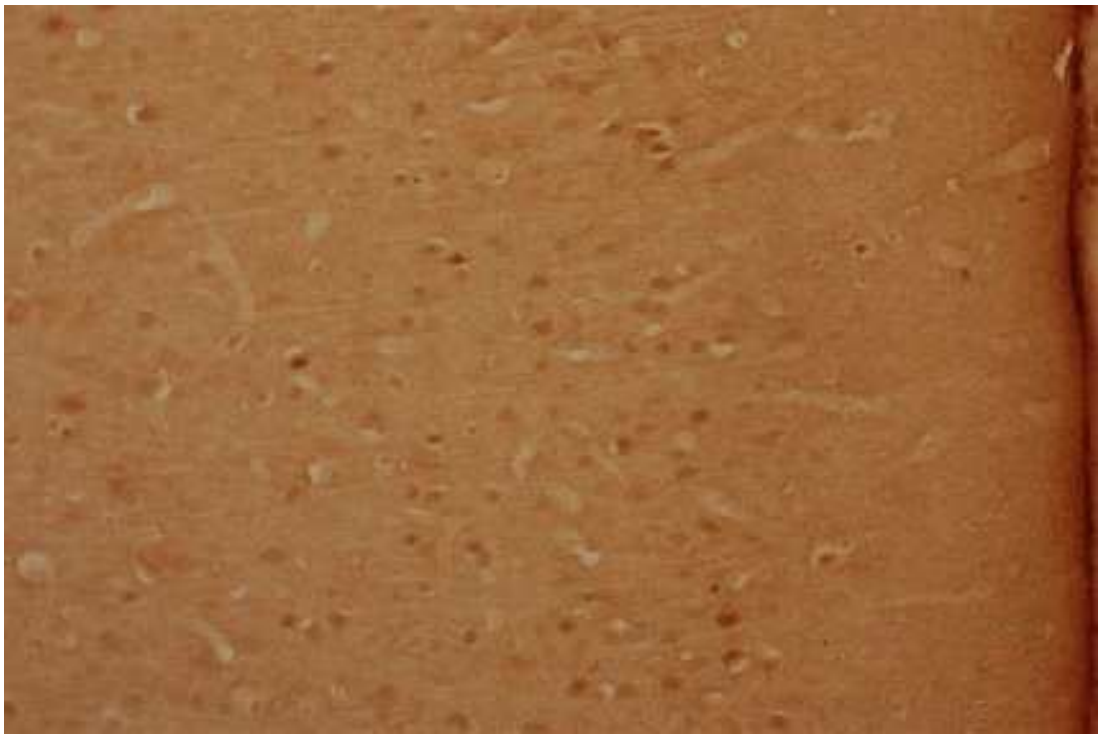


Figure 7. D1 immunopositive staining in the right ILC of representative male (A) SAL-treated and (B) DOM-treated subjects.

A



B

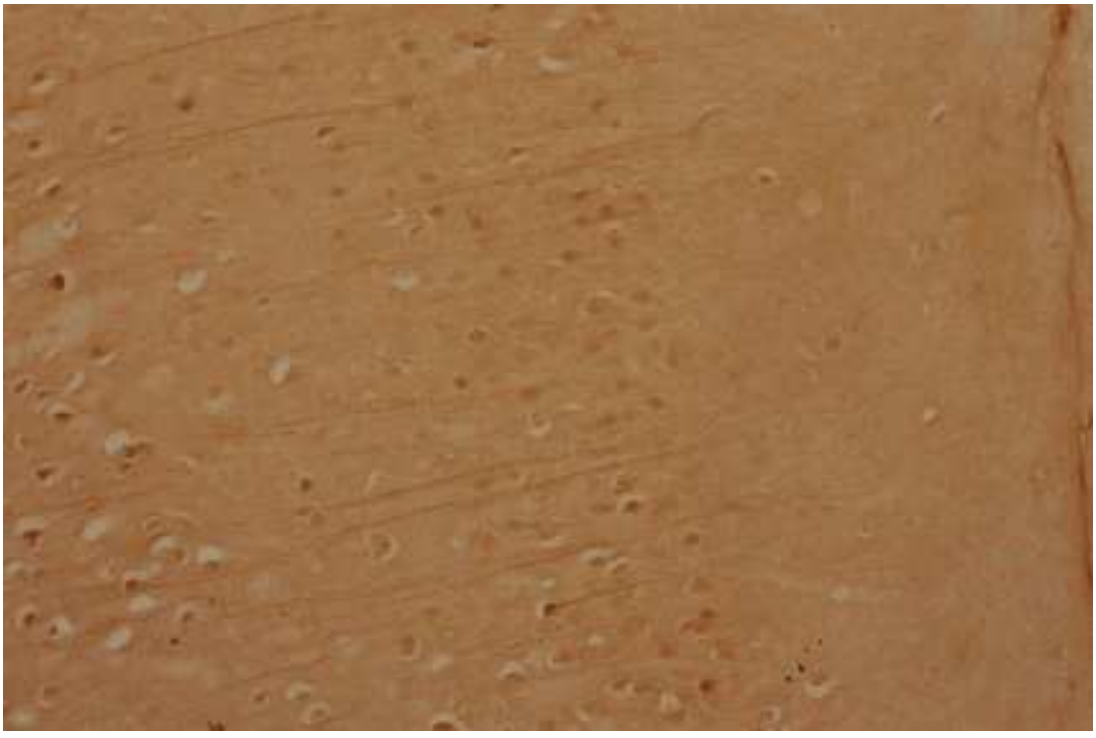


Figure 8. D1 immunopositive staining in the right PRL of representative male (A) SAL-treated and (B) DOM-treated subjects.

A

B

Figure 9. D2 immunopositive staining in the right ILC of representative male (A) SAL-treated and (B) DOM-treated subjects.

A

B

Figure 10. D2 immunopositive staining in the right PRL of representative male (A) SAL-treated and (B) DOM-treated subjects.

A

B

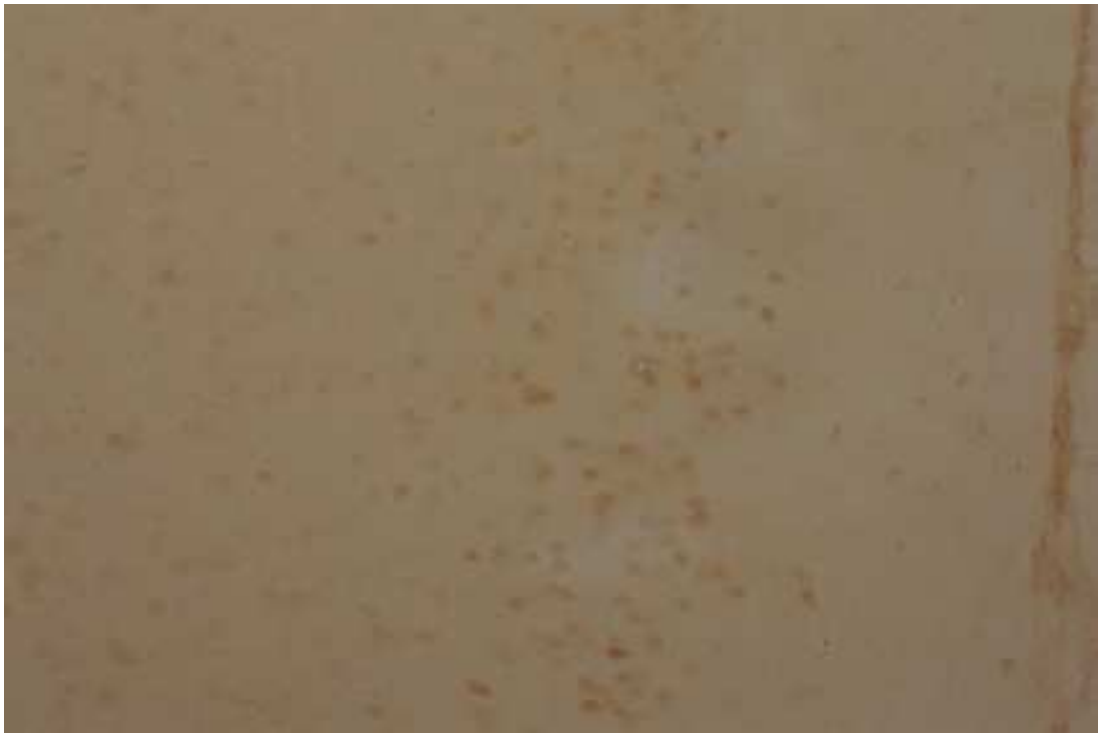
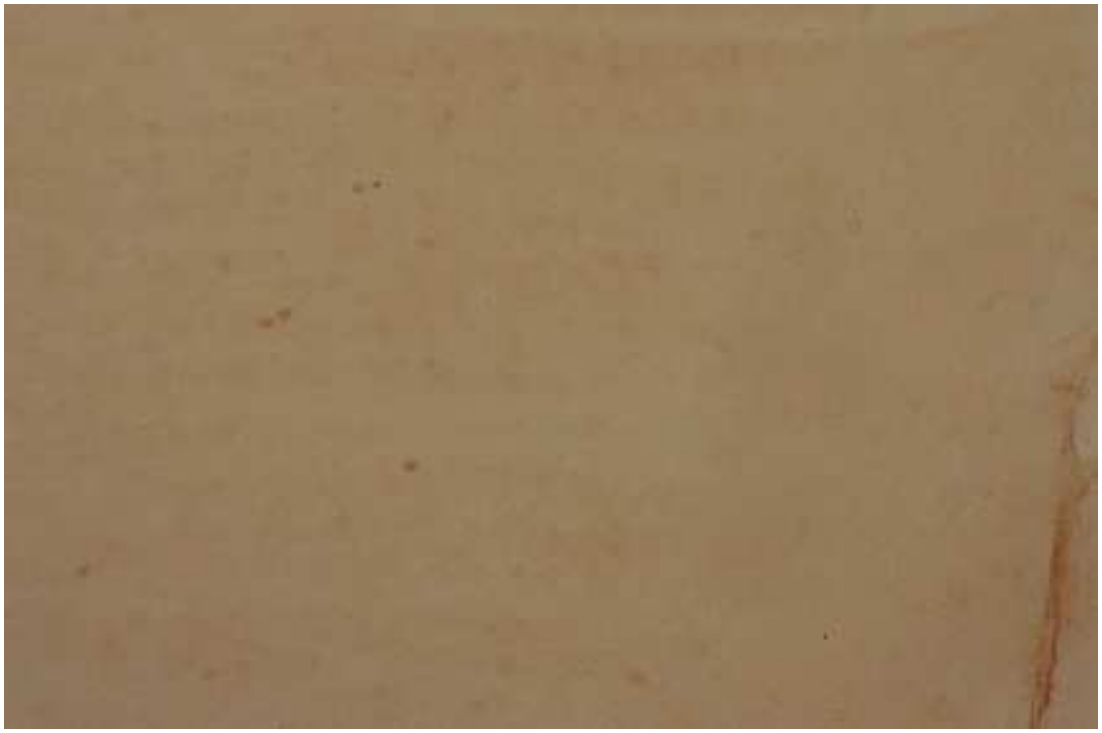


Figure 11. DAT immunopositive staining in the right ILC of representative male (A) SAL-treated and (B) DOM-treated subjects.

A



B

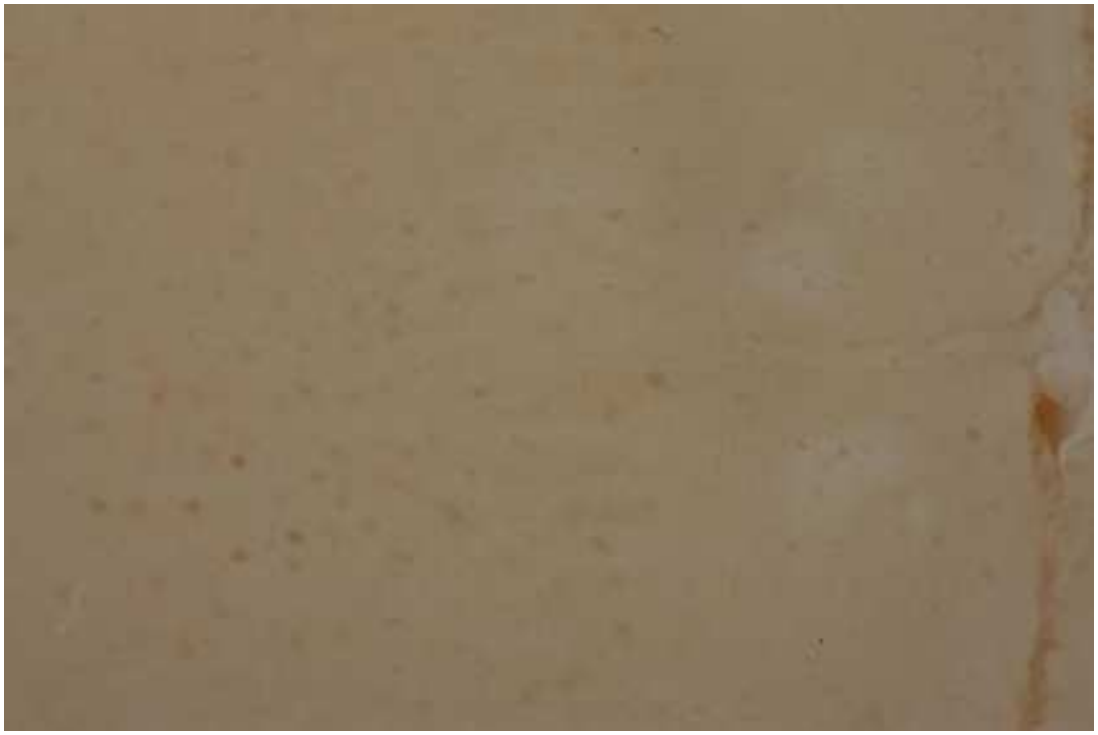


Figure 12. DAT immunopositive staining in the right PRL of representative male (A) SAL-treated and (B) DOM-treated subjects.

A

B

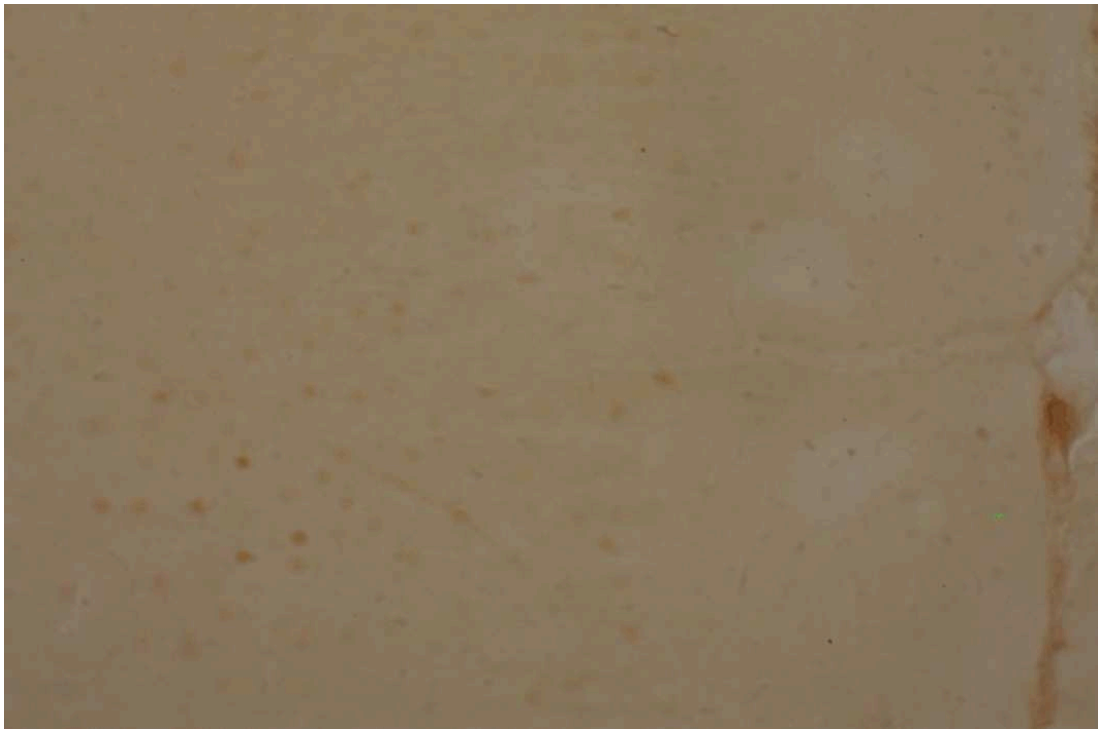


Figure 13. DAT immunopositive staining in the right PRL of representative female (A) SAL-treated and (B) DOM-treated subjects.

Instances of immunoreactivity were counted in a standard counting square that was positioned in layers I-VI of the prelimbic cortex (PRL) and infralimbic cortex (ILC) of the mPFC. The PRL of the mPFC was identified as located at the midline of the brain, exactly equidistant from the most dorsal and ventral regions of each section. The ILC of the mPFC was identified as one-third of the distance from the most ventral region of the section. Hemispheres were photographed separately and two images were acquired for each hemisphere in order to ensure that the 1 mm thickness of the cortex was visible.

3.2.5 Data analyses

Based on previous studies of the DOM model showing extensive sex-specific effects (Doucette et al., 2007; Adams et al., 2008; Adams 2009b; Burt et al., 2008a; Robbins, 2010; Ryan et al., 2011) and hemisphere-specific effects (Adams, 2009a; Robbins, 2010; Greenan, 2011), DA marker data was separated for sex, hemisphere, and region of the mPFC and was analyzed using Student's *t*-tests (PASW Statistics 18). A result of $p \leq 0.05$ indicated statistical significance.

3.3 Results

Results of Student's *t*-tests revealed a statistically significant treatment effect [$t(10) = 2.277$, $p = 0.046$] with DOM-treated females demonstrating increased DAT staining in the right PRL (see Figure 14). No other statistically significant effects were observed in terms of DAT, D1, or D2 staining. See Tables 7-9 for mean and SEM values for D1, D2, and DAT staining.

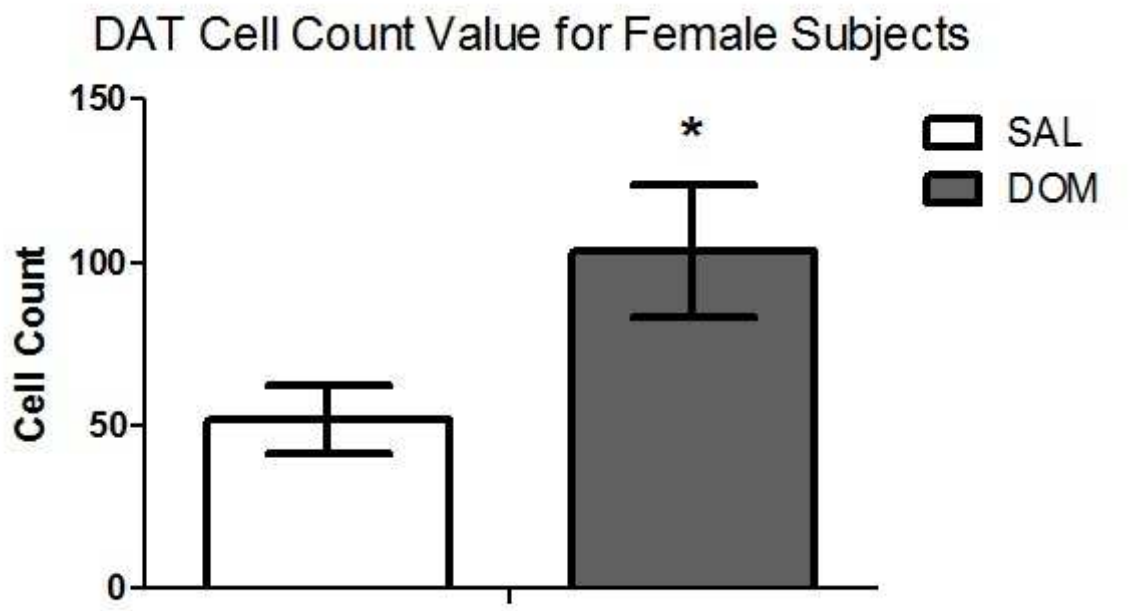


Figure 14. Mean ± SEM cell count measurement of DAT staining in the right PRL or DOM- and SAL-treated female subjects. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

Table 7. Mean ± SEM for D1 cell counts in the PRL and ILC of rats treated neonatally with DOM and SAL.

Brain Area	Male		Female	
	SAL	DOM	SAL	DOM
right PRL	155.67 ± 18.91	165.83 ± 24.63	178.00 ± 7.31	164.33 ± 14.82
left PRL	176.00 ± 24.15	167.83 ± 19.11	168.50 ± 11.74	165.83 ± 24.51
right ILC	184.83 ± 21.61	161.83 ± 37.264	191.33 ± 16.58	182.80 ± 25.78
left ILC	180.83 ± 26.97	188.33 ± 19.92	207.83 ± 16.54	193.17 ± 14.80
mPFC (PRL and ILC)	873.33 ± 105.17	851.67 ± 103.73	914.17 ± 38.51	849.80 ± 69.70

Table 8. Mean ± SEM for D2 cell counts in the PRL and ILC of rats treated neonatally with DOM and SAL.

Brain Area	Male		Female	
	SAL	DOM	SAL	DOM
right PRL	50.17 ± 9.10	60.33 ± 10.33	54.33 ± 11.71	51.00 ± 3.27
left PRL	89.67 ± 55.81	78.50 ± 19.56	79.50 ± 39.37	108.50 ± 36.77
right ILC	49.83 ± 13.89	60.17 ± 15.09	73.33 ± 14.63	72.50 ± 6.78
left ILC	81.50 ± 34.02	78.83 ± 14.08	102.50 ± 43.17	122.83 ± 33.79
mPFC (PRL and ILC)	271.17 ± 95.30	277.83 ± 33.57	309.67 ± 82.79	354.83 ± 71.31

Table 9. Mean ± SEM for DAT cell counts in the PRL and ILC of rats treated neonatally with DOM and SAL. Asterisk indicates a statistically significant difference between treatment groups ($p \leq 0.05$).

Brain Area	Male		Female	
	SAL	DOM	SAL	DOM
right PRL	56.67 ± 16.38	96.33 ± 20.33	51.67 ± 10.39	103.50 ± 20.25*
left PRL	127.00 ± 67.63	111.33 ± 35.03	122.17 ± 64.62	129.83 ± 23.47
right ILC	77.17 ± 15.80	124.83 ± 27.46	78.83 ± 17.32	107.80 ± 35.01
left ILC	133.33 ± 51.40	167.00 ± 30.95	158.33 ± 59.47	182.50 ± 35.83
mPFC (PRL and ILC)	394.17 ± 143.87	499.50 ± 70.42	411.00 ± 131.37	527.80 ± 54.94

However, a pattern was observed in terms of higher DAT in right and left PRL and ILC of female and male DOM-treated rats as compared to SAL-treated counterparts, with the exception of the left PRL.

3.4 Discussion

Results indicated that statistically significant differences between groups in terms of D1, D2, or DAT staining the PRL, ILC, or when combined to give an mPFC value were limited to an increase in DAT staining in the right PRL of female DOM-treated subjects.

Based on the hypodopaminergia in the mPFC, which is associated with negative and cognitive symptoms in schizophrenia, an increase in DAT staining could be interpreted as a contributing factor to such decreases in DA tone, as increased DAT could function to remove DA from the synapse at an increased rate, thereby depleting synaptic DA levels. This is also supported by the observation that modafinil, which functions through mechanism that is not yet completely understood, has been shown to inhibit DAT functioning (Zokowska et al., 2009) and, also, to improve attentional set-shifting ability (Turner et al., 2004). The effectiveness of decreasing DAT activity in improving cognitive symptomatology could support the proposition that increased DAT staining, which may reflect increased DAT levels, functions to contribute to the variety of cognitive symptoms that have been observed in the current study and in previous studies of the DOM model (Burt et al., 2008a; Adams et al., 2009b; Robbins, 2010). Mechanistically, it is possible that the observed increase in DAT staining is the result of a compensatory increase in DAT

following neonatal overactivation of the mesocortical DA system. It has been previously noted that KARs serve to modulate the mesocortical DA system (Kalivas et al., 1988), therefore stimulation of this pathway from PND 8-14 via DOM administration may have served to promote an upregulation of DAT in order to remove excess DA from the synapse, and this compensatory upregulation may have become imprinted, thereby persisting into adulthood (Rice and Baroni, 2000).

Regardless, the current findings in terms of DA alterations in the mPFC are relatively modest and are, in fact, surprisingly modest based on the variety of negative and cognitive symptoms, which are believed to be associated with decreased prefrontal DA signalling, that have been observed in the DOM model of schizophrenia. These include spatial memory abnormalities (McQuaid, 2009), social withdrawal (McInnis, 2009; Ryan et al., 2009), and temporal memory dysfunction (Robbins, 2010). Additionally, previous studies have shown DA alterations in the prefrontal cortex of DOM-treated males, as evidenced through decreased staining of tyrosine hydroxlase, the rate-limiting enzyme in DA synthesis (Robbins, 2010). Finally, more extensive statistically significant alterations were expected based on previously mentioned studies of the clinical population which implicate D1, D2, and DAT (Arinami et al., 1997; Okubo et al., 1997; Geddes, 2000; Abi-Dargham et al., 2002; Kane et al., 2003; Turner et al., 2004; Floresco et al., 2005; Schmidt et al., 2012). However, despite the lack of extensive statistically significant results, the information gained by the current study is important as it complements the previous findings of altered tyrosine hydroxylase by further characterizing potential DA system

alterations in the mPFC.

While statistically significant treatment effects were limited to an increase in DAT staining in the right PRL of female DOM-treated subjects, a consistent pattern was observed in terms of higher DAT in right and left PRL and ILC of female and male DOM-treated rats as compared to SAL-treated counterparts, with the exception of the left PRL. It is possible that immunohistochemical methods of analyses were sufficiently sensitive to detect trends in staining, but insufficiently sensitive to completely detect statistically significant alterations. It is important to consider that while immunohistochemistry is a semi-quantitative method that provides excellent information in terms of protein location, other semi-quantitative methods, such as Western blots and high-performance liquid chromatography, may provide better information in terms of protein content. Immunohistochemistry involves quantifying the number of immunoreactive areas in a particular 50 micron section of tissue. Thus, a very limited portion of the brain area of interest is being assessed. Also, Western blots and HPLC are also limited in their utility, in part, since a significant increase in protein level provides no information on whether the increase is due to a normal number of cells expressing an increased amount of the protein and/or an increased number of cells expressing a normal level of the protein. However, simultaneous immunohistochemical and Western blot analyses could allow researchers to assess the entire volume of a given brain area and also obtain information on the anatomical location of protein levels. Perhaps future studies in the laboratory will use these complementary techniques in order to gain more information on given

neurochemical markers.

Also, it is important to consider while there no were no statistically significant findings in terms of D1 or D2 in the mPFC, the mPFC receives diverse innervation outside of DA, and alterations in the noradrenaline, serotonin, acetylcholine, GABA, or other neurotransmitter systems may underlie the behavioural findings that have been previously described. In fact, previous studies have shown abnormalities in the GABA system, as evidenced through decreased glutamic acid decarboxylase 67 in the left CA3 of female DOM-treated subjects (Adams, 2009). Further examination of other neurotransmitter systems associated with schizophrenia, in order to complement the findings related to the DA system, are an important future direction for the DOM model.

Chapter 4

Alterations in caspase-3 in rats treated neonatally with domoic acid

Summary

The symptomatology of schizophrenia involves a variety of symptoms such as altered PPI, LI, hallucinations, and delusions, which have been speculated to share a common underlying process related to an impaired ability to filter out irrelevant information (Braff et al., 1977; Adler et al., 1982). This suggests the possibility of a contribution by altered synaptic connections, which could include decreased numbers of synapses resulting from increased synaptic pruning, an increase in synaptic connections resulting in abnormal connnectivity, or microscopic abnormalities in synaptic anatomy. In fact, decreased prefrontal white matter (Breier et al., 1992), increased prefrontal neuronal apoptosis inhibitor protein (Vawter et al., 2002), abnormalities in prefrontal synaptic proteins such as N-ethylmaleimide sensitive factor fusion protein and synapsin II (Mirnics et al., 2000), and decreased prefrontal dendritic spine density (Garey et al., 1998) have been shown in the clinical population, thereby supporting the concept of abnormal prefrontal synapse anatomy and function. The current study was conducted in order to determine the effects of neonatal treatment with DOM on normal developmental apoptotic processes by assessing caspase-3, an executioner caspase, in the prefrontal cortex of subjects at PND 21. Results revealed a statistically significant decrease in optical density measurement in the right PRL among DOM-treated males and a statistically significant decrease in cell count measurements in the right PRL among DOM-treated females. No other statistically significant effects were observed in terms of caspase-3 staining.

4.1 Introduction

Apoptosis is a form of programmed cellular death which, unlike necrosis, does not involve the release of harmful substances into the extra-cellular environment (as reviewed in Elmore, 2007). There are three main pathways involved in apoptotic signalling: the extrinsic pathway, the intrinsic pathway, and the perforin/granzyme pathway (as reviewed in Elmore, 2007). The extrinsic apoptotic pathway involves stimuli such as Fas ligand and tumour necrosis factor α (TNF-α) stimulating death receptors which, in turn, are bound by adapter proteins (as reviewed in Elmore, 2007). These adapter proteins are then able to associate with procaspase 8, leading to the formation of death-inducing signalling complex and the auto-catalytic activation of procaspase 8, which converts inactive procaspase 3 to caspase 3, marking the beginning of the execution pathway, which is the common end-point of the intrinsic, extrinsic, and perform/granzyme pathway (as reviewed in Elmore, 2007). The intrinsic apoptotic pathway involves stimuli such as excess intracellular calcium ions, which can be due to excessive Glu stimulation, which opens mitochondrial permeability transition pores and leads to a loss of mitochondrial transmembrane potential (as reviewed in Elmore, 2007). Subsequently, cytochrome C is released from the mitochondria, serving as a stimulus for the activation of apoptotic protease activating factor-1 and procaspase 9, thereby forming an apoptosome which yields active caspase 9 (as reviewed in Elmore, 2007). Finally, caspase 9 is able to convert inactive procaspase 3 to caspase 3, the common end-point of the intrinsic, extrinsic, and perforin/granzyme pathway (as reviewed in Elmore, 2007). The perforin/granzyme pathway involves cytotoxic T cell-dependent release of perforin, granzyme A, and granzyme B (as reviewed

in Elmore, 2007). Perforin and granzyme A are able to activate DNAase, thereby increasing DNA cleavage and cellular death while granzyme B is able to activate caspase 10 which, in turn, activates caspase 3 to stimulate the execution pathway (as reviewed in Elmore, 2007). The execution pathway, which begins with caspase 3 activation and be triggered through the intrinsic, extrinsic, or perforin/granzyme pathways, involves various mechanisms of cellular death including chromosomal degradation, cytoskeletal degradation, and phagocytic uptake of apoptotic bodies (as reviewed in Elmore, 2007).

High levels of apoptosis occur during development as a consequence of an initial overproduction of neurons. It is estimated that up to half of the original neuronal population is then pruned through apoptosis (Oppenheim, 1981; Burek and Oppenheim, 1999) with neurons which receive adequate stimulation through trophic support from glial cells, presynaptic cells, and steroid hormones being retained and neurons not receiving proper stimulation being pruned through apoptosis (Lindsay, 1979; Okada and Oppenheim, 1984; Nordeen et al., 1985). This process is critical and tightly regulated as excessive levels can result in over-pruning of synaptic connections while insufficient levels can result in under-pruning of synaptic connections, thereby leading to excessive synaptic connections.

Apoptosis has been linked with several neurodegenerative disorders such as Huntington's disease (Hickey and Chesselet, 2003), Alzheimer's disease (Su et al., 1994), and amyotrophic lateral sclerosis (Martin, 1999). More recently, the role of apoptosis in

schizophrenia has been investigated. This is due to the subtle findings of reduced neuropil, as evidenced through reduced number of dendritic spines and reduced total dendritic length (Garey et al., 1998; Glantz and Lewis, 2000) and reductions in presynaptic markers such as synaptophysin and synaptosomal-associated protein 25 (Karson et al., 1999) in the cortex of those with schizophrenia. Additionally, findings include layer-specific reductions in interneurons in layer II of prefrontal cortex and in layers II–VI of the anterior cingulate cortex (Benes et al., 1991) and pyramidal neurons in layer IV of anterior cingulate cortex (Benes et al., 2001), reductions in neuronal number in the NAcc (Pakkenberg, 1990) and mediodorsal thalamus (Pakkenberg, 1987), and reductions in oligodendrocytes and glial cells in general in the PFC (Cotter et al., 2002; Hof et al., 2003) in those with schizophrenia. These findings suggest either increased cellular apoptosis or increased synaptic apoptosis, the latter referring to a form of apoptosis that targets synapses and/or distal neurites without affecting the remaining portion of a neuron (Mattson et al., 1998).

Therefore, the current study was conducted in order to examine apoptotic processes in the DOM animal model of schizophrenia with the hypothesis that DOM-treated animals would demonstrate altered caspase-3 staining in the mPFC.

4.2 Materials and methods

4.2.1 Experimental animals and injection procedure

Refer to section 2.2.1

4.2.2 Tissue collection and sectioning

At approximately PND 21 subjects (n = 20; 5 of each sex x treatment group) were deeply anesthetized with sodium pentobarbitol (s.c. injection), decapitated, and brains were dissected from the skull and stored in 4% paraformaldehyde for 10 days. Brains were then transferred to 0.08% sodium azide in phosphate-buffered saline until sectioning. Prior to sectioning on a vibratome, a small notch was placed in the right hemisphere of each brain to allow the identification of each hemisphere. Coronal sections (50 μm) were then produced in the area of the PRL (3.7 to 2.2 relative to bregma, respectively) (Ramachandra and Subramanian, 2011). In addition to every fourth section being kept for caspase-3 immunohistochemistry, a control slice was kept for each brain. Tissue was stored 24-well plates containing 0.08% sodium azide in PBS until the implementation of immunohistochemical procedures.

4.2.3 Immunohistochemical analyses

The immunohistochemical procedure used to assess active caspase-3, an executioner caspase in cellular apoptosis, was adapted from a study by Lanshakov et al. (2009) and began with a series of rinses (3 x 5 min unless otherwise stated) in PBS followed by a 30 minute incubation in 1% hydrogen peroxide in PBS. Sections were then re-rinsed in PBS

prior to being incubated for 30 minutes in 1% sodium borohydride in PBS. Another series of rinses in PBS was then conducted prior to a 30 minute incubation in blocking solution (5% NHS, 2% BSA, 0.2% milk, 0.5% Triton X-100 in TBS). Following incubation, blocking solution was removed and sections were treated with the primary antibody to active caspase-3 (rabbit anti-cleaved caspase-3; polyclonal) (Millipore, Billerica MA, USA) at a working dilution of 1:1000 in PBS for approximately 60 hours at 4°C. Rinses (5 x 5 min) in TBS were then performed prior to incubation for 2 hours in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame CA, USA) in TBS at a dilution of 1:100. Tissue was then rinsed in TBS followed by a 2 hour incubation period in avidin-biotin complex (Vector Laboratories, Burlingame CA, USA), after which the final set of rinses in TBS was performed. Sections were then floated in PBS onto slides and the peroxidase reaction was developed using 0.2% hydrogen peroxide in DAB for 6 minutes. Following treatment with DAB, sections were rinsed with approximately 2 mL of both PBS and distilled water and were left to dry overnight. The following day, sections were dehydrated by undergoing a series of 3 minute rinses in 70% EtOH, 95% EtOH, 100% EtOH, 100% EtOH, xylene, and xylene again before a final 45 minute rinse in xylene. Permount was then administered and cover slips were placed on sections.

4.2.4 Quantification of staining

Equivalent sections from separate subjects were identified using the forceps minor of the corpus callosum and lateral ventricle as landmarks. The PRL of the mPFC was identified as located at the midline of the brain, exactly equidistant from the most dorsal and ventral

regions of each section. Photographs (1000 x magnification) were taken of the left and right hemispheres of representative sections of the PRL using a digital Cannon Rebel EOS xSi camera (Canon Canada Inc., ON, Canada) mounted on a MEIJI trinocular ML5000 series microscope (MEIJI Techno America, CA, USA). Instances of immunoreactivity in the PRL, defined as dark, punctated somata which represent sites of caspase-3, were both manually counted and digitally quantified using optical density measurements using ImageJ software (1.41d, NIH, USA) (see Figure 15). Optical density values were obtained by mean staining intensity over each pixel in the areas of interest and subtracting the minimum staining intensity, as a method of controlling for differences in background staining, from the mean value.

4.2.5 Data analyses

Based on previous studies of the DOM model showing extensive sex-specific effects (Doucette et al., 2007; Adams et al., 2008; Adams 2009b; Burt et al., 2008a; Robbins, 2010; Ryan et al., 2011) and hemisphere-specific effects (Adams, 2009a; Robbins, 2010; Greenan, 2011), caspase-3 data was separated for sex, hemisphere, and region of the mPFC and was analyzed using Student's *t*-tests (PASW Statistics 18). A result of $p \leq 0.05$ indicated statistical significance.

4.3 Results

Results of Student's *t*-tests revealed statistically significant treatment effects with DOM-treated males demonstrating a decreased optical density measurement in the right PRL

A

B

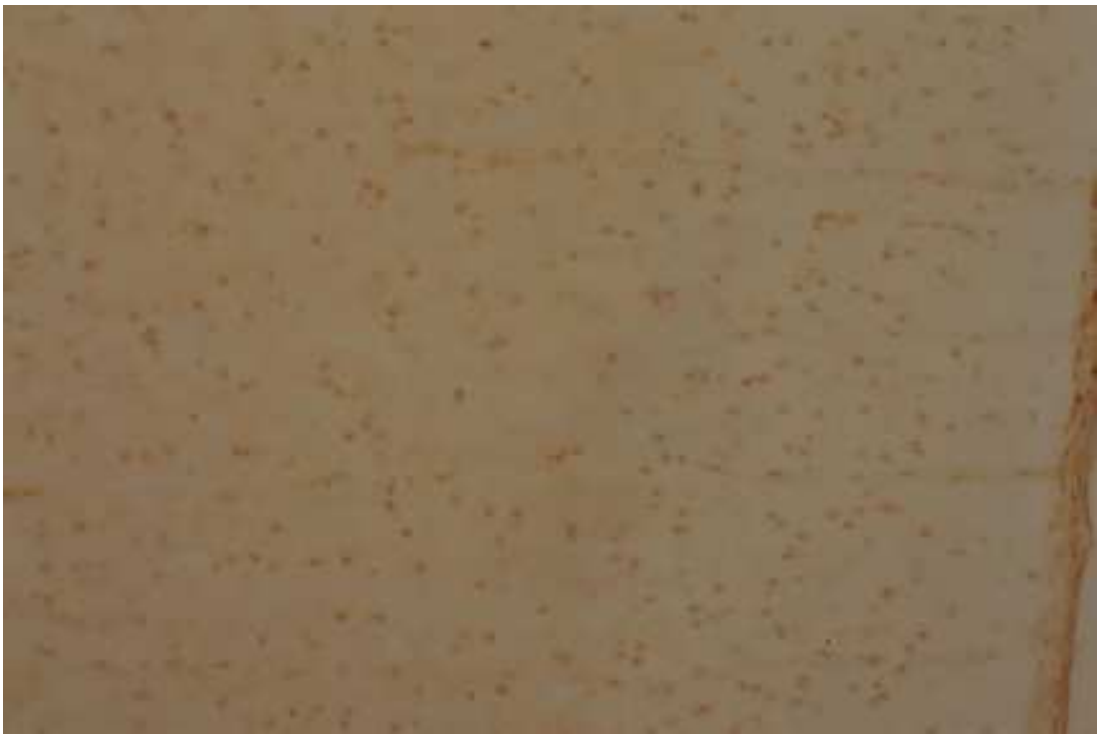


Figure 15. Caspase-3 immunopositive staining in the right PRL of representative male (A) SAL-treated and (B) DOM-treated subjects.

[t(8) = -2.838, p = 0.022] (see Figure 16) and with DOM-treated females demonstrating a decreased cell count measurement in the right PRL [t(8) = -2.307, p = 0.050] (see Figure 17). See Table 10 for mean and SEM values for caspase-3 staining.

4.4 Discussion

The current study revealed that neonatal treatment with DOM from PND 8-14, a critical period of brain development which is approximately equivalent to the third trimester of human development, resulted in decreased caspase-3 staining in the right PRL of DOM-treated males, as demonstrated by optical density measurements, and of DOM-treated females, as demonstrated by cell count measurements. In addition to the statistically significant findings, there is a consistent trend of decreased caspase-3 staining, using both cell counting and optical density methods, in each hemisphere of the PRL in both genders. The current findings of decreased caspase-3 in DOM-treated subjects are somewhat in contrast to findings of unaltered levels of caspase-3 in the temporal cortex of post-mortem human subjects (Jarskog et al., 2004) and unaltered levels of caspase-3 in organotypic hippocampal slice cultures that were treated for 24 hours with concentrations up to 20 μm of DOM (Pérez-Gómez and Tasker, 2012).

However, it is difficult to translate findings from post-mortem brains and *in vitro* hippocampal brain slices to the mPFC of subjects at PND-21. Alternatively, studies have shown increased caspase-3 activity and caspase-3 positive cells in rat frontal cortex

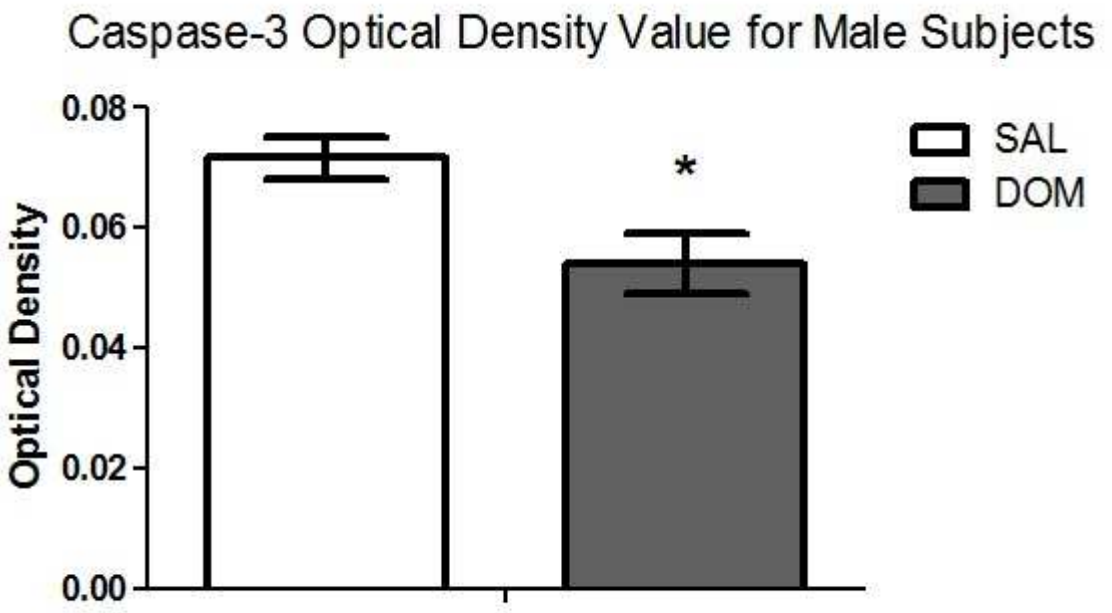


Figure 16. Mean ± SEM for the intensity of caspase-3 staining in the right PRL or DOM- and SAL-treated male subjects. Scores can range from 0 (lightest staining) to 1 (darkest staining). The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

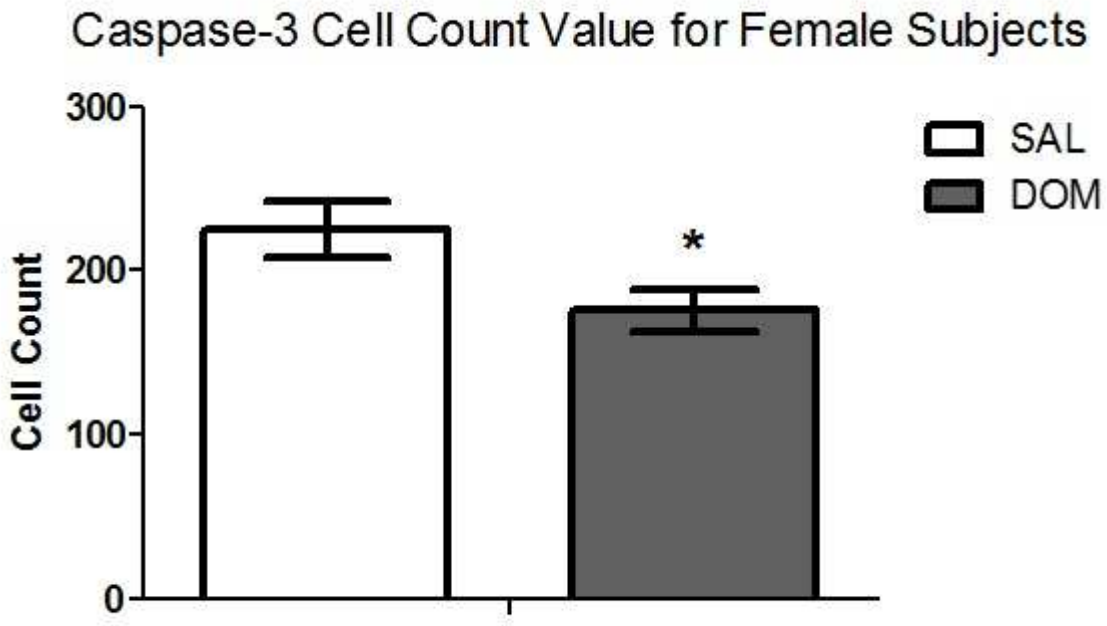


Figure 17. Mean ± SEM cell count measurement of caspase-3 staining in the right PRL or DOM- and SAL-treated female subjects. The asterisk indicates a statistically significant difference from controls ($p \leq 0.05$).

Table 10. Mean ± SEM for caspase-3 staining intensity, as quantified by optical density, and cell counts in the PRL of rats treated neonatally with DOM and SAL. Asterisk indicates a statistically significant difference between treatment groups ($p \leq 0.05$).

	Male		Female	
Measure	SAL	DOM	SAL	DOM
Right hemisphere cell count	273.40 ± 21.81	226.20 ± 24.46	225.40 ± 17.084	176.00 ± 12.92*
Left hemisphere cell count	271.60 ± 34.25	227.80 ± 39.17	249.40 ± 19.40	205.00 ± 20.70
Right hemisphere optical density	0.072 ± 0.003	0.050 ± 0.005*	0.080 ± 0.007	0.071 ± 0.012
Left hemisphere optical density	0.060 ± 0.004	0.059 ± 0.006	0.081 ± 0.010	0.070 ± 0.007

following treatment with atypical antipsychotics (Jarskog et al., 2007) and decreased Bcl-2 and an increased Bax/Bcl-2 ratio in the temporal cortex of those with schizophrenia (Jarskog et al., 2004). These findings, combined with the finding of unaltered caspase-3 in the temporal cortex, may signify that while apoptosis is not increased in chronic schizophrenia, the system is primed to over-respond to pro-apoptotic stimuli.

The observed decrease in staining through optical density measurements could have been due to either less cells staining positive for caspase-3 or due to a similar number of cells staining more weakly for caspase-3. By combining optical density measurements with cell counting methods, which both show trends of decreased staining, it is made more likely that the decreased caspase-3 staining was due to fewer cells staining positive for caspase-3, since decreased cell counts were observed. However, it remains possible, but less likely, that the underlying abnormality was, in fact, that a similar number of cells were merely staining more weakly for caspase-3. This remains possible since some cells may have become faint to the point of being non-detectable by visual cell counting methods.

Alternatively, it is possible that DOM treatment resulted in increased cell death at a point prior to when animals were euthanized and brains were dissected for immunohistochemical processing. This allows for the possibility that a normal percentage of the remaining cells in DOM-treated animals stained positive for caspase, but a lower overall cell number resulted in the appearance that either less cells were staining positive

for caspase-3 or the staining intensity was diminished. This possibility could be examined by euthanizing animals at various times throughout the PND 8-14 treatment period to assess the progression of caspase-3 staining or by counter-staining serial sections with cresyl violet stain to gain an appreciation for the total number of cells present. Thus, decreased caspase-3 staining could indicate lower levels of apoptosis, perhaps as a result of low doses of DOM providing survival signals, or could be the result of an earlier period of increased cell death.

Regardless, the staining intensity and cell counts for caspase-3 were lower in DOM-treated animal, which is significant finding based on the importance of cell population size. Cell populations are controlled through the normal pruning of neurons and synapses which occurs during development and is due in large part to inadequate survival signals. These survival signals can include nerve growth factor, BDNF, and Glu signalling (Bahr et al., 2002; Davies, 2003). Somewhat counter-intuitively, based on the role of Glu signalling in excitotoxicity and cell death, Glu signalling at less than toxic doses is essential for maintaining neuronal survival (McDonald and Johnston, 1990). Thus, while an increase in apoptotic markers may be expected following neonatal treatment with DOM, namely through calcium-induced pathways leading to mitochondrial release of cytochrome C, decreased caspase-3 staining could be due to increased pro-survival signalling. This is supported by both the fact that the current study involved only low doses of DOM administration and the fact that there are no subsequent DOM-induced alterations in developmental milestones as evidenced through eye opening, weight gain, and other

measures (Doucette et al., 2003).

The finding of decreased prefrontal caspase-3 in DOM-treated subjects could also provide insight into previously described behavioural abnormalities following neonatal treatment with DOM. As previously described, decreased caspase-3 staining could indicate lower levels of apoptosis, perhaps as a result of low doses of DOM providing survival signals, or could indicate an earlier period of increased cell death, perhaps as a result of Glu hyperactivity. Either of these scenarios would alter PFC structure and, in turn, function, thereby potentially resulting in alterations in behaviours which depend on PFC circuitry and which have been shown to be altered in the current model, include altered PPI (Adams et al., 2008), abnormal LI (Adams, 2009), social withdrawal (McInnis et al., 2009; Ryan et al., 2009), emotionality (Doucette et al., 2007), and temporal memory ability (Robbins, 2010).

However, while there are obvious links to be made between abnormalities in behaviours which are dependent on the prefrontal cortex, decreased prefrontal caspase-3, which is the executioner caspase in apoptosis, and the potential for increased cellular survival in the prefrontal cortex, the complexities of apoptosis must also be considered. For example, while the pro-apoptotic caspase-3 was decreased in DOM-treated subjects, the possibility remains that there were reciprocal alterations in anti-apoptotic proteins such as Bcl-2, Bcl-x, Bcl-xl, Bcl-xs, Bcl-w, or BAG, or potentially decreased levels of other pro-apoptotic proteins such as other caspases, Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, or Blk (as reviewed

in Jarskog et al., 2005).

An additional focus of future research could involve other proteins that have the potential to be altered based on the possibility that DOM-induced calcium-influx may alter gene expression. It is interesting to note that increased intracellular calcium serves to activate protein kinase C, thereby cleaving phosphatidylinositol 4,5-bisphosphate (PIP2) into IP3 and DAG (as reviewed in Purves et al, 2008). Subsequently, DAG triggers a signalling pathway which includes Raf, MEK, ERK, fos, and finally AP-1, a transcription factor (as reviewed in Purves et al, 2008). Genes that are affected by AP-1 include tyrosine hydroxylase (Gizand-Ginsberg and Ziff, 1990), DA β-hydroxylase (Nankova et al., 1994), choline acetyltransferase (Hahn et al., 1992), among many others. Assessment of DA β-hydroxylase and choline acetyltransferase could serve as the next targets of investigation. Rationale for this choice includes not only clinical relevance, as DA β-hydroxylase polymorphisms have been associated with symptom severity and response to treatment (Yamamoto et al., 2003) and lower levels of choline acetyltransferase have in found in the pontine tegmentum of subjects with schizophrenia (Karson et al., 1993), but also from potential mechanistic connections between DOM treatment, previous laboratory findings, calcium influx, and the AP-1 transcription factor. First, neonatal exposure to DOM has been previously shown to indirectly activate NMDARs, thereby potentially contributing to calcium influx (Tasker et al., 2005). Previous laboratory results have also shown altered tyrosine hydroxylase staining in various brain areas including the PFC (Robbins, 2010). The transcription of the tyrosine hydroxylase gene is controlled, in part, by AP-1, a

transcription factor that is inducible through a pathway that begins with increased intracellular calcium levels (Gizand-Ginsberg and Ziff, 1990). Therefore, it is possible that neonatal treatment with DOM produces increases in intracellular calcium which results in altered AP-1 and subsequent alterations in AP-1 targeted genes, such as tyrosine hydroxylase, DA β-hydroxylase, and choline acetyltransferase.

Regardless, while the current findings suggest that further examination of the apoptotic pathways is the DOM-model of schizophrenia is warranted, the current result is significant not only in relation to previously reported cognitive abnormalities in DOM-treated subjects but also in terms of providing further etiological validity for the model. Etiological validity involves recapitulating the clinical etiology of the disorder in question. As with the DISC-1 model, NMDAR antagonism models, and the neonatal VH lesion model, the neonatal DOM-model has inherent etiological validity due to its neurodevelopmental nature, and this validity is further strengthened through findings such as altered caspase-3 in the PRL which suggests abnormal prefrontal connections.

In summary, altered caspase-3 in the PRL suggests abnormalities in synaptic structure and function, a finding that is relevant in terms of improving the etiological validity of the DOM-model of schizophrenia, providing a frame-work for understanding the cognitive abnormalities that are observed in the neonatal DOM-model, and suggesting further research in pro-apoptotic proteins, anti-apoptotic proteins, and proteins which under transcriptional control of AP-1 (e.g. DA β-hydroxylase and choline acetyltransferase).

Chapter 5

General discussion

The current study was conducted with the aim of further characterizing a novel animal model of schizophrenia which involves neonatal exposure to DOM, a Glu agonist, during a critical period of CNS development. This was accomplished by assessing the face validity, construct validity, and etiological validity of the model using the attentional-set shifting and puzzle box paradigms, immunohistochemical analyses of D1, D2, and DAT, and immunohistochemical analysis of caspase-3, respectively.

5.1 Face validity of low dose-domoic acid exposure as an animal model of schizophrenia

Face validity, which refers to the phenomenological similarity in behavioural abnormalities observed in the model and in the clinical population, is a key form a validity for animal models of psychiatric disorders (Geyer and Markou, 2000). Face validity, in terms positive symptoms, negative symptoms, and cognitive symptoms, has been shown in the current model (see Table 10). The current study further characterized the face validity of the model by assessing short-term memory, long-term memory, and problem solving ability using the puzzle box paradigm, and by assessing attentional set-shifting ability using the digging task.

Statistically significant results were obtained for both of the behavioural tests of cognition. These include improved cognitive flexibility on the IDS for DOM-treated males and improved short-term memory and problem solving ability among DOM-treated females. While these results not only fail to demonstrate cognitive impairment in DOM-treated

subjects, they actually show what appears to be cognitive improvement. However, as discussed previously, many of the statistically significant findings can be interpreted as the result of an increased stress-response in DOM-treated subjects, a finding which has previously been demonstrated (Gill et al., 2012). Also, the working memory component of the puzzle box task is much more simple than in previous studies (McQuaid, 2009). Our current understanding of the deficits in the cognitive domain in schizophrenia is currently far from complete and the current findings may in fact provide insight into the complexities of the cognitive alterations. Whether the enhanced performance in the working memory and problem-solving components of the puzzle box task is a byproduct of increased stress, a faster (i.e. more impulsive) reaction time, or is in fact the result of enhanced cognitive ability is intriguing. Impulsivity is a key behavioural characteristic of DA dysfunction and has been implicated in schizophrenia (Dursun et al., 2000; Hutton et al., 2002) while at the same time a link between creativity and schizophrenia has been proposed (de Manzano et al., 2010). In either case, the findings highlight the fact that impairments in one component of cognitive function does not necessitate generalized deficits. In addition to the potential influence of stress effects, differences in terms of working memory demand, and impulsivity, the lack of cognitive impairments is not entirely unexpected, stemming from the considerable symptomatic heterogeneity of schizophrenia, which consists of residual, disorganized, catatonic, paranoid, and undifferentiated subtypes, all of which involve subtle differences in symptoms expression (DSM-IV-TR, 2000). Since not all behavioural symptoms are manifested in each patient, and the severity of symptoms also varies between patients, not all animal models are

expected to exhibit all behavioural components of schizophrenia. However, animal models should ideally exhibit the "gold-standard" behavioural of altered PPI, altered LI, and social withdrawal, all of which have been exhibited in the DOM model (Adams et al., 2009a; Ryan et al., 2011). To this end, the face validity of the DOM model has already been largely established (see Table 11), but it remains important to assess a wide-range of behaviours that are associated with schizophrenia in order to more fully characterize the persistent behavioural impacts of the proposed etiological insult.

5.2 Construct validity of low dose-domoic acid exposure as an animal model of schizophrenia

Construct validity, which refers to the accuracy with which a test measures that which it is intended to measure, is another key form a validity for animal models of psychiatric disorders and is often established through analyses of underlying neurobiology (Geyer and Markou, 2000). Construct validity of the current model of schizophrenia has previously been established through hippocampal findings including altered mossy fiber sprouting and TrkB receptor expression (Bernard et al., 2007), altered BDNF and cell counts (Doucette et al., 2004), altered GAD67 in the left CA3 region (Adams, 2009a), and through mPFC findings including altered tyrosine hydroxylase staining (Robbins, 2010). The current study involved further characterizing the construct validity of the DOM model by assessing DA markers such as D1, D2, and DAT in the mPFC in an attempt to establish a mechanism for the manifestation of previous behavioural findings including altered PPI, LI, and temporal memory ability.

Table 11. Summary of characteristics of schizophrenia which have been observed in the current animal model of schizophrenia.

Type of Validity	Displayed in Clinical Population	Displayed in Model
Face		
Positive symptoms	Drug and reward seeking	Burt et al., 2008a
	Psychomotor agitation	Burt et al., 2008b
Negative symptoms	Emotional changes	Doucette et al., 2007
	Social withdrawal	Ryan et al., 2011
Cognitive symptoms	Executive function deficits:	
	Altered response to novelty	Burt et al., 2008a
	Reversal learning	Adams et al., 2009b
	Working memory	Adams et al., 2009b
	Temporal memory dysfunction	Robbins, 2010
	Decreased PPI	Adams et al., 2009a
	Decreased LI	Adams et al., 2009a
Construct		
	Hippocampal changes:	
	Elevated BDNF	Doucette et al., 2004
	Mossy fiber sprouting	Bernard et al., 2007
	TrkB receptors	Bernard et al., 2007
	Decreased GAD67 in left CA3	Adams et al., 2009a
	Evidence of DA dysfunction	Burt et al., 2008b, Robbins 2010
Etiological		
	Acts on Glu system	All references
	Developmental in origin	All references

Again, statistically significant treatment effects were limited and manifested only as an increase in DAT staining in the right PRL of DOM-treated females. This finding was has impact as increased DAT staining may indicate a net increase in DAT functioning which could be the result of compensatory upregulation of DAT due to overstimulation of the mesocortical DA system by DOM and could, in turn, contribute to the cognitive abnormalities that have been previously demonstrated (Burt et al., 2008a; Adams et al., 2009b; Robbins, 2010) by decreasing DA tone in the mPFC through increased synaptic removal of neurotransmitter.

These relatively modest findings are, again, not completely unexpected since the strongest studies linking the chosen markers to schizophrenia in the clinical population and other animal models are limited to successful treatment with selective D1-like receptor partial agonists (Abi-Dargham et al., 2002) and selective D2R antagonists (Schmidt et al., 2012), decreased prefrontal D1 receptors via PET imaging in drug-naive patients (Okubo et al., 1997), and improvement of attentional set-shifting ability following treatment with modafinil, which inhibits DAT functioning (Turner er al., 2004). Regardless, the markers were chosen based on the significant association between schizophrenia and altered prefrontal DA signalling (Howes and Kapur, 2009) and the association between previous behavioural findings (Doucette et al., 2004; Doucette et al., 2007; Burt et al., 2008a; Burt et al., 2008b; Adams et al., 2009a; Adams et al., 2009a; Ryan et al., 2011) and findings of DA alterations (Robbins, 2010) in the current model. The construct validity of the current model is not weakened by the lack of significant findings in terms of D1 and D2 receptors

but, instead, these findings serve to guide further studies into the underlying neurochemical alterations which are responsible for the behavioural abnormalities manifested following neonatal DOM exposure. The complex mPFC circuitry, which involves many neurotransmitter systems including DA, Glu, GABA, serotonin, noradrenaline, opiates, and neurotensin (as reviewed in Steketee, 2003), allows for many potential avenues of future analyses, with previous studies of the clinical population and of animal models indicating that the Glu (Dracheva et al., 2001), GABA (Beasley and Reynolds, 1997), and serotonin (Hashimoto et al., 1991) systems are most strongly implicated.

5.3 Etiological validity of low dose-domoic acid exposure as an animal model of schizophrenia

Etiological validity, which involves recapitulating the clinical etiology, is another key form a validity for animal models of psychiatric disorders (Geyer and Markou, 2000). While etiological validity is not an essential component of all animal models, since adequate face validity and predictive validity can allow for the effective assessment of symptom manifestation and treatment, ensuring etiological validity can allow for the confidence that behavioural symptoms are due to the same underlying process in the model as they are in the clinical population. However, etiological validity is often difficult to establish since the etiology of the human condition is often poorly understood, as is the case for schizophrenia. In terms of current animal models of schizophrenia, inherent etiological validity exists in the DISC-1 model, NMDAR antagonism models, and the

neonatal VH lesion model. However, the etiological validity of each of these models appears limited as the DISC-1 model does not incorporate any non-genetic contributions to schizophrenia, the NMDAR antagonism models involve relatively high-dose exposure to NMDAR antagonists, and the neonatal VH lesion model involves an unrealistically severe brain lesion. While there is significant difficulty associated with establishing etiological validity in a model of a disorder with an unknown etiology, it also allows for the implementation of current understanding and knowledge of the etiology of schizophrenia in an attempt to create a realistic model, as was done in the current and previous studies employing neonatal exposure to DOM, a Glu agonist (see Table 10). The current study further characterized the etiological validity of the DOM model, which involves developmental exposure to low-doses of a Glu agonist, by assessing immunohistochemical staining of caspase-3, an executioner caspase involved in apoptosis, in the mPFC. The assessment of mPFC caspase-3 staining in DOM-treated subjects allowed for further insight into previously described behavioural abnormalities following neonatal treatment with DOM. These include various behaviours which depend on prefrontal circuits, include altered PPI (Adams et al., 2008), abnormal LI (Adams, 2009), social withdrawal (McInnis et al., 2009; Ryan et al., 2009), emotionality (Doucette et al., 2007), and temporal memory ability (Robbins, 2010).

Results revealed a statistically significant decrease in optical density measurement in the right PRL among DOM-treated males and a statistically significant decrease using cell count measurement in the right PRL among DOM-treated females. These findings could

stem from an early upregulation of apoptotic pathways due to overactivity of KARs which, in turn, could have reduced the available cell population, thereby producing low caspase-3 staining. Alternatively, it is possible that the amount by which Glu tone was increased by DOM administration was insufficient to activate apoptosis but instead served as a survival signal to reduce the amount of apoptosis, thereby producing low caspase-3 staining.

In summary, the etiological validity of the DOM model is further strengthened by the results of the caspase-3 analysis despite the unclear explanation for the findings, as they indicate that DOM treatment is having a yet unclear effect on apoptosis, a key process in brain maturation, which has relevance for a neurodevelopmental disorder such as schizophrenia.

5.4 Future directions

The current thesis focussed on further establishing the face validity, construct validity, and etiological validity of a novel animal model of schizophrenia, and future avenues of investigation could involve focussing on any combination of these areas and, additionally, could focus on establishing predictive validity.

In terms of the three types of validity that were assessed in the current model, construct validity may be the area which requires the most investigation in the future. This stems largely from the fact that a number of behavioural findings have been observed, but only limited neurochemical studies have been conducted (see Table 11). However, a difficulty

arises in whether the targets of neurochemical studies should be markers that are related to previously established behavioural abnormalities, or whether markers should be chosen based on previous findings of alterations in the clinical population. The choice between these two alternatives is best taken upon considering the goals for the model and whether they are more directed at generating novel findings that can guide clinical research, or whether they are more directed at attempting to establish the degree to which the model recreates each aspect of the clinical manifestation of schizophrenia. An additional difficulty with the former alternative is that there is no single marker that, alone, explains complex behavioural findings, so the number of relevant neurochemical markers is vast, making the firm establishment of construct validity a daunting task. However, it seems as though, at this point in the model, establishing potential links between neurochemical markers and established behavioural abnormalities would provide a more clear, concise picture than would examining disjointed systems in an attempt to replicate clinical findings. Based on the behavioural findings of social withdrawal (Ryan et al., 2011), altered PPI and LI (Adams et al., 2009a), and temporal memory dysfunction (Robbins, 2010), which point to both mPFC and hippocampal abnormalities, various protein markers of the Glu, GABA, and serotonin systems, such as synthetic enzymes, receptors, transporters, and degradative enzymes, should be assessed in both of these brain structures.

Further studies on the face validity of the model could focus on the reassessment of attentional set-shifting ability using the elevated plus maze procedure as previously described (Stefani et al., 2003), as this could eliminate a number of the factors which are

believed to have impaired subject performance in the current study. However, an alternative view is that since the major "gold-standard" behavioural alterations have been observed in the model, spanning each of the major symptoms classes, perhaps the previously established face validity should now be used in order to assess predictive validity. Predictive validity, which is considered by some to be the most important form of validity, is often used to refer to the ability of a model to identify novel therapeutic compounds (Geyer and Markou, 2000). Therefore, an interesting future avenue for research would involve assessing the ability of currently used atypical antipsychotics, such as clozapine or risperidone, to reverse the previously observed findings of altered PPI, altered LI, and social withdrawal. Should these results reveal that atypical antipsychotics are able to effectively treat subjects in the model, more novel treatment regimes could be assessed.

Alternatively, while further assessments of construct validity and predictive validity are both obvious future directions for research, one of the most unique features of the current model is its strong etiological validity due to its neurodevelopmental nature and its mechanistic links to abnormal Glu signalling during development, which is speculated to contribute to schizophrenia (as reviewed in Goff and Coyle, 2001). Many of the current animal models of schizophrenia either have little etiological validity or, as previously described, have inherent weaknesses in their etiological validity. For this reason, further examination of the etiological validity of the current model may be the most effective way to further distinguish the DOM model providing unique information in terms of the

etiology of schizophrenia. Possible avenues of research include further assessment of caspase-3 staining at various time-points with concurrent assessment of pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2 in the mPFC and hippocampus, performing the terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine, 5′-triphosphate nick end-labeling method of assessing double-stranded DNA breaks associated with apoptosis, and silver staining for cell death.

Prior to the decision of whether to further pursue studies on construct validity, face validity, predictive validity, and/or etiological validity, it is important to carefully assess the strengths of the current model, the strengths of the major currently accepted animal models, and the goal for the current model in order to determine how to best proceed with the current model in order to further our understanding of schizophrenia.

5.5 Conclusion

The current study employed both behavioural analyses of mPFC functioning and immunohistochemical analyses of mPFC cell death and DA system integrity in order to further assess the face, construct, and etiological validity of the DOM model of schizophrenia.

While only modest treatment effects were seen, the construct validity and etiological validity of the model was nonetheless further strengthened. Face validity was not furthered by the current study, although treatment effects were observed, but this can be

accounted for by the considerable behavioural and neurochemical heterogeneity the clinical population. Additionally, the model retains considerable face validity, construct validity, and etiological validity based on previous behavioural studies, neurochemical studies, and features of the developmental insult that is administered. Future studies should be conducted in order to further establish the neurochemical alterations that are responsible for behavioural abnormalities in the model, to establish the predictive validity of the model with the goal of making contributions to the development of novel treatment regimes, and to further examine the mechanism whereby neonatal treatment with DOM produces persistent behavioural and neurochemical alterations.

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