PAOPA: A POTENTIAL NOVEL DRUG FOR THE TREATMENT OF
SCHIZOPHRENIA

PAOPA, A POTENT DOPAMINE D2 RECEPTOR ALLOSTERIC MODULATOR,

PREVENTS AND REVERSES BEHAVIOURAL AND BIOCHEMICAL

ABNORMALITIES IN AN AMPHETAMINE-SENSITIZED PRECLINICAL MODEL

OF SCHIZOPHRENIA

By

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TITLE: PAOPA, a potent dopamine D2 receptor allosteric modulator, prevents and

reverses behavioural and biochemical abnormalities in an amphetamine-sensitized

preclinical model of schizophrenia

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ABSTRACT

Allosteric modulators are emerging as a new class of therapeutics for the treatment of complex disorders, including psychiatric illnesses such as schizophrenia. The disease is marked by hyperdopaminergic signaling in the striatum, which plays a role in the development of positive symptoms like delusions, hallucinations, and paranoia. Conventional antipsychotic drug therapy typically employs dopamine D2 receptor antagonists that compete with endogenous dopamine at the orthosteric, or dopamine-binding site, in an attempt to normalize these psychotic symptoms. However, they are often associated with adverse motor and metabolic side effects. Furthermore, only some antipsychotic drugs are able to treat the negative symptoms of schizophrenia, which include social withdrawal and anhedonia, and there is currently no treatment for the cognitive impairment associated with the disease.

Allosteric modulators are safer alternatives to conventional orthosteric therapeutics as they interact with their receptor at a novel binding site and their mechanism involves modulation of endogenous signaling. Therefore, levels of endogenous ligand limit the activity of an allosteric modulator. Our lab has synthesized and evaluated over 185 compounds for their activity at the dopamine D2 receptor. Of these compounds, PAOPA is the most potent allosteric modulator, and has been shown to be effective in treating the MK-801 induced preclinical animal model of schizophrenia without causing the adverse effects induced by currently prescribed antipsychotic drugs. The objective of this study was to evaluate PAOPA's ability to treat behavioural abnormalities in an amphetamine-sensitized model of schizophrenia.

Four groups (n=10/group) of male Sprague Dawley rats received intraperitoneal

injections three days per week on alternate days over three weeks. Group A received saline, group B received D-amphetamine (1mg/kg during week one, 2mg/kg during week two, 3mg/kg during week three), group C received PAOPA (1mg/kg), and group D received the same doses of amphetamine as group B with PAOPA (1mg/kg). Following a three-week withdrawal, each group was tested for prepulse inhibition, social interaction, and locomotor activity. Amphetamine-sensitized rats were subjected to the same tests following PAOPA administration (1mg/kg). To assess whether behavioural changes were associated with changes in brain chemistry, post-mortem dopamine levels were measured in the striatum, nucleus accumbens, and medial prefrontal cortex. Data were analyzed by one-way ANOVA or paired t test where appropriate.

Amphetamine sensitization induced schizophrenic-like behavioural abnormalities, including deficits in prepulse inhibition and social interaction, as well as increased locomotor activity and sensitivity to amphetamine challenge. Concurrent amphetamine and PAOPA treatment prevented all amphetamine- induced behavioural abnormalities. Furthermore, amphetamine-induced deficits in prepulse inhibition and social interaction were reversed one hour following PAOPA treatment. PAOPA treatment alone had no effect on behaviour or post-mortem striatal dopamine. Behavioural changes in amphetamine-sensitized rats were accompanied by a reduction in post-mortem striatal dopamine levels. In correlation with behavioural results, PAOPA administration during amphetamine sensitization prevented this biochemical change.

These results demonstrate that PAOPA can prevent and reverse behavioural and associated biochemical abnormalities in amphetamine-sensitized rats. PAOPA is a candidate for the development of treatments for schizophrenia.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

3-MT – 3-methyoxytyramine

5-HT – 5-hydroxytrypatmine

6-OHDA – 6 hydroxydopamine

7TMR – 7 transmembrane receptor

AADC – L-amino acid decarboxylase

ANOVA – analysis of variance

APD – antipsychotic drug

ATP – adenosine triphosphate

CAF – Central Animal Facility

CaMK – calmodulin-dependent kinase

cAMP – cyclic adenosine triphosphate

CCAC – Canadian Council on Animal Care

COMT – catechol-O-methyltransferase

D2L – dopamine D2 receptor (long isoform)

D2S – dopamine D2 receptor (short isoform)

DA – dopamine

DAG – diacyl glycerol

DAT – dopamine transporter

dB – decibel

DHX - dihydrexidine

DOPAT – 3,4-dihydroxyphenylacetic acid

ECD – electrochemical detector

g - gram

GABA - γ-aminobutyric acid

GDP – guanosine diphosphate

GPCR – G protein coupled receptor

GRK – G protein coupled receptor kinase

GSK3 – glycogen synthase kinase 3

GTP – guanosine triphosphate

IP - intraperitoneal

IP_3 – inositol 1,4,5-trisphosphate

kg – kilogram

L-DOPA – L-3,4-dihydroxyphenylalanine

MAO – monoamine oxidased

mg - milligram

mPFC – medial prefrontal cortex

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

ms – millisecond

NAc – nucleus accumbens

ng - nanogram

NMDA – N-methyl-D-aspartate

nVH – neonatal ventral hippocampus

PAOPA – 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide

PCP - phencyclidine

PFC – prefrontal cortex

PIP$_2$ – phosphatidylinositol 4,5-bisphosphate

PKA – protein kinase A

PKB – protein kinase B

PKC – protein kinase C

PLC – phospholipase C

PLG – L-prolyl-L-leucyl-glycinamide

PP2A – protein phosphatase 2A

PPI – prepulse inhibition

RGS – regulators of G protein signaling

s – second

TH – tyrosine hydroxylase

VTA – ventral tegmental area

1   INTRODUCTION

Allosteric modulators have recently emerged as promising candidates for the treatment of more complex disease states, including mental disorders. Their unique properties and mechanism of action may result in safer alternatives to conventional antipsychotics, which bind at a receptor's orthosteric site (Wang *et al.*, 2009).   Although their specific receptor binding profiles and pharmacological mechanisms vary, most currently prescribed antipsychotic drugs are dopamine D2 receptor antagonists that compete with endogenous dopamine to reduce hyperdopaminergic neurotransmission in the striatum (Meltzer, 1991; Mukherjee *et al.*, 2001; Howes and Kapur, 2009). However, blocking the orthosteric site can lead to an accumulation of synaptic dopamine, and nonspecific drug-receptor interactions are often unpredictable. These properties often result in undesirable side effects following long-term antipsychotic drug use, including movement and metabolic disorders (Daumit *et al.*, 2008; Meyer *et al.*, 2008; Miller *et al.*, 2008).

Allosteric modulators, on the other hand, are more specific for their target and do not compete with endogenous ligand. Furthermore, their activity is entirely dependent on physiological signaling, creating a ceiling to their effect (Conn *et al.*, 2009). By inducing conformational changes, allosteric modulators alter how their target receptor will respond to endogenous ligand (Kenakin, 2010). Therefore, higher doses of an allosteric compound are more tolerable than orthosteric compounds.

Several allosteric modulators have recently been proposed for the treatment of schizophrenia, by targeting the metabotropic glutamate receptor subtypes mGluR2 (Galici *et al.*, 2006; Benneyworth *et al.*, 2007) and mGluR5 (Lecourtier *et al.*, 2007),

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nicotinic acetylcholine receptors (Buchanan *et al.*, 2008) as well as the muscarinic M4 receptor (Brady *et al.*, 2008; Chan *et al.*, 2008). However, these compounds may rely on indirect reestablishment of normal dopaminergic neurotransmission for their desired pharmacological effect (Schilstrom et al, 2007; Gill *et al.*, 2011). Therefore it is conceivable that direct allosteric modulation of dopamine receptors would be more suitable for treating schizophrenia. However, despite the close association between the dopamine D2 receptor and schizophrenia (Howes and Kapur, 2009), few allosteric modulators of the dopamine receptors have been reported (Hoare and Strange, 1996; Hoare *et al.*, 2000; Soriano *et al.*, 2009; Soriano *et al.*, 2010). Our lab has developed several novel allosteric modulators of the dopamine D2 receptor, based on the structure of endogenous brain peptide PLG (L-prolyl-L-leucyl-glycinamide) . Among them, PAOPA (3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide) (Figure 1) is one of the most potent (Verma *et al.*, 2005).

PAOPA is able to allosterically enhance agonist binding to bovine and human dopamine D2 receptors, while having no effect on antagonist binding (Mishra *et al.*, 1990; Verma *et al.*, 2005). By increasing agonist-induced GTPase activity, PAOPA is able to maintain D2Rs in high affinity states, ultimately causing increased inhibition of adenylyl cyclase activity through increased D2 receptor stimulation (Mishra *et al.*, 1999). It has also been demonstrated that PAOPA can increase dopaminergic sensitivity in the nigrostriatal pathway. This is presumably the mechanism by which PAOPA can prevent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and haloperidol-induced movement disorders (Marcotte *et al.*, 1998; Ott *et al.*, 2000; Sharma *et al.*, 2003) and modulate rotational behavior in the 6-hydroxydopamine (6-OHDA) lesioned rat model of

Parkinson's disease (Mishra *et al.*, 1997). Interestingly, PAOPA is also able to prevent deficits in social interaction in a preclinical animal model of schizophrenia induced by chronic treatment with the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (Dyck *et al.*, 2011), although the mechanism by which it improves schizophrenic-like behavior is not yet known.

The objective of this study was to determine whether PAOPA, a dopamine D2 receptor allosteric modulator, is effective in preventing and reversing behavioral abnormalities in an amphetamine-sensitized rodent model of schizophrenia. The amphetamine-sensitized model is a widely accepted and well-studied preclinical animal model of schizophrenia because it induces behavioral and biochemical abnormalities similar to those observed in the disease state. Dopaminergic supersensitivity likely occurs via similar mechanisms of excessive stimulation of dopamine D2 receptors in schizophrenia and as a result chronic amphetamine administration (Seeman and Kapur, 2000; Peleg-Raibstein *et al.*, 2009). Furthermore, biochemical abnormalities were explored by measuring post-mortem dopamine levels in the striatum, nucleus accumbens (NAc), and medial prefrontal cortex (mPFC) in all treatment groups to determine whether amphetamine sensitization disrupted dopamine levels in these key brain regions.

1.1   Schizophrenia

Schizophrenia is among the most debilitating mental illnesses, affecting roughly 1% of the global population (van Os and Kapur, 2009). Symptoms of the disease are divided into three categories: positive symptoms, negative symptoms, and cognitive dysfunction (van Os and Kapur, 2009; Crow, 1980). Positive symptoms are behaviours that are present in individuals with the disease, but absent in the healthy population,

including paranoia, hallucinations, and delusions (Crow, 1980). Negative symptoms, on the other hand, are behaviours that are present in the healthy population, but are lacking or absent in patients with schizophrenia, including apathy, anhedonia, and social withdrawal (Crow, 1980). Cognitive dysfunction in schizophrenia includes deficits in memory, attention, problem solving, and capacity for language. The best predictor of long-term prognosis seems to be the severity of cognitive dysfunction, rather than the severity of positive or negative symptoms (Simpson *et al.*, 2010).

The disease usually manifests fully late in adolescence or early in adulthood, although cognitive and negative features may appear much earlier in prodromal patients. There is currently no biological marker for diagnosis, despite common physical characteristics among patients including increased ventrical size, decreased temporal lobe volume, and increased striatal dopamine storage and release (Karam *et al.*, 2010).

The pathology of schizophrenia is not yet fully understood. It is very complex, and several neurotransmitters, such as dopamine, glutamate, and γ-aminobutyric acid (GABA), have been implicated in its development (Seeman and Kapur, 2000; Carlsson *et al.*, 2001; Reynolds *et al.*, 2001). Positive symptoms are thought to be a result of dopaminergic hyperactivity in the striatum, while negative and cognitive symptoms are though to be caused by hypoactivity in the cortex (Karam *et al.*, 2010). Recently, it has been proposed that the aberrant dopaminergic signaling in the cortex could actually be a result of excessive striatal signaling (Simpson *et al.*, 2010).

1.2   Antipsychotic Drugs

There is currently no cure for schizophrenia, however symptoms are treated with three generations of antipsychotic drugs (APDs). Although their specific

pharmacological mechanisms vary, most antipsychotic drugs are dopamine D2 receptor antagonists that compete with endogenous dopamine to reduce hyperdopaminergic neurotransmission in the striatum (Meltzer, 1991; Mukherjee *et al.*, 2001).

First generation APDs include the phenzothiazines, butyrophenones, and thioxanthenes, such as chlorpromazine and haloperidol. Known as "typical" APDs, they interact almost exclusively with dopamine D2 receptors, acting as antagonists that compete with endogenous dopamine (Schultz *et al.*, 2007). Although they can be effective in treating the positive symptoms and preventing psychotic relapse, they have little or no effect on negative and cognitive symptoms. Furthermore, many patients are resistant or only partially responsive to these drugs, and long term-use can result in extrapyramidal movement disorders, resulting in low patient compliance (Jeste *et al.*, 1999).

Second generation APDs, also known as "atypical" APDs, were developed with the goal of improved efficacy for the treatment of negative and cognitive symptoms. The first wave of second generation APDs was based on the structure of the benzodiazapine clozapine. These drugs, which are dopamine D2 receptor and serotonin 5-HT$_{2A}$ receptor antagonists, seem to be somewhat effective in controlling negative symptoms and rarely cause extrapyramidal effects, although they can induce severe metabolic side effects such as agranulocytosis, seizures, sedation, hypotension, and weight gain (Citrome *et al.*, 2004; Parsons *et al.*, 2009). A second wave of atypical drugs followed with the hopes of reducing adverse effects. These benzamides are D2 receptor antagonists that also bind non-specifically to a wide array of receptors. Their widespread binding profile is thought

to be the mechanism by which these APDs improve some negative and cognitive aspects of schizophrenia (Mailman and Murthy, 2010).

Third generation APDs, such as aripiprazole, were initially characterized as partial dopamine D2 receptor agonists. Partial D2 receptor agonists stimulate the receptor to a lower extent than dopamine. Therefore they theoretically compete with dopamine in regions of high dopaminergic transmission, but also stimulate dopamine receptor signaling in hypodopaminergic regions (Perreault et al, 2011). However, studies have shown that aripiprazole's agonism varies between cell lines, and that it can act as a full agonist for D2-mediated inhibition of dopamine synthesis (Shapiro *et al.*, 2003). It has been proposed that a key aspect of aripiprazole's pharmacology may be that it has different effects on presynaptic and postsynaptic dopamine receptor signaling. This differential signaling at the same receptor is known as "functional selectivity", and may be a key component of third generation APD pharmacology (Mailman and Murthy, 2010).

1.2.1   Functional Selectivity

Classical concepts of receptor-mediated signaling involve coupling of a receptor to fixed signaling pathways, regardless of cellular context. Ligands have traditionally been viewed as agonists, antagonists, or inverse agonists, depending on how they influence those fixed pathways (Mailman and Murthy, 2010). A full agonist acting on a receptor should fully activate all pathways of the endogenous ligand, while an antagonist should prevent an agonist from activating all of those pathways. Since the effects of a ligand on a receptor would be a product of the ligand's affinity and efficacy, differences

in a ligand's effects between cells and tissues would ascribed to differences in signal intensity (Urban *et al.*, 2007).

However, it is rarely the case that a drug's response will be the same in all systems. It is becoming increasingly clear that receptors can assume multiple conformational states, and ligands can induce receptor conformations that favour various signaling pathways (Perez *et al.*, 1996; Audet *et al.*, 2008). This may occur because of the association and dissociation of signaling molecules, including G proteins. Alternatively, several signaling cascades may be activated by one ligand, with some being stimulated more strongly than others (Mailman, 2007). A receptor can therefore couple to various signaling pathways, depending on the signaling partners that are favoured by the activating ligand. In this way, two ligands can stimulate the same receptor to signal through completely independent pathways. This concept is known as "functional selectivity" (Urban *et al.*, 2007).

Several functionally selective ligands have been characterized, including the dopamine D2 receptor functionally selective ligand, dihydrexidine (DHX). This compound was initially characterized as a full dopamine D1 receptor agonist (Mottola *et al.*, 1992). However, unique signaling events observed *in vitro* and *in vivo* lead to the realization that the pharmacology of DHX was not so simple. Further studies have revealed that DHX can induce unique signaling events at the dopamine D1 and D2 receptors within and across several cell lines, confirming it is acting in a functionally selective manner (Mottola *et al.*, 2002; Kilts *et al.*, 2002).

1.3 Dopamine

Dopamine (Figure 1) is a catecholamine neurotransmitter that binds and activates the five classes of dopamine receptors – D1, D2, D3, D4, and D5. It is produced in the brain and the adrenal gland, and serves as a precursor to the catecholamines norepinephrine and epinephrine (Beaulieu and Gainetdinov, 2011). In the brain, dopamine functions as a slow-acting modulator of the faster-acting neurotransmitters glutamate and GABA to influence cognition, movement, mood, and reward (Berridge, 2006). In the periphery, dopamine is involved in the senses, regulation of blood pressure, as well as immune system and kidney function (Velasco and Luchsinger, 1998).

Dopamine is synthesized from the amino acid tyrosine in a series of chemical reactions. Initially, tyrosine is taken up by neuronal cells and converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) in the rate-limiting step of dopamine synthesis. L-DOPA is quickly converted by L-amino acid decarboxylase (AADC) to yield dopamine. In neurons that use dopamine as a neurotransmitter, this is the final step, and dopamine can be packaged into vesicles by vesicular monoamine transporter (VMAT) for release into the synapse (Miyake *et al.*, 2010). However, cells that use the catecholamine norepinephrine as a neurotransmitter also contain the enzyme dopamine β hydroxylase, which utilizes dopamine to produce norepinephrine. Norepinephrine can be further metabolized phenylethanolamine N-methyltransferase to yield epinephrine (Fernstrom and Fernstrom, 2007). Synaptic dopamine is inactivated via reuptake by the dopamine transporter (DAT) and is subsequently metabolized by monoamine oxidase (MAO) and catechol O-methyltransferase (COMT) to yield homovanillic acid, with the intermediates 3,4-

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dihydroxyphenylacetic acid (DOPAT) and 3-methoxytyramine (3-MT) (Velasco and Luchsinger, 1998). The synthetic and metabolic pathways of dopamine are shown in Figure 2.

Dopamine is implicated in several disease states. Schizophrenia is believed to be a result of dysregulated dopamine signaling, as almost all APDs block dopamine D2 receptors. Parkinson's and Huntington's diseases involve loss of specific dopaminergic neurons in the striatum, while other illnesses that have been linked to dopamine include attention deficit and hyperactivity disorder, bipolar disorder, depression, hypertension, and kidney disease (Beaulieu and Gainetdinov, 2011).
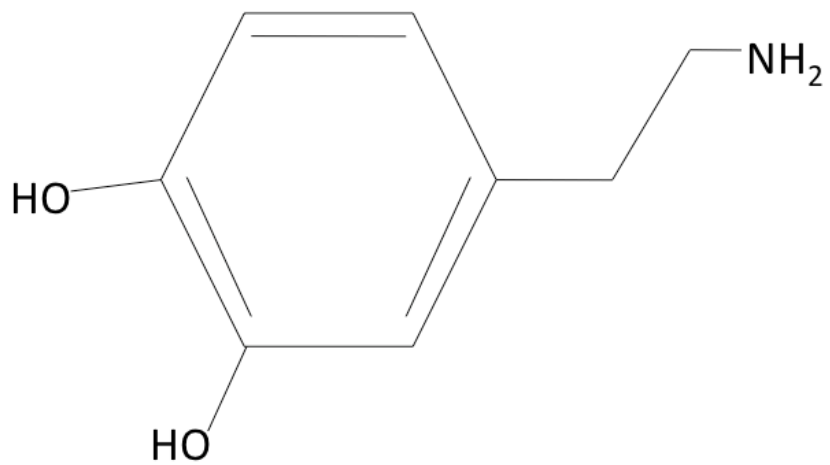
Figure 1. The chemical structure of dopamine.
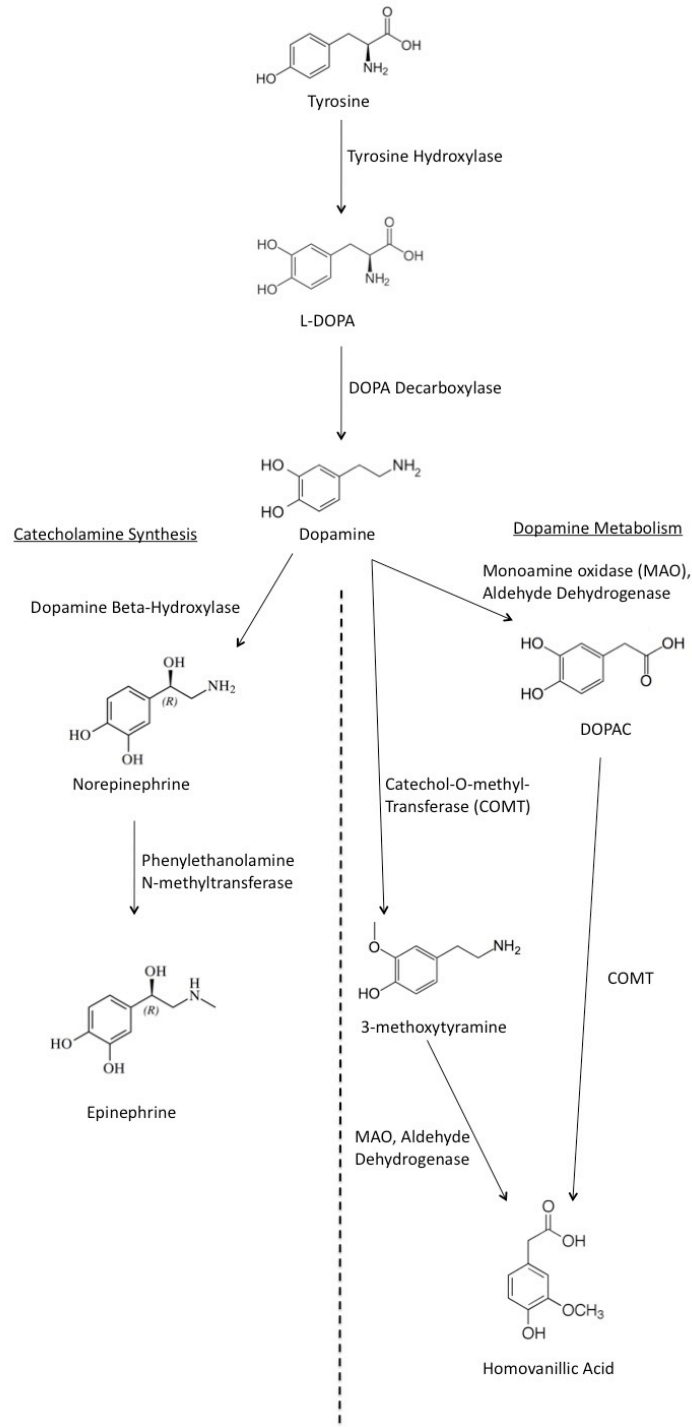
Figure 2. The synthetic and metabolic pathways of dopamine. Tyrosine is converted to L-DOPA by TH. L-DOPA is then converted to dopamine by AADC. Dopamine β hydroxylase uses dopamine to produce norepinephrine, which can be further metabolized by phenylethanolamine N-methyltransferase to yield epinephrine. Synaptic dopamine is inactivated via reuptake by DAT and is subsequently metabolized by MAO and COMT to yield homovanillic acid, with the intermediates DOPAT and 3-methoxytyramine 3-MT.

1.3.1   Dopamine Pathways in the Brain

There are four major neuronal circuits in the brain involved in dopaminergic neurotransmission (Figure 3): mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular. For the purposes of this thesis, only the mesolimbic and mesocortical pathways will be discussed in great detail, as they are both closely associated with schizophrenia.

The mesolimbic pathway transmits dopaminergic signals from the ventral tegmental area (VTA) to the NAc. Dopaminergic neurons from the VTA extend to and stimulate medium spiny neurons within the NAc to release GABA into the ventral pallidum. This pathway is involved in motivation and reward through modulation of wanting behaviour, known as incentive salience (Berridge, 2006). Neurons within the VTA fire in response to new stimuli and based on the prediction error of a reward. If a reward to a stimulus is greater than predicted, these neurons respond by firing. However, if a reward was predicted but not achieved, there is a depression in firing of these neurons. Over time these neurons fire in anticipation of reward, instead of firing in response to the reward itself (Barch and Dowd, 2010). The mesolimbic system is also believed to be involved in addiction, as drugs of abuse like amphetamine and cocaine increase synaptic dopamine levels and dopaminergic stimulation of neurons in this pathway (Pierce and Kumaresan, 2006).

The mesocortical pathway transmits dopaminergic signals from the VTA to the prefrontal cortex (PFC). Deregulation of this pathway is though to give rise to the cognitive and negative symptoms of schizophrenia. These neurons, which mediate behaviour, motivation, and cognition, converge on pyramidal neurons and interneurons

within the PFC, where there is a high expression of D1 receptors and to a lower extend, D2 receptors (Rodrigues *et al.*, 2011). Mesocortical dopamine neurotransmissions are not believed to carry information about the new stimulus. Instead, glutamatergic transmissions relay information from the VTA to the PFC, which responds by entering a state of persistent activity. Mesocortical dopaminergic signaling mediates the level of activity in the PFC. Activity in this region depends on dopaminergic stimulation of D1 and D2 receptors and follows an inverted U-shaped curve. Therefore, too little or too much dopaminergic stimulation hinders higher cognitive functions, especially working memory (Seamans and Yang, 2004). Hypodopaminergic function within this pathway is believed to give rise to the negative and cognitive symptoms of schizophrenia. It has recently been proposed that this hypodopaminergic signaling in the PFC may arise from dysregulation of striatal-cortical circuitry (Simpson et al, 2010).

Mesolimbic Pathway	Mesocortical Pathway	Nigrostriatal Pathway	Tuberoinfundibular Pathway
VTA → N.Ac.	VTA → PFC	S.N. → Striatum	Hypothalamus → Pituitary
Involved in reward, "wanting", and addiction	Involved in working memory	Involved in movement	Involved in prolactin secretion
Associated with positive symptoms of schizophrenia	Associated with negative and cognitive symptoms of schizophrenia	Associated with Parkinson's disease	

Figure 3. Major dopamine pathways in the brain. There are four major dopamine pathways in the brain: (1) the mesolimbic pathway; (2) the mesocortical pathway; (3) the nigrostriatal pathway; and (4) the tuberoinfundibular pathway. Adapted from Rodrigues *et al*., (2011).

1.4   G Protein-Coupled Receptors

In order to understand dopamine receptor physiology, signaling and regulation of G protein-coupled receptors (GPCRs) must be discussed. GPCRs are a family of transmembrane proteins that bind extracellular ligand and transmit a signal across the cell membrane to stimulate intracellular signaling events. Purification and characterization of such hydrophobic proteins is difficult, so the structures of few GPCRs have been resolved. The crystal structure of the rhodopsin and has been extended to the entire GPCR family (Figure 4) (Palczewski *et al.*, 2000).

GPCRs consist of a single polypeptide chain that extends from the extracellular amino (N)-terminus, through the cell membrane seven times, to the cytoplasmic carboxy (C)-terminus. Their distinct structure gives rise to the alternative name seven transmembrane receptors (7TMRs). The seven membrane-spanning helices, named I through VII, are arranged in a barrel. Helices are connected by three extracellular loops (E-I through E-III) and three cytoplasmic loops (C-I through C-III), which contain sites for modifications that influence ligand binding, receptor signaling, and receptor trafficking. The orthosteric ligand-binding site of most GPCRs is embedded in the transmembrane region within the helices, and access to the site is regulated by E-II (Palczewski *et al.*, 2000).

Heterotrimeric G proteins, composed of α, β, and γ subunits, mediate canonical G protein signaling. In the absence of ligand, the G protein's α subunit is bound to guanosine diphosphate (GDP), rendering it inactive, and is also associated with the tightly bound β/γ subunits. Upon ligand binding, conformational changes cause the exchange of GDP for guanosine triphosphate (GTP), allowing α subunit to dissociate

from the β/γ subunits. Both the active α and the β/γ subunits are free to diffuse throughout the membrane and initiate signaling events (Beaulieu and Gainetdinov, 2011).

The α subunit determines coupling events, and is classified as $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, or $G\alpha_{12}$. Most GPCRs signal through $G\alpha_s$, $G\alpha_{i/o}$, or $G\alpha_{q/11}$. $G\alpha_s$ couples to adenylyl cyclase, simulating it to convert adenosine triphosphate (ATP) to cyclic AMP (cAMP), which regulates protein kinase A (PKA) and various ion channels. Conversely, $G\alpha_{i/o}$ is inhibitory to adenylyl cyclase, resulting in decreased cAMP production. $G\alpha_{q/11}$ activates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to yield the second messengers diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). This results in the release of Ca^{2+} from the endoplasmic reticulum, and activation of protein kinase C (PKC) and calmodulin-dependent kinase (CaMK). β/γ subunits also influence signaling through modulation of ion channels and various kinases. (Beaulieu and Gainetdinov, 2011).

The name GPCR can be misleading, as these receptors also signal via G protein-independent pathways. These non-classical signaling pathways vary greatly depending on the receptor and ligand involved. Phosphorylation by GPCR kinases (GRKs) and the subsequent association of arrestin proteins are important for late signaling events, as arrestins can recruit signaling molecules (Luttrell *et al.*, 1999).
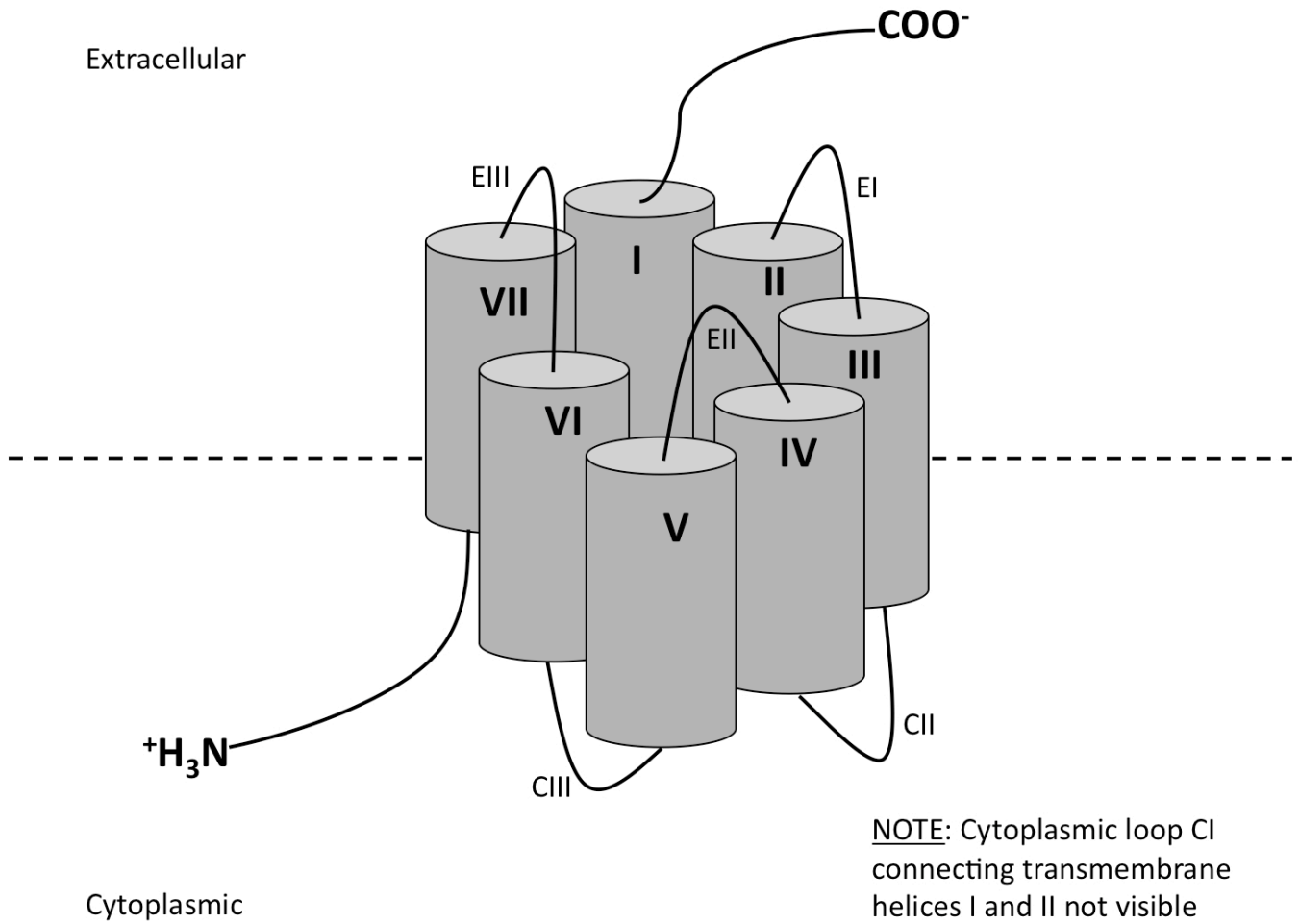
Figure 4. Cartoon representation of a G protein coupled receptor (GPCR). Adapted from

Palczewski *et al.*, (2000).

1.4.1 GPCR Regulation

Regulation of GPCR signaling is achieved at various levels. At the intracellular level, $G\alpha$ subunits of G proteins are able to hydrolyze GTP to GDP resulting in termination of signaling at and the reassociation of β/γ sunbunits. This process is usually slow, but can be sped up by a family of proteins called regulators of G protein signaling (RGS), which increase the rate of GTP hydrolysis of $G\alpha_{i/o}$ and $G\alpha_q$ alpha subunits (Ross and Wilkie, 2000).

Signal regulation can also occur at the level of GPCRs, which are subject to desensitization, internalization, and downregulation. Homologous desensitization occurs when a ligand desensitizes its own receptor. This process involves the phosphorylation of an activated receptor's intracellular regions by GRKs and the recruitment of arrestin proteins. The recruitment of arrestins sterically hinders further activation of downstream signaling, even in the presence of agonist (Pierce *et al.*, 2002). Heterologous desensitization occurs when activation of a receptor causes the desensitization of other receptors in the same cell. This may be a feedback mechanism mediated by kinases in downstream signaling pathways (Ferguson, 2001).

Arrestin proteins can also promote receptor internalization through the recruitment of beta-adaptin and clathrin proteins, promoting endocytosis. Internalization of a receptor removes it from the cell surface and eliminates access to ligand, although signaling events can still be initiated by an internalized receptor (Laporte *et al.*, 2002). An internalized receptor is processed based on the extent of phosphorylation and modification, and either recycled to the cell surface or subject to lysosomal degradation (Claing *et al.*, 2002).

1.4.2 GPCR Oligomerization

Although classical models of GPCR signaling focus on activation of a monomeric receptor, it has been demonstrated that receptor oligomerization is a critical aspect of GPCR function. Receptors may exist in simple dimers or in higher order complexes to exert cooperative effects. Such cooperativity has profound effects on signaling and regulation of GPCRs (George *et al.*, 2002).

Receptors can oligomerize with like receptors (homo-oligomerization) or different receptors (hetero-oligomerization), a process that likely occurs during protein synthesis and folding. Monomers, dimers, and higher-order oligomers are selective for different ligands, and can activate different pathways depending on the receptors involved in the complex (Milligan, 2009). GPCR oligomerization can influence agonist binding, modulation of partner receptors, and association of proteins and signaling partners (Maurice *et al.*, 2011).

Cells regulate and promote the association of specific receptors for the formation of complexes that regulate cell-specific signaling events. Oligomeric complexes can signal through very different pathways than their constituent receptors (George *et al.*, 2002). For example, dopamine D1 and D2 receptors are known to form a functional heterodimer (Dziedzicka-Wasylewska *et al.*, 2006). These two receptors, which couple to $G\alpha_s$ and $G\alpha_{i/o}$, respectively, act together as a dimer to modulate calcium-dependent signaling by coupling to $G\alpha_q$ (Hasbi *et al.*, 2009).

1.5   Dopamine Receptors

Dopamine receptors are a family of GPCRs found throughout the body, which bind the neurotransmitter dopamine. In the brain they are involved in several key neurological functions including mood, emotion, cognition, movement, reward, and prolactin secretion (Beaulieu and Gainetdinov, 2011). Dopamine receptors are also found throughout the vascular system where they control contraction and relaxation of endothelial smooth muscle, and in the kidney where they modulate excretion of various ions (Velasco and Luchsinger, 1998).

There are 5 different dopamine receptors that can be placed into two categories: D1-like and D2-like. D1-like receptors, which include D1 and D5 receptors, are entirely postsynaptic, couple to $G\alpha_s$ to stimulate adenylyl cyclase, and increase the production of cAMP (Beaulieu and Gainetdinov, 2011). In the brain, D1-like receptors are highly expressed in regions controlling movement, cognition, and reward, with D1 receptors being expressed at much higher levels than D5 receptors (Beaulieu and Gainetdinov, 2011).

D2-like receptors, which include D2, D3, and D4 receptors, can be pre- or post-synaptic, and inhibit adenylyl cyclase via coupling to $G\alpha_{i/o}$ (Beaulieu and Gainetdinov, 2011). D2 receptors can be further divided into short (D2S) and long (D2L) splice variants. D2L is a classical postsynaptic receptor, while D2S and D3 receptors are predominantly presynaptic autoreceptor that controls the synthesis, storage, and release of dopamine. The expression of D3 receptors is limited to the limbic regions, such as the striatum and nucleus accumbens (NAc) (Beaulieu and Gainetdinov, 2011).

The combined effects of D1 and D2 receptor stimulation influences several key brain processes. Dopamine plays a critical role in locomotor activity (mediated by D1, D2, and D3 receptors in limbic regions) and learning and memory (mediated by D1 and D2 receptors) (Beaulieu and Gainetdinov, 2011). This thesis will focus on dopamine D2 receptors and their known role in schizophrenia.

1.5.1 Dopamine D2 Receptors

Dopamine D2-like receptors are very highly expressed in the mesolimbic regions of the brain, including the striatum and the NAc (Beaulieu and Gainetdinov, 2011). These receptors have been historically linked to the G protein $G\alpha_{i/o}$, the inhibition of adenylyl cyclase, and modulation of various ion channels. However, this quick signal is only the first response to D2 receptor stimulation, lasting only minutes (Mailman and Murthy, 2010).

The second response is much slower, taking hours, and involves G protein-independent activation of glycogen synthase kinase 3 (GSK3)-mediated events (Beaulieu and Gainetdinov, 2011). Activated D2 receptors recruit beta-arrestin 2, which in turn recruits protein kinase B (PKB). Protein phosphatase 2A (PP2A) is also recruited to cleave phosphate groups from and inhibit PKB. Since PKB phosphorylation is required for phosphorylation and inhibition of GSK, activation of D2 receptors prevents the inhibition of GSK3, and frees up this kinase to initiate gene transcription events (Mailman and Murthy, 2010).

However, the response of dopamine D2 receptors is complex, and also relies on differences between presynaptic and postsynaptic populations. Dopamine D2 receptors, unlike the D1-like family of dopamine receptors, undergo alternative splicing to yield

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two major splice variants, the short (D2S) and long (D2L) isoforms, which differ only in a 29 amino acid stretch on the third extracellular loop (Figure 5). Despite their high sequence homology, the two isoforms of dopamine D2 receptors have distinct functions and localization (Beaulieu and Gainetdinov, 2011).

The D2S and D3 receptor isoforms are almost exclusively presynaptic, acting as autoreceptors to regulate dopamine synthesis, storage, and release at presynaptic terminals. D2L, on the other hand, acts as a classical neurotransmitter receptor on postsynaptic terminals (Beaulieu and Gainetdinov, 2011). The balance of these receptors is not even as there are more presynaptic D2S and D3 autoreceptors than there are postsynaptic D2L receptors, which is why D2 receptor agonists have higher potencies on presynaptic terminals. This also explains why low concentrations of D2 agonists have a sedative effect, while higher concentrations stimulate the limbic regions (Meller *et al.*, 1987; Mailman, 2007; Mailman and Murthy, 2010).
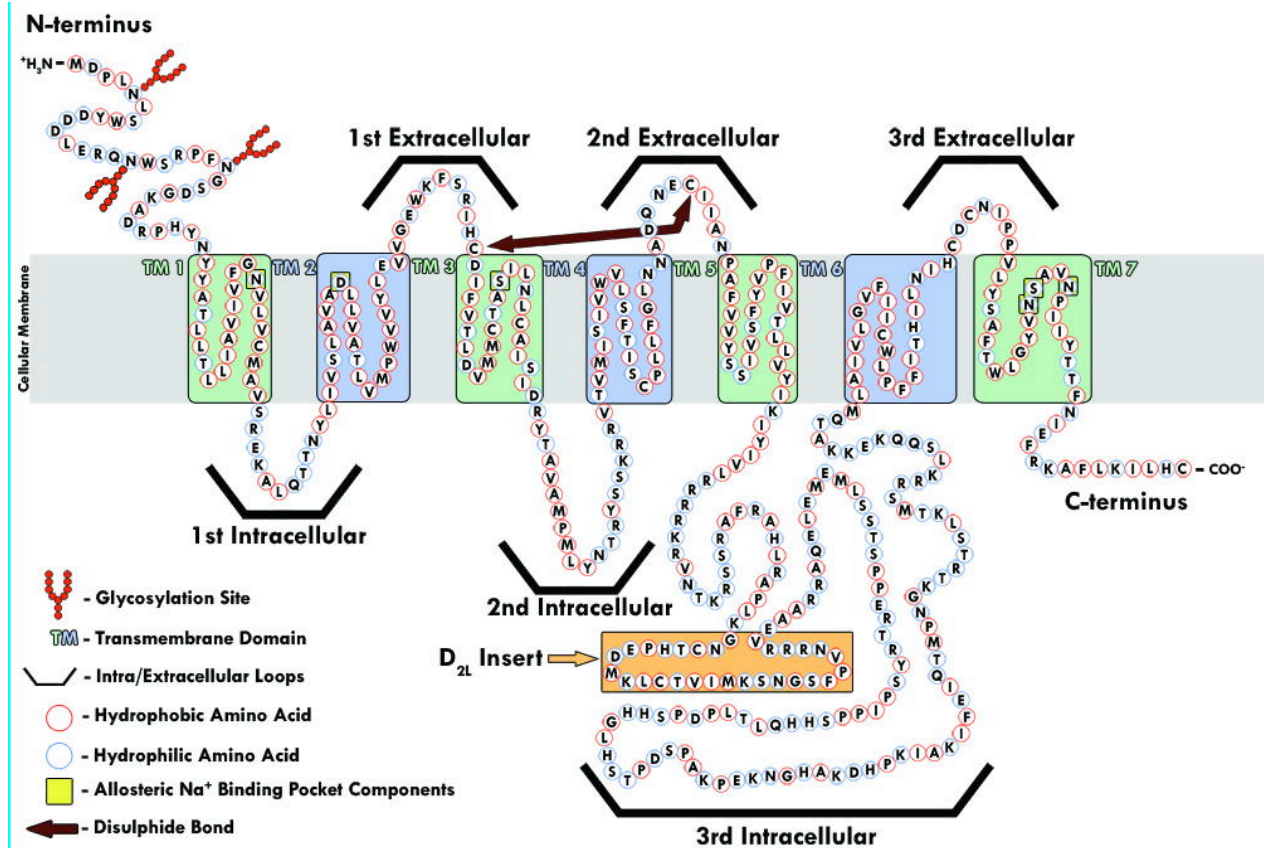
Figure 5. The sequence and structure of dopamine D2 receptors. String diagram of showing amino acid composition and structural features of dopamine D2S and D2L receptors, including differences in sequence, and glycosylation sites. Figure appears courtesy of Kevin Skoblenick.

1.6   Dopamine Hypothesis of Schizophrenia

One widely accepted theory that attempts to explain the link between schizophrenia and modified brain function is the DA hypothesis. This hypothesis, which originated from the observation that positive symptoms could be alleviated by D2 receptor blockade (Stone *et al.*, 2007), states that psychosis is a result of dysregulated dopamine signaling. It is believed that patients with schizophrenia have alterations in dopamine regulation, culminating in hyperdopaminergic signaling in the striatum and hypodopaminergic signaling in the PFC (Seeman, 1980; Howes and Kapur, 2009).

There appears to be a link between mesolimbic dopaminergic supersensitivity and the positive symptoms of schizophrenia, which explains why treatment of these symptoms is possible with dopamine receptor antagonists (Miyake *et al.*, 2010). Several lines of evidence point to striatal hyperactivity in schizophrenia. Studies have demonstrated that dopamine synthesis is increased in patients with schizophrenia (Reith *et al.*, 1994; McGowan *et al.*, 2004). This finding has been replicated in drug-naïve (Hietala *et al.*, 1999) and prodromal patients (Howes *et al.*, 2009). Additionally, *in vivo* D2 receptor radioligand displacement assays have demonstrated that dopamine release in the striatum is exaggerated in response to the dopamine-releasing agent amphetamine in patients with schizophrenia (Laruelle *et al.*, 1996; Breier *et al.*, 1997; Abi-Dargham *et al.*, 1998). The relationship between schizophrenia and striatal dopamine release has since been confirmed in drug-naïve patients (Abi-Dargham *et al.*, 2009), and appears to be related to disease phases, as dopamine release is even more sensitive to amphetamine during psychotic episodes (Laruelle *et al.*, 1999). Furthermore, striatal responses to wanting behaviour and reward mediated by mesolimbic pathways, are also altered in

schizophrenia (Barch and Dowd, 2010).

There also appear to be changes in dopamine D2 receptor physiology in schizophrenia, as researchers have reported increased dopamine D2 receptor occupancy, indicating dopamine D2 receptor expression in schizophrenia (Abi-Dargham *et al.*, 2000). There may also be changes in receptor affinity states, with increased proportions of D2 receptors in a high affinity state in schizophrenia (Seeman *et al.*, 2006). These findings, along with the increased synthesis and release of dopamine, have led researchers to believe that the disease is marked with more dopamine release and more dopamine D2 receptors in the striatum (Seeman and Kapur, 2000), causing increased dopamine neurotransmission (Figure 6).

In contrast to striatal hyperactivity, hypodopaminergic function in the PFC has been linked to the negative and cognitive aspects of the disease (Abi-Dargham and Moore, 2003), which explains why simple dopamine receptor antagonism does not improve these symptoms (Miyake *et al.*, 2010). Evidence suggests that decreased dopamine D1 receptor expression in the PFC may contribute to decreased cognitive function (Okubo *et al.*, 1997). However, it has recently been proposed that deficits in cognition and hypofrontality may be a result of dysregulated striatal-cortical circuitry involved in cognition (Simpson *et al.*, 2010).

Normal Striatal Dopamine Activity

Tyr → L-DOPA → Dopamine

Presynaptic Neuron

Postsynaptic Neuron

Striatal Dopamine Activity in Schizophrenia

Tyr → L-DOPA → Dopamine

⇧ Dopamine Synthesis

Presynaptic Neuron

⇧ Dopamine Release

⇧ Dopamine D2 Receptor Expression

Postsynaptic Neuron
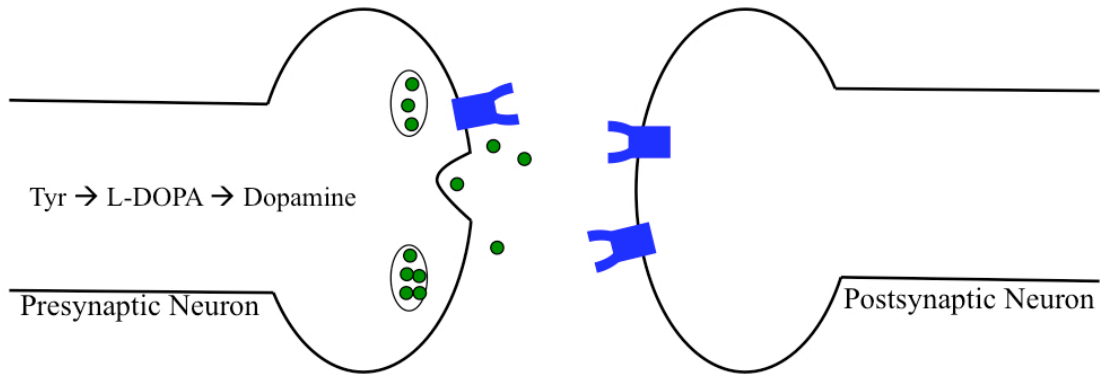
● Dopamine

Dopamine D2 Receptor
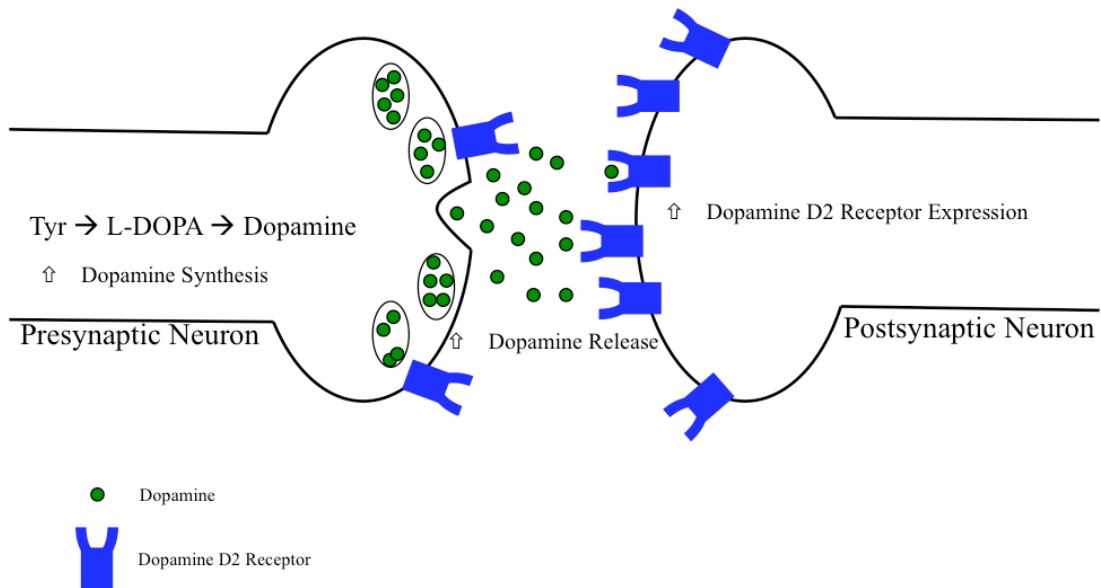
Figure 6. Striatal dopamine activity in schizophrenia. Schizophrenia is characterized by increased dopamine synthesis, increased dopamine release, and increased dopamine D2 receptor expression in the striatum. These all contribute to mesolimbic dopamine supersensitivity.

1.6.1 Potential Role of The Striatum in Negative and Cognitive Aspects of Schizophrenia

There are several direct and indirect connections between the striatum and the PFC, which together mediate working memory and executive function (Simpson *et al.*, 2010). Therefore, cortical dopamine neurotransmission and function can be influenced by dysregulated striatal neurotransmission. This is believed to be a large factor in the cognitive and negative aspects of the disease (Miyake *et al.*, 2010; Simpson *et al.*, 2010). As proposed by Simpson *et al.*, (2010), Figure 7 depicts three striatal-cortical circuits which could potentially be disrupted in schizophrenia.

Pathway #1 involves direct projections from the PFC to the striatum. Excessive activity in the striatum could disrupt information coming in directly from the PFC. Striatal-cortical loops modulate cognitive processes such as working memory, so disruption in these loops could have a negative impact on cognitive function. Pathway #2 involves indirect striatal-cortical connections through the VTA. Neurons projecting from the striatum feed back to the VTA and control firing of the dopaminergic neurons of the mesocortical pathway. Hyperdopaminergic signaling in the striatum could alter neurotransmission between the striatum and the VTA, and subsequently cause changes in dopaminergic projections extending from the VTA to the PFC. Finally, Pathway #3 involves another indirect connection between the striatum and the PFC through the substantia nigra (SN) and the thalamus. Striatal neurons project onto GABAergic neurons of the SN, which in turn project to the PFC via the thalamus. Excessive dopamine signaling in this pathway, like the other two proposed mechanisms, would feed back to the PFC and alter cortical neurotransmission.

Pathway #1: Prefrontal Cortex - Striatum
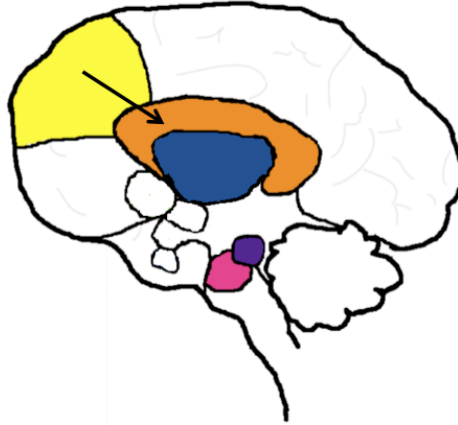
Pathway #2: Striatum - Ventral Tegmental Area - Prefrontal Cortex
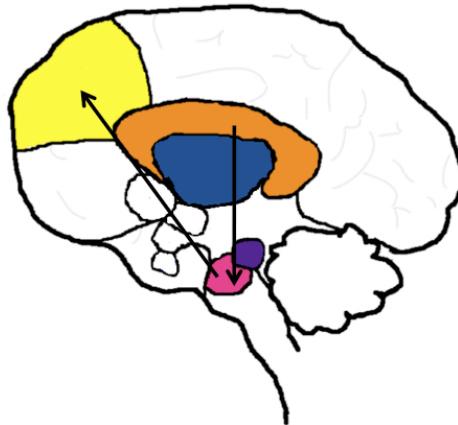
Pathway #3: Striatum - Substantia Nigra - Thalamus - Prefrontal Cortex

Legend

Prefrontal Cortex

Striatum

VTA

Substantia Nigra

Thalamus
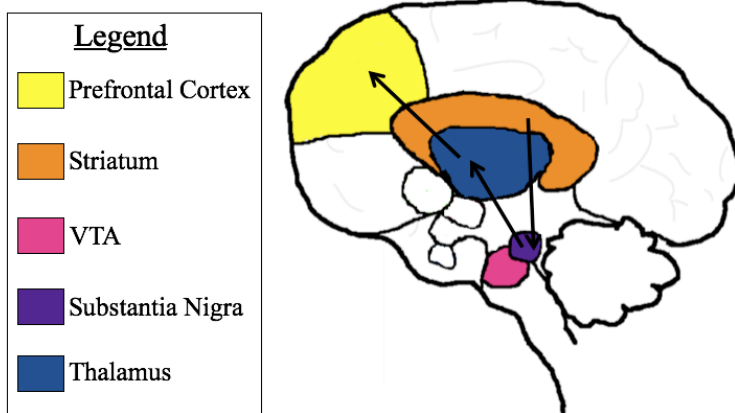
Figure 7. Neuronal circuitry connecting the striatum and prefrontal cortex. These pathways have been proposed to be altered in schizophrenia, leading to the cognitive and negative aspects of the disease. Adapted from Simpson *et al.*, (2010).

1.7 Amphetamine Model of Schizophrenia

The amphetamine-sensitized rodent model is a widely accepted and well-studied animal model of schizophrenia because it induces behavioural and biochemical abnormalities similar to those observed in the disease state. This model was first tested based on the observation that chronic amphetamine users often exhibit psychotic-like symptoms that are indistinguishable from paranoid schizophrenia. Therefore, dopaminergic supersensitivity likely occurs via similar mechanisms of excessive stimulation of dopamine D2 receptors in schizophrenia and as a result chronic amphetamine use (Seeman and Kapur, 2000; Peleg-Raibstein *et al.*, 2009).

Amphetamine has a complex pharmacology, although its effects on the synthesis, metabolism, and synaptic levels of dopamine in the striatum can explain how it mimics dopaminergic states in schizophrenia. (1) Amphetamine causes an increase in dopamine synthesis in presynaptic terminals through the activation of TH, the rate-limiting enzyme in dopamine synthesis. (2) Amphetamine is able to slow down dopamine metabolism by inhibiting MAO. (3) Amphetamine interacts with DAT and reverses the transport of dopamine, causing a flood of synaptic dopamine (Sulzer, 2011).

A sensitization regimen involving repeated, intermittent administration of escalating doses of amphetamine followed by a withdrawal period has been shown to induce schizophrenic-like mesolimbic dopaminergic supersensitivity (Robinson and Becker, 1986; Paulson *et al.*, 1991; Tenn *et al.*, 2003; Tenn *et al.*, 2005; Peleg-Raibstein *et al.*, 2006). Following a withdrawal period, amphetamine-sensitized animals express a number of schizophrenic-like behavioural abnormalities, including deficits in prepulse inhibition (PPI) (Tenn *et al.*, 2003; Tenn *et al.*, 2005; Peleg-Raibstein *et al.*, 2006). PPI

is commonly used as a robust indicator of schizophrenic-like behaviour, as patients with the schizophrenia consistently display deficits in sensorimotor gating behaviour (Grillon *et al.*, 1992).

Upon acute amphetamine challenge, amphetamine-sensitized animals also exhibit increased locomotor activity, as well as increased stereotyped behaviour such as sniffing and limb movement (Robinson and Becker, 1986; Paulson and Robinson, 1991; Tenn *et al.*, 2003). Although patients with schizophrenia do not exhibit increased locomotor activity, it is generally viewed that increased locomotor activity is indicative of positive symptoms in animal models. Studies have also reported deficits in attentional set shifting (Fletcher *et al.*, 2005; Featherstone *et al.*, 2008) and visual attention (Fletcher *et al.*, 2007), indicating that the amphetamine model of psychosis may also mimic the cognitive impairment observed in schizophrenia. Some studies also suggest that amphetamine sensitization may induce deficits in social behaviour in the rat (Gambill and Kornetsky, 1976; Ellison *et al.*, 1978; Beatty *et al.*, 1984; Steinpreis *et al.*, 1994), although this finding remains controversial (Sams-Dodd, 1995; Sams-Dodd, 1998). For a comparison of behavioural abnormalities observed in four preclinical animal models of schizophrenia, see Table 1.

Behavioural abnormalities observed in the amphetamine-sensitized model correlate with biochemical changes. Chronic amphetamine exposure leads to increased levels of D2^{High} receptors (Seeman, 2009a), which can be normalized following treatment with the typical antipsychotic drug haloperidol (Seeman, 2009b). Acute amphetamine challenge also causes exaggerated dopamine release and D2 receptor occupancy in the striatum of rats (Paulson *et al.*, 1991), much like in human patients (Breier *et al.*, 1997;

Abi-Dargham *et al.*, 2000). Furthermore, resting levels of striatal dopamine in the rat

have been shown to decrease *in vivo* over the course of chronic amphetamine treatment

(Cass *et al.*, 1989), much like human subjects following chronic methamphetamine

exposure (Wilson *et al.*, 1996).

Table 1. Comparison of behavioural abnormalities observed in four precinical animal models of schizophrenia.

Preclinical Animal Model	Sensorimotor Gating	Social Interaction	Locomotor Activity
Amphetamine	Tenn *et al., (*2003) Tenn *et al.*, (2005) Peleg-Raibstein *et al.*, (2006)	*Deficit* Gambill and Kornetsky, (1976) Ellison *et al.*, (1978) Beatty *et al.*, (1984) Steinpreis *et al.*, (1994) *No Change* Sams-Dodd, (1995) Sams-Dodd, (1998)	Robinson and Becker, (1986) Paulson and Robinson, (1991) Tenn et *al.*, (2003) Tenn et *al.*, (2005)
NDMA Receptor Antagonist (PCP, MK-801)	Bast *et al.*, (2000) Linn and Javitt, (2001) Li *et al.*, (2011)	Sams-Dodd, (1995) Sams-Dodd, (1998) Audet *et al.*, (2009) Dyck *et al.*, (2011)	Sams-Dodd, (1995)
Neonatal Ventral Hippocampus Lesion	Le Pen and Moreau, (2002)	Silva-Gomez *et al.*, (2003) Flores *et al.*, (2005)	Silva-Gomez *et al.*, (2003)
Maternal Immune Activation	Wolff and Bilkey, (2010)	Smith *et al.*, (2007)	*No Data*

1.8 Allosteric Modulators for the Treatment of Schizophrenia

Allosteric modulators are ligands that do not bind to the same site as endogenous ligand, known as the orthosteric site. Instead, these compounds bind elsewhere on the receptor and induce conformational changes that may influence either the orthosteric site or coupling to signaling molecules (Figure 8). Allosteric ligands may also favour specific signaling pathways in a functionally selective manner. While these mechanisms can occur independently or cooperatively, the most commonly observed effect of allosteric modulation is the modulation of orthosteric ligand binding affinity (Wang et al., 2009)

Allosteric modulators are highly specific for their target and do not compete with endogenous ligand. Furthermore, their activity is entirely dependent on physiological signaling, creating a ceiling to their effect (Conn *et al.*, 2009). By inducing conformational changes, allosteric modulators alter how their target receptor will respond to endogenous ligand. Therefore, higher doses of an allosteric compound are more tolerable than orthosteric compounds (Conn *et al.*, 2009).

Allosteric modulators have recently emerged as promising candidates for the treatment of more complicated disease states, including mental disorders. Their unique properties and mechanism of action result in a safer alternative to conventional antipsychotics, which bind an orthosteric site. Several allosteric modulators have been proposed for the treatment of schizophrenia, by targeting the metabotrobic glutamate receptor subtypes mGluR2 (Galici *et al.*, 2006; Benneyworth *et al.*, 2007) and mGluR5 (Lecourtier *et al.*, 2007), nicotinic acetylcholine receptors (Buchanan *et al.*, 2008) as well as the muscarinic M4 receptor (Brady *et al.*, 2008; Chan *et al.*, 2008).

However, several of these compounds rely on indirect normalization of

dopaminergic neurotransmission for their desired pharmacological effect (Schilstrom *et al.*, 2007; Wang *et al.*, 2007; Gill *et al.*, 2011). Therefore it is conceivable that direct allosteric modulation of dopamine receptors would be more suitable for treating schizophrenia. However, few allosteric modulators of the dopamine receptors have been reported (Hoare and Strange, 1996; Hoare *et al.*, 2000; Soriano *et al.*, 2010), despite the close association between the dopamine D2 receptor and schizophrenia.
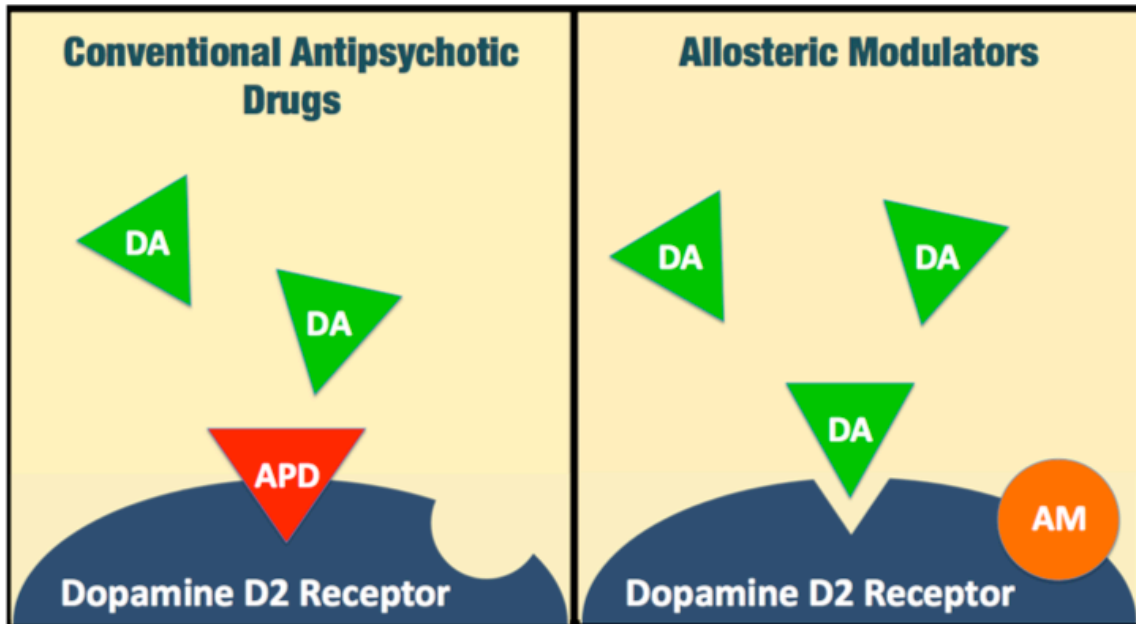
Figure 8. Comparison between conventional antipsychotic drugs and allosteric modulators. Conventional antipsychotic drugs (APD) are dopamine D2 receptor antagonists, and compete with endogenous dopamine (DA) for binding to the orthosteric site. In contrast, allosteric modulators (AM) bind elsewhere on the receptor and can influence the receptor's affinity to endogenous ligand in a more subtle, safer way.

1.8.1 PLG

L-prolyl-L-leucyl-glycinamide (PLG) (Figure 9), also known as melanocyte inhibiting factor, is an endogenous hypothalamic tripeptide first established as an inhibitor of the release of melanocyte stimulating hormone from the pituitary gland (Celis *et al.*, 1982). It has also been shown to potentiate the effects of L-DOPA (Huidobro-Toro et al., 1974) and temporarily improve symptoms of Parkinson's disease in clinical trials (Barbeau et al., 1976).

PLG interacts with the other drugs in a therapeutic manner. Its ability to antagonize morphine-induced catalepsy (Chiu and Mishra, 1979) and antipsychotic drug-induced dyskinesias (Chiu *et al.*, 1981; Bhargava, 1984; Chiu *et al.*, 1985; Mycroft *et al.*, 1987; Sharma *et al.*, 2003) highlight the therapeutic potential of this tripeptide. Based on the results of earlier clinical trials, other studies have focused on PLG's ability to alleviate behavioural abnormalities in animal models of Parkinson's disease, such as the 6-hydroxydopamine-lesioned model (Smith and Morgan, 1982; Mishra *et al.*, 1997) and MPTP-induced model (Sheng *et al.,* 1987; Marcotte *et al.*, 1998).

The pharmacological mechanism of PLG's actions began to unfold when it was discovered that PLG binds with a high affinity to human striatal tissue (Chiu *et al.*, 1983) and is able to reduce the sensitivity of striatal dopamine receptors induced by haloperidol treatment (Rajakumar *et al.*, 1987). More recent work has demonstrated that PLG in fact acts as a positive allosteric modulator of dopamine D2 receptors (Verma *et al.*, 2005), and is able to increase the dopamine D2 receptor's inhibitory effect on adenylyl cyclase (Mishra *et al.*, 1999). Interestingly, PLG has recently been shown to upregulate c-Fos expression in dopaminergic regions (Khan *et al.*, 2010), although it is also able to reduce

haloperidol-induced upregulation of c-Fos (Ott *et al.*, 2000). This raises the possibility

that modulation of D2 receptors via PLG may cause functionally selective signaling

through non-classical dopamine D2 receptor pathways, depending on cellular context and

dopaminergic tone.
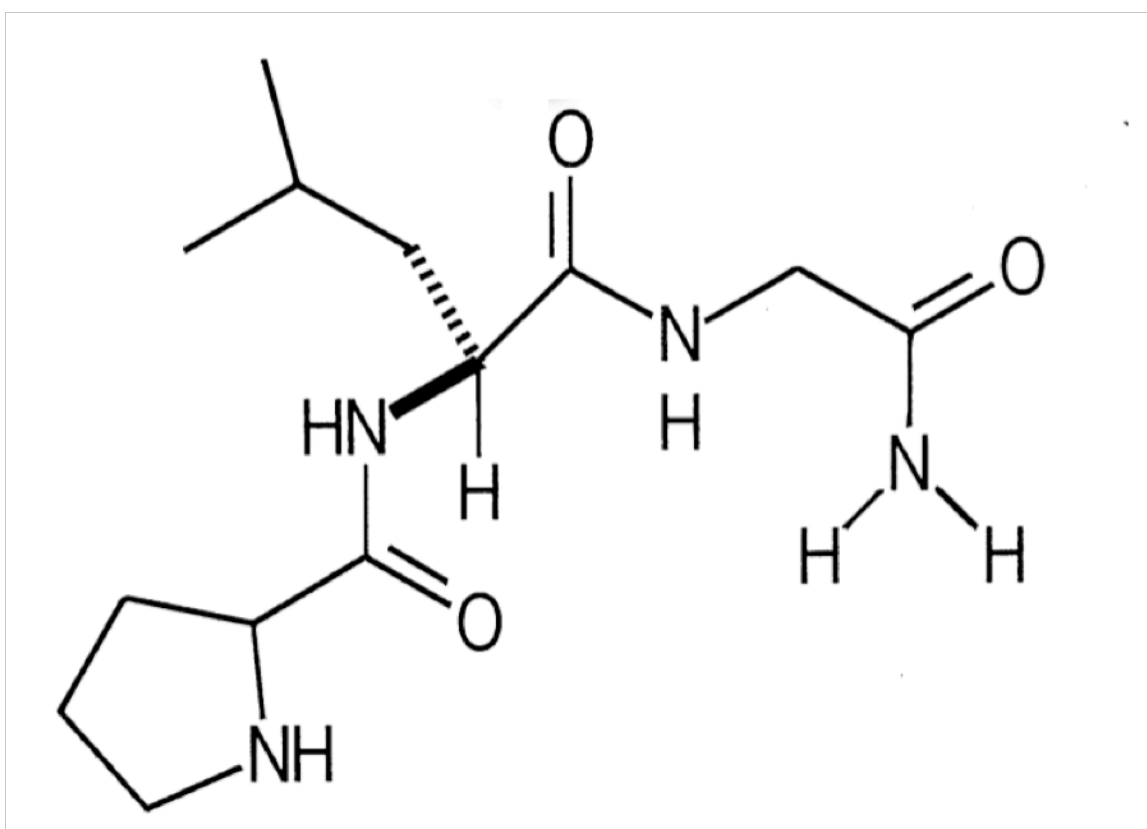
Figure 9. Chemical structure of endogenous tripeptide L-prolyl-L-leucyl-glycinamide (PLG).

1.9.2 PAOPA

The potential of PLG in preclinical models and clinical trials was hindered by the tripeptide's potency and half-life. Several analogues of PLG have since been synthesized and tested for their ability to modulate the dopamine D2 receptor *in vitro*. Among them, PAOPA (Figure 10) (3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide) is the most potent positive allosteric modulator.

PAOPA is able to allosterically enhance agonist binding to bovine and human D2Rs, while having no effect on antagonist binding (Mishra *et al.*, 1990; Verma *et al.*, 2005). By increasing agonist-induced GTPase activity, PAOPA is able to maintain D2Rs in high affinity states, ultimately causing increased inhibition of adenylyl cyclase activity through increased D2 receptor stimulation (Mishra *et al.*, 1999).

PAOPA is also able to increase dopaminergic sensitivity in the nigrostriatal pathway. This is presumably the mechanism by which PAOPA can prevent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and haloperidol-induced movement disorders (Marcotte *et al.*, 1998; Ott *et al.*, 2000; Sharma *et al.*, 2003) and modulate rotational behaviour in the 6-hydroxydopamine (6-OHDA) lesioned rat model of Parkinson's disease (Mishra *et al.*, 1997). Interestingly, PAOPA is also able to prevent deficits in social interaction in a model of schizophrenia induced by chronic exposure to the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (Dyck *et al.*, 2011), although the mechanism by which it improves schizophrenic-like behaviour is not yet known.
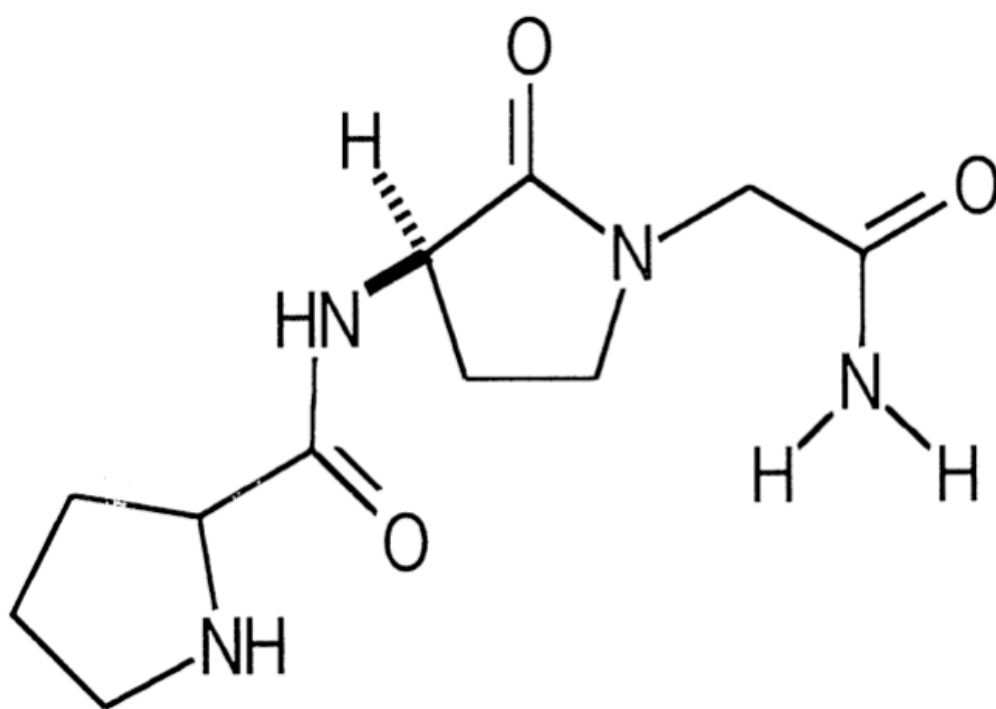
Figure 10. Chemical structure of the potent dopamine D2 receptor allosteric modulator PAOPA.

2   OBJECTIVES AND HYPOTHESES

The therapeutic potential of PAOPA has been demonstrated several times in animal models of Parkinson's disease (Marcotte *et al*., 1998; Mishra *et al*., 1997) and neuroleptic-induced neurological disorders (Ott *et al.*, 2000; Sharma *et al.*, 2003). Previous work from our lab has shown that PAOPA is also able to reverse negative symptoms in an MK-801-induced model of schizophrenia (Dyck *et al.*, 2011). However, to date, no studies have investigated PAOPA's potential in alleviating behavioural abnormalities following a rigorous amphetamine sensitization regimen.

Therefore, the overall objective of this study was to determine whether the dopamine D2 receptor allosteric modulator PAOPA is effective in preventing and reversing behavioural abnormalities in an amphetamine-sensitized model of schizophrenia. This was achieved by measuring behaviours in a battery of tests following amphetamine sensitization with and without concurrent PAOPA administration, and following administration of a dose of PAOPA to amphetamine-sensitized rats. Furthermore, biochemical abnormalities were explored by measuring post-mortem dopamine levels in all treatment groups to determine whether amphetamine sensitization disrupted dopamine levels in these key brain regions.

2.1   Effects of Amphetamine Sensitization and PAOPA Treatment on Prepulse Inhibition

Disruptions in prepulse inhibition (PPI) are a robust indicator of sensorimotor gating deficits that can be observed in patients with schizophrenia (Grillon *et al.*, 1992) and in several preclinical animal models including phencyclidine (PCP)- and MK-801-induced models (Bast *et al.*, 2000; Linn and Javitt, 2001; Li *et al.*, 2011), the neonatal

ventral hippocampus (nVH) lesioned model (Le Pen and Moreau, 2002), and the maternal immune infection model (Wolff and Bilkey, 2010). The amphetamine-sensitized model also induces robust deficits in sensorimotor gating (Tenn *et al.*, 2003; Tenn *et al.*, 2005), and was chosen to test the effects of PAOPA on this reproducible behavioural abnormality.

It was hypothesized that: (1) amphetamine sensitization would disrupt PPI; (2) administration of PAOPA concurrently with amphetamine would prevent disruptions in PPI; (3) PAOPA treatment alone would have no effect on PPI; and (4) PAOPA treatment following sensitization would reverse amphetamine-induced deficits in PPI.

2.2   Effects of Amphetamine Sensitization and PAOPA Treatment on Social Interaction

Negative symptoms in schizophrenia are measured in preclinical animal models as abnormalities in social interaction, and have been observed in the PCP- and MK-801-induced models (Audet *et al.*, 2009; Dyck *et al.*, 2011), the nVH lesioned model (Silva-Gomez *et al.*, 2003; Flores *et al.*, 2005), and the maternal immune infection model (Smith *et al.*, 2007). Although several researchers have demonstrated that amphetamine sensitization can induce deficits in social behaviour (Gambill and Kornetsky, 1976; Ellison *et al.*, 1978; Beatty *et al.*, 1984; Steinpreis *et al.*, 1994), this phenomenon remains controversial (Sams-Dodd, 1995; Sams-Dodd, 1998). To date there have been no reports measuring social interaction after a sensitization regimen followed by a withdrawal period. Therefore, the objectives of this experiments were to determine whether a sensitization regimen involving intermittent, escalating doses of amphetamine can induce

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deficits in social behaviour after a three week withdrawal, and whether PAOPA can prevent and reverse such deficits.

It was hypothesized that: (1) amphetamine sensitization would disrupt social interaction; (2) administration of PAOPA concurrently with amphetamine would prevent disruptions in social interaction; (3) PAOPA treatment alone would have no effect on social interaction; and (4) PAOPA treatment following sensitization would reverse amphetamine-induced deficits in social interaction.

2.3 Effects of Amphetamine Sensitization and PAOPA Treatment on Locomotor Activity

Although patients with schizophrenia do not generally display hyperlocomotor activity, this behavioural abnormality is used as a model of positive symptoms schizophrenia, because positive symptoms in humans and locomotor activity in rats are both related to mesolimbic dopaminergic sensitivity. Furthermore, several well-established preclinical animal models display increased locomotion upon challenges with dopamine D2 receptor agonists or dopamine releasing agents, including NMDA receptor antagonist models (Sams-Dodd, 1998), the nVH lesioned model (Silva-Gomez *et al.*, 2003), and the amphetamine model (Robinson and Becker, 1986; Paulson and Robinson, 1991; Tenn *et al.*, 2003). Therefore, the preventative effects of PAOPA on amphetamine-induced hyperlocomotion were examined.

It was hypothesized that: (1) amphetamine sensitization would cause increased locomotor activity following a saline challenge; (2) administration of a low dose of amphetamine to amphetamine-sensitized would exaggerate the increased locomotor activity; (3) administration of PAOPA concurrently with amphetamine would prevent

increases in locomotor activity; and (4) PAOPA treatment alone would have no effect on locomotor activity.

2.4 Effects of Amphetamine Sensitization on Post-Mortem Brain Dopamine Levels

Changes in dopamine levels have been shown in human brain tissue following chronic methamphetamine use (Wilson *et al.*, 1996), and in rats following exposure to amphetamine (Cass *et al.*, 1989). To assess whether behaviour abnormalities observed in amphetamine-sensitized rats were associated with changes in dopamine, high performance liquid chromatography was employed to determine post-mortem levels of dopamine in three brain regions: the striatum, the NAc, and the medial prefrontal cortex (mPFC).

It was hypothesized that: (1) amphetamine sensitization would increase post-mortem dopamine levels in the striatum; (2) amphetamine sensitization would increase post-mortem dopamine levels in the NAc; (3) amphetamine sensitization would decrease post-mortem dopamine levels in the mPFC; (3) PAOPA treatment alone would have no effect on PPI; (4) administration of PAOPA concurrently during amphetamine sensitization would prevent changes in post-mortem dopamine levels; and (5) PAOPA treatment alone would have no effect on post-mortem dopamine levels.

3   METHODOLOGY

3.1   Animals

Male Sprague-Dawley rats were obtained at a weight of 250-300g from Charles River Laboratories (Wilmington, MA), and individually housed at the McMaster University Central Animal Facility (CAF) in accordance with the Canadian Council on Animal Care (CCAC). Male rats were chosen to avoid the effects of the female estrus cycle, and because previous research from our lab on PAOPA has used male rats. Animals were maintained at a 12 hour : 12 hour dark/light cycle, with *ad libidum* access to food and water. Prior to testing, animals were randomly divided into the following four groups for drug sensitization (n=10/group): Saline, Amphetamine, PAOPA, and Amphetamine+PAOPA. General health and weight of the rats were monitored daily.

3.2   Drugs

Saline solution (0.9%) was obtained from McMaster University's Health Sciences Stores. D-amphetamine was obtained from Sigma through McMaster University's CAF and prepared fresh in 0.9% saline each week as follows: 1mg/mL for week one, 2mg/mL for week two, and 3mg/mL for week three. PAOPA was synthesized by Dr. Rodney Johnson (University of Minnesota) as previously described (Yu *et al.*, 1988; Baures *et al.*, 1994) and prepared fresh as a 1mg/mL solution in saline each week. For the Amphetamine+PAOPA solutions, both D-amphetamine (1-3mg/mL) and PAOPA (1mg/mL) were prepared in the same solution as described above. Isofluorane was obtained from McMaster University's CAF.

3.3 Sensitization Regimen

The doses of amphetamine were adapted from Tenn *et al.*(2003), while the effective doses of PAOPA were determined from previous studies (Dyck *et al.*, 2011). All rats received 3 intraperitoneal (IP) injections on alternate days (Monday, Wednesday, Friday) over 3 weeks, for a total of 9 injections. Saline rats were administered 0.9% saline (1mL/kg). Amphetamine rats were administered escalating doses of D-amphetamine (1mg/kg for week one, 2mg/kg for week two, 3mg/kg for week 3). Amphetamine+PAOPA rats were administered identical escalating doses of D-amphetamine (1-3mg/kg) concurrently with 1mg/kg doses of PAOPA. PAOPA rats were administered PAOPA (1mg/kg). A 3-week drug withdrawal period followed the injections, during which the rats were not handled. To test whether PAOPA could reverse behavioural sensitization, amphetamine-sensitized rats were administered a single I.P. injection of PAOPA (1mg/kg). One hour following the injection, behavioural tests were again performed on PAOPA-treated amphetamine-sensitized rats. General health and weight gain were monitored daily. For a time line of sensitization and behavioural testing, see Figure 11.

3.4 Behavioural Tests

The following behavioural tests were performed: PPI, social interaction, and locomotor activity. To reduce stress on the animals, no two tests were performed on the same day.

3.4.1 Prepulse Inhibition

Startle responses were measured with the SR-Lab Startle Response System (San Diego Instruments, San Diego, CA). Methods and selection of startle and prepulse intensities were adapted from Tenn *et al.* (2003). Each rat was placed in the startle

apparatus for a 5min acclimatization period, with a 65-decibel (dB) background of white

noise. After this period, a series of five startle pulse-alone (110dB, 40ms) trials was

presented. This series of stimuli was followed by 65 randomized trials consisting of no

pulse (0dB, no additional stimuli other than background noise present), a startle pulse

(110dB, 40ms), one of three prepulse intensities (68dB, 71dB, or 77dB; 20ms) presented

100ms preceding the startle pulse, or one of three prepulse intensities alone. Another

series of five startle pulse-alone trials was presented. The time between trials ranged

from 10s to 20s with an average of 15s. Startle responses were measured every 1ms for a

100ms period after presentation of the startle stimulus.

3.4.2 Social Interaction

Methods were adapted from File (1980) and Sams-Dodd (1995). An open test

arena (100 x 100 x 40cm) constructed from smooth black polyvinyl chloride was used.

Two days prior to testing, each rat was habituated alone to the testing area for two 5-

minute trials. For the interaction experiment, rats were paired with their own treatment

group and by similar body weight (within 20 grams). Members of each pair were not

familiar with one another, as no two rats were ever paired more than once. Each pair was

placed in the arena for a 5-minute period. An interaction was defined as grooming,

sniffing, biting, fighting, playing, or a passive interaction during which rats were

touching or in close proximity, but not actively engaging one another. Rats were scored

for the total number of interaction episodes and the total interaction time in seconds.

3.4.3 Locomotor Activity

AccuScan computerized cages (AccuScan Instruments, Columbus, OH) were

utilized, and multidirectional movements were recorded by the computerized system.

Rats were familiarized with the system 3 days prior to the actual day of testing. On the

day of testing, rats were placed in the chambers for an hour of habituation, after which

the effects of saline (1mL/kg) and amphetamine (1mg/kg) challenge were tested and

recorded for one hour.

3.5 Sacrifice

Following behavioural testing, rats were left undisturbed for one week before

sacrificing. Rats were anaesthetized with isofluorane and decapitated in accordance with

McMaster University's Central Animal Facility (CAF). The brain was removed, and the

striatum, NAc, and mPFC were dissected out on ice and stored at -80°C until use.

3.6 Determination of Post-Mortem Brain Dopamine Levels

A Waters 2695 separations module coupled to a Waters 2465 electrochemical

detector (ECD) was used to determine dopamine levels in dissected striatal and nucleus

accumbens tissue from rats of each treatment group via high performance liquid

chromatography (HPLC). The mobile phase consisted of 50mM sodium acetate, 20mM

citric acid, 2mM sodium octyl sulfate, 1mM Di-N-butylamine, 100M

ethylenediaminetetraacetic acid, 4% methanol, and 2M potassium chloride. Tissue

samples were weighed and immediately placed on ice before being homogenized by hand

in ice-cold 0.1 M perchloric acid containing 2.5×10^{-4} mg/mL 2,3-Dihydroxybenzoic acid

(DHBA) as an internal standard. Homogenates were sonicated three times for three

seconds each time, and then centrifuged at 4°C for 20 minutes at 13000rpm.

Supernatants were collected and passed through a 0.2um glass fiber filter. Twenty (20)

μL of filtrates were injected into the HPLC-ECD system and passed through a Waters

Nova-Pak C-18 column (4μm; 3.9x150mm) at a flow rate of 1mL/minute. The

electrochemical detector current was set to 200nA for striatal samples, 50nA for nucleus accumbens samples, and 20nA for medial prefrontal cortex samples. The ratio of DA:DHBA peak area for each sample was compared to a set of standards and used to determine the mass of dopamine in the sample. Dopamine levels were expressed as nanograms of DA per milligram wet tissue (ng DA/mg tissue).

3.7   Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Before analyses, outlier detection was performed using the GraphPad Outlier Tool. Significance was defined as $p < 0.05$.

3.7.1   PPI

PPI data were analyzed according to Tenn *et al.* (2003). Percent PPI was calculated by the following formula: %PPI = 100 – (P+S)/S) * 100, where P+S is the mean response amplitude for prepulse-plus-startle pulse trials, and S is the mean response amplitude for the startle pulse-alone trials. To examine the effects of the sensitization regimen, %PPI results at each prepulse intensity were analyzed using a one-way analysis of variance (ANOVA) with by Tukey's post-hoc test. To examine the effects of PAOPA administration on amphetamine-sensitized rats, %PPI results from before and after PAOPA administration were analyzed by means of a t-test at each prepulse intensity.

3.7.2   Social Interaction

Analysis of social interaction was adapted from Dyck *et al.* (2011). Prior to analysis of social interaction recordings, the parameters defining an interaction were set. Rats were evaluated for each parameter by three scorers blind to treatment group. The number of interactions and time spent interacting for each rat were calculated by taking

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the average of all three scorers' observations. To examine the effects of the sensitization regimen, the total number of interactions and the total time spent interacting were analyzed by means of one-way ANOVA with Tukey's post-hoc test. To examine the effects of PAOPA administration on amphetamine-sensitized rats, the same parameters were compared before and after PAOPA administration and analyzed by means of a paired t-test.

3.7.3 Locomotor Activity

Locomotor activity was analyzed using total activity counts. To examine the effects of the sensitization regimen, activity counts for each 1-hour time period were analyzed across all treatment groups by means of one-way ANOVA followed by Tukey's post-hoc test.

3.7.4 Brain Dopamine Levels

Dopamine levels, as determined through HPLC, were expressed as nanograms of dopamine per milligram of tissue. To examine the effects of the sensitization regimen, dopamine levels across all treatment groups were analyzed by means of one-way ANOVA followed by Tukey's post-hoc test.
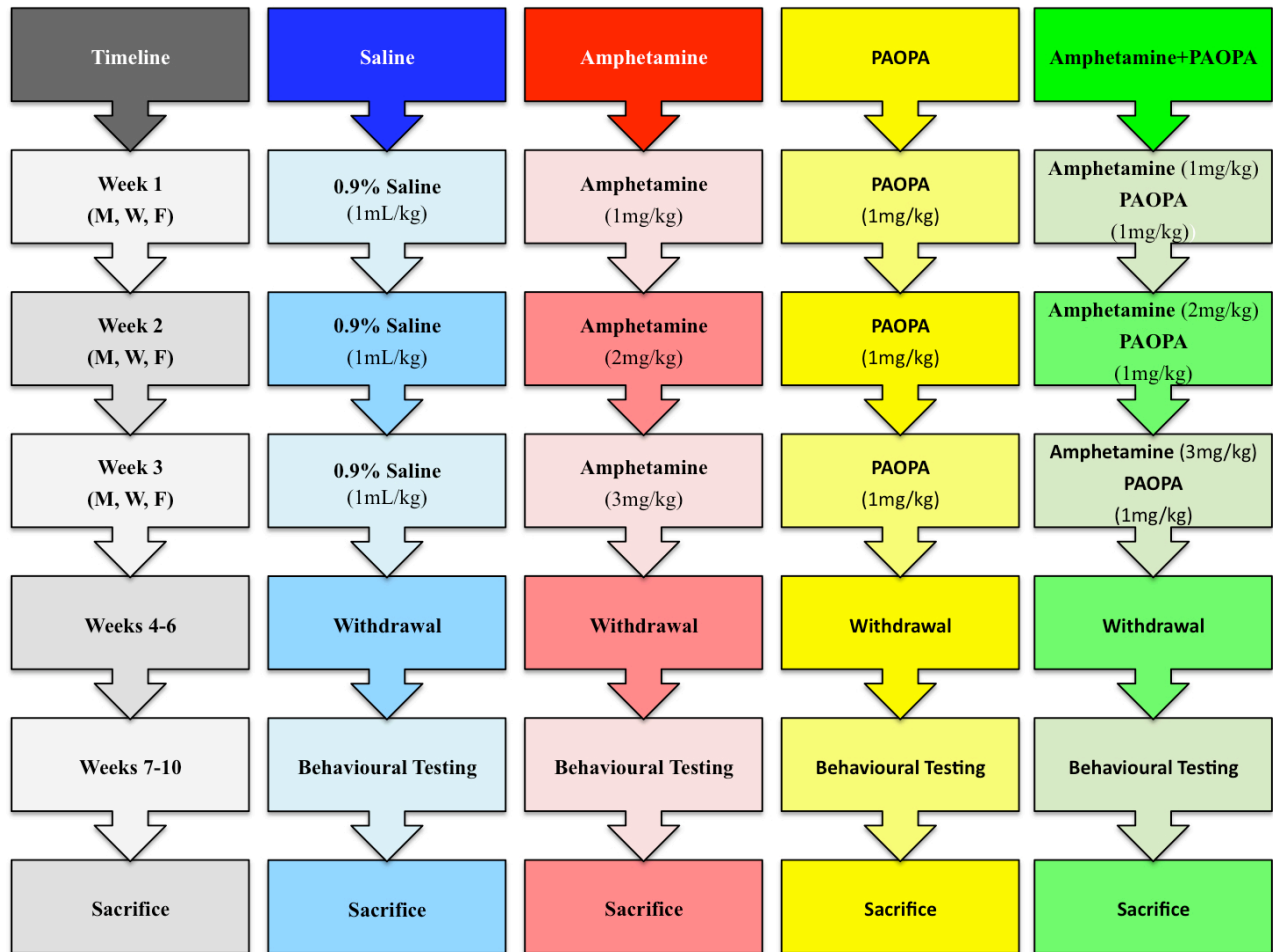
Figure 11. Timeline of experiments. Treatment groups were subjected to a sensitization regimen involving repeated, intermittent doses of their respective treatments from Week 1 through Week 3. Following a three-week sensitization, all groups were subjected to a three-week withdrawal period from Week 4 to Week 6. Behavioural testing took place during Weeks 7 through 10, and rats were left untouched for one week before they were sacrificed.

4 RESULTS

4.1 Effects of Amphetamine Sensitization and PAOPA Treatment on Pre-Pulse Inhibition

Following the 3-week sensitization and 3-week withdrawal periods, amphetamine-sensitized amphetamine-sensitized rats had a significant reduction in %PPI following a 71 dB prepulse when compared to saline-treated control rats (Figure 14). When PAOPA was administered concurrently with amphetamine, it was able to prevent the deficit in PPI (Figure 14). Rats treated chronically with PAOPA alone had no significant difference in PPI when compared to saline-treated rats (Figure 14). ANOVA results for the 71 dB prepulse intensity (Figure 14) are as follows: $F(3,32) = 4.771$; **$p = 0.0074$; Post-Hoc results: Saline vs. Amphetamine $p < 0.05$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.01$.

When amphetamine-sensitized rats were challenged with saline, the disruption in PPI at the 71 dB prepulse persisted. This deficit was reversed 1 hour following a single dose of PAOPA (1mg/kg) (Figure 15; ***$p = 0.0003$).
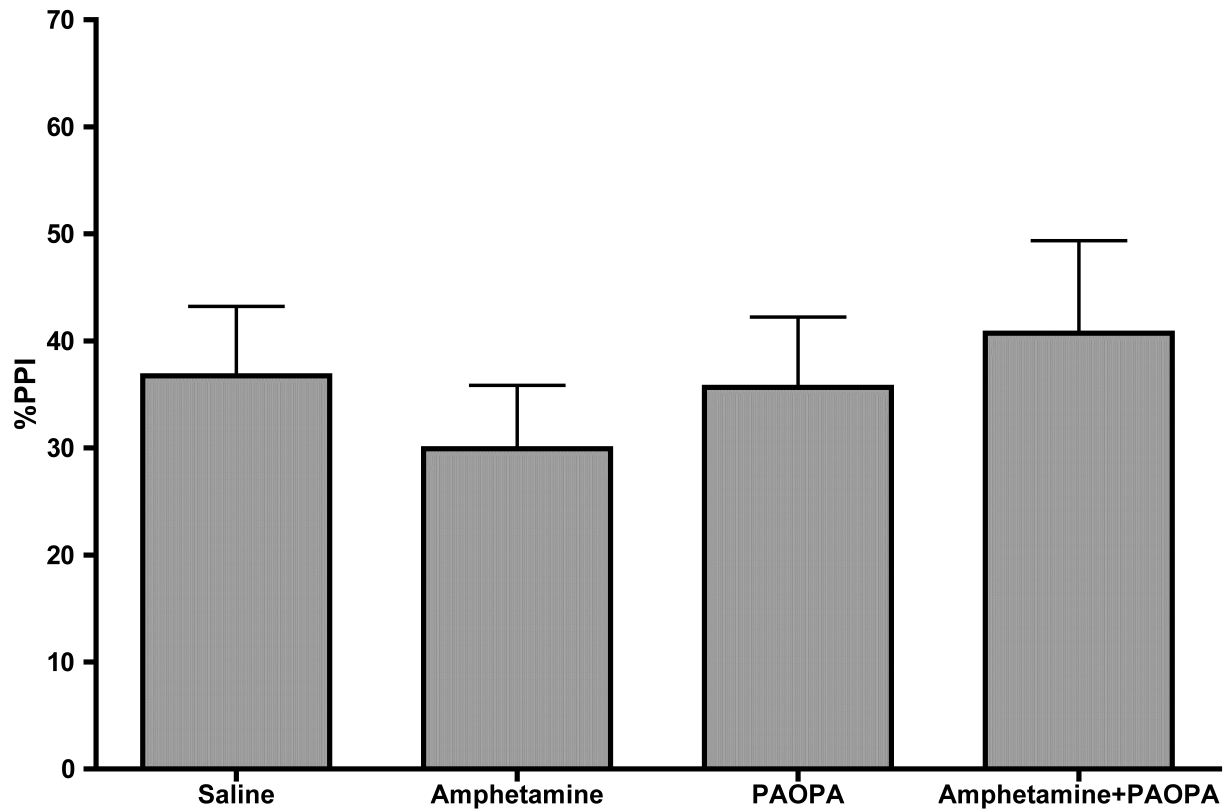
Figure 12. Effects of amphetamine and PAOPA on prepulse inhibition following a 68dB prepulse. Graph depicts %PPI (mean ± SEM) following a 68dB prepulse for each treatment group. $F(3,32) = 0.4178$; $p = 0.7414$.
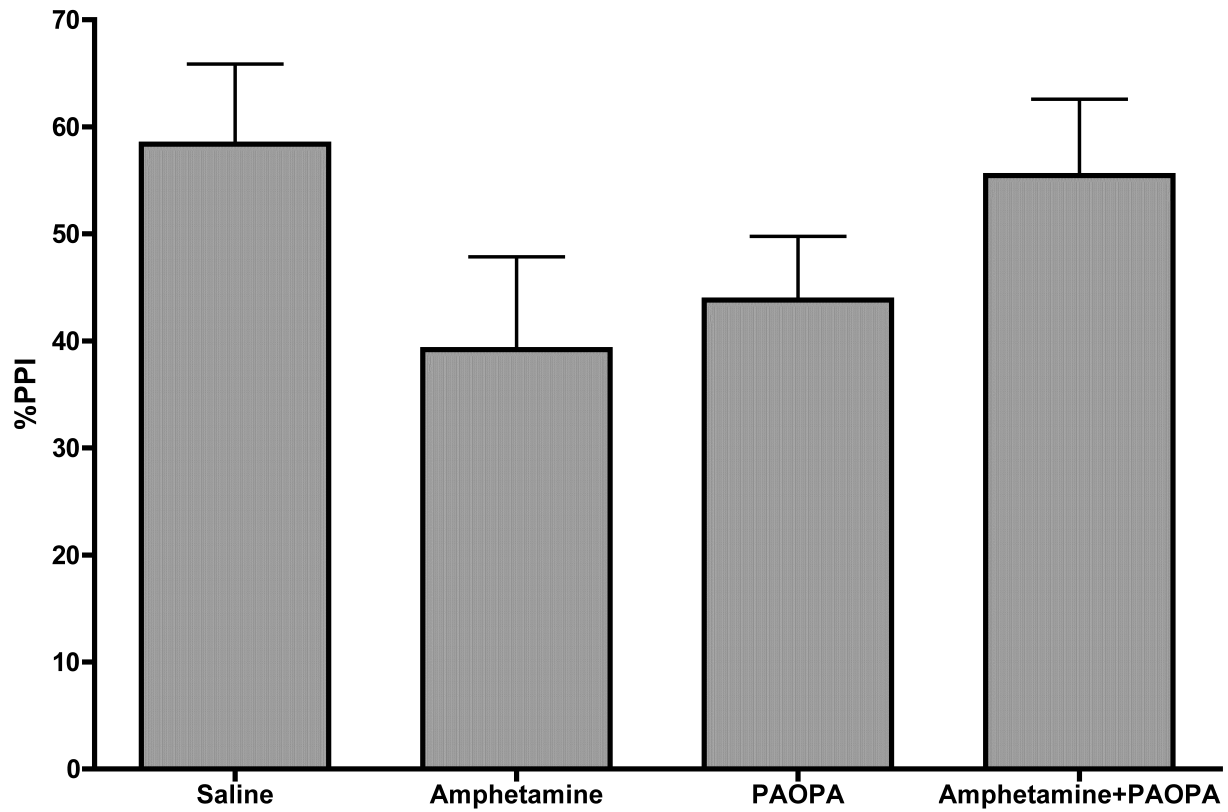
Figure 13. Effects of amphetamine and PAOPA on prepulse inhibition following a 77dB prepulse. Graph depicts %PPI (mean ± SEM) following a 77dB prepulse for each treatment group. $F_{(3,33)} = 1.536$; $p = 0.2236$.
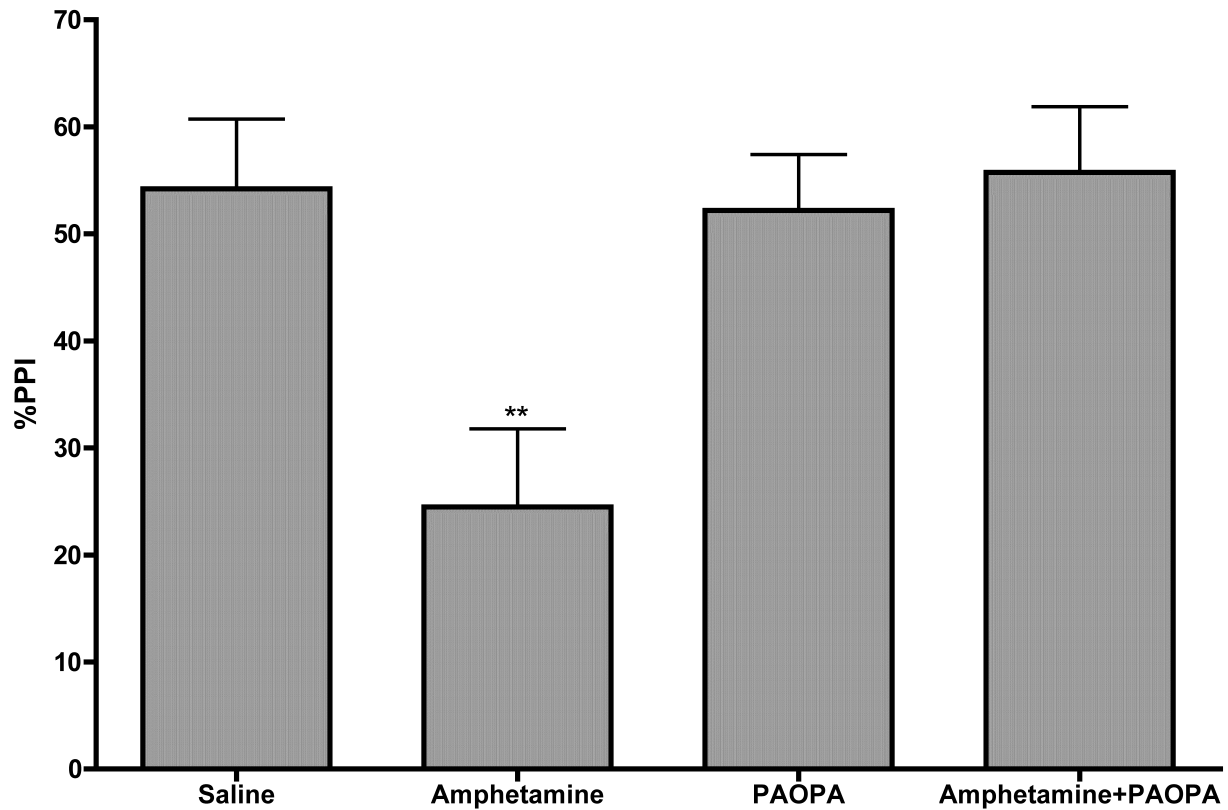
Figure 14. Effects of amphetamine and PAOPA on prepulse inhibition following a 71dB prepulse. Graph depicts %PPI (mean ± SEM) following a 71dB prepulse for each treatment group. Amphetamine sensitization significantly disrupted %PPI. Chronic PAOPA treatment had no effect on %PPI, while concurrent administration of PAOPA during amphetamine sensitization prevented the development of this behavioural abnormality. $F(3,32) = 4.771$; **$p = 0.0074$; Post-Hoc results: Saline vs. Amphetamine $p < 0.05$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.01$.
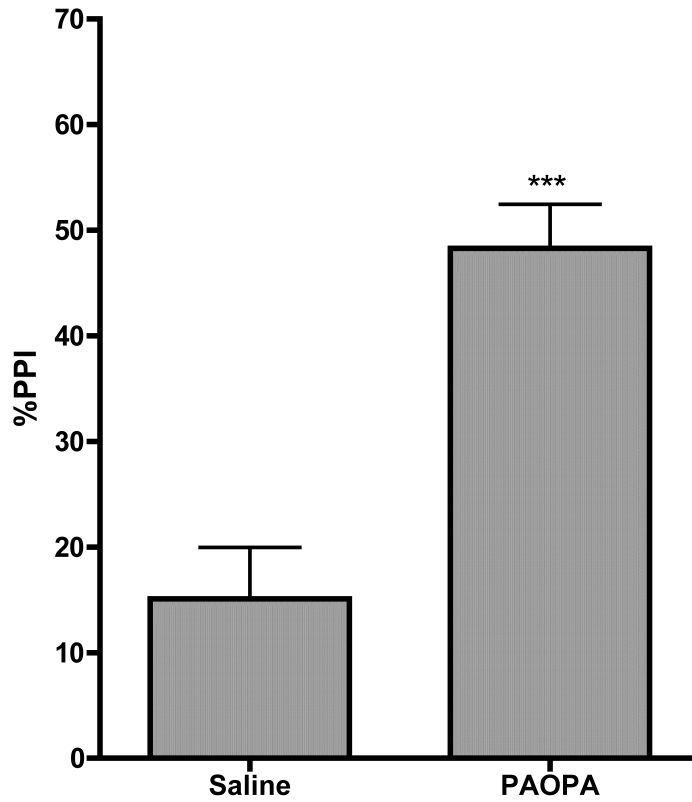
Figure 15. Effects of PAOPA treatment on prepulse of amphetamine-sensitized rats following a 71dB prepulse. Graph depicts %PPI (mean ± SEM) following a 71dB prepulse for amphetamine-sensitized rats one hour following administration of Saline (0.9%; 1mL/kg) and PAOPA (1mg/kg). PAOPA administration significantly increased %PPI at the 71dB prepulse intensity compared to saline. ***p = 0.0003.

4.2   Effects of Amphetamine Sensitization and PAOPA Treatment on Social Interaction

Amphetamine-sensitized rats engaged in fewer interaction episodes (Figure 16) and spent less time interacting (Figure 17) than saline-treated rats, indicating a deficit in social behaviour. Rats treated concurrently with amphetamine and PAOPA showed no deficit in either parameter (Figure 16; Figure 17). Rats treated with PAOPA alone showed no significant difference in the number of interactions (Figure 16) or the time total interaction time (Figure 17) when compared to saline-treated rats. ANOVA results for the number of interaction episodes (Figure 16) are as follows: $F(3,34) = 5.195$; $**p = 0.0046$; Post-Hoc results: Saline vs. Amphetamine $p < 0.05$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.05$. ANOVA results for the time spent in interaction (Figure 17) are as follows: $F(3,35) = 9.595$; $***p < 0.0001$; Post-Hoc results: Saline vs. Amphetamine $p < 0.01$, Amphetamine vs. PAOPA $p < 0.01$, Amphetamine vs. Amphetamine+PAOPA $p < 0.001$.

Finally, 1 hour following a single dose of PAOPA (1mg/kg), the social interaction deficit seen in amphetamine-sensitized rats was reversed, with an increase in the total number of interaction episodes (Figure 18 ; $***p < 0.0001$) and total interaction time (Figure 19 ; $**p= 0.0046$).
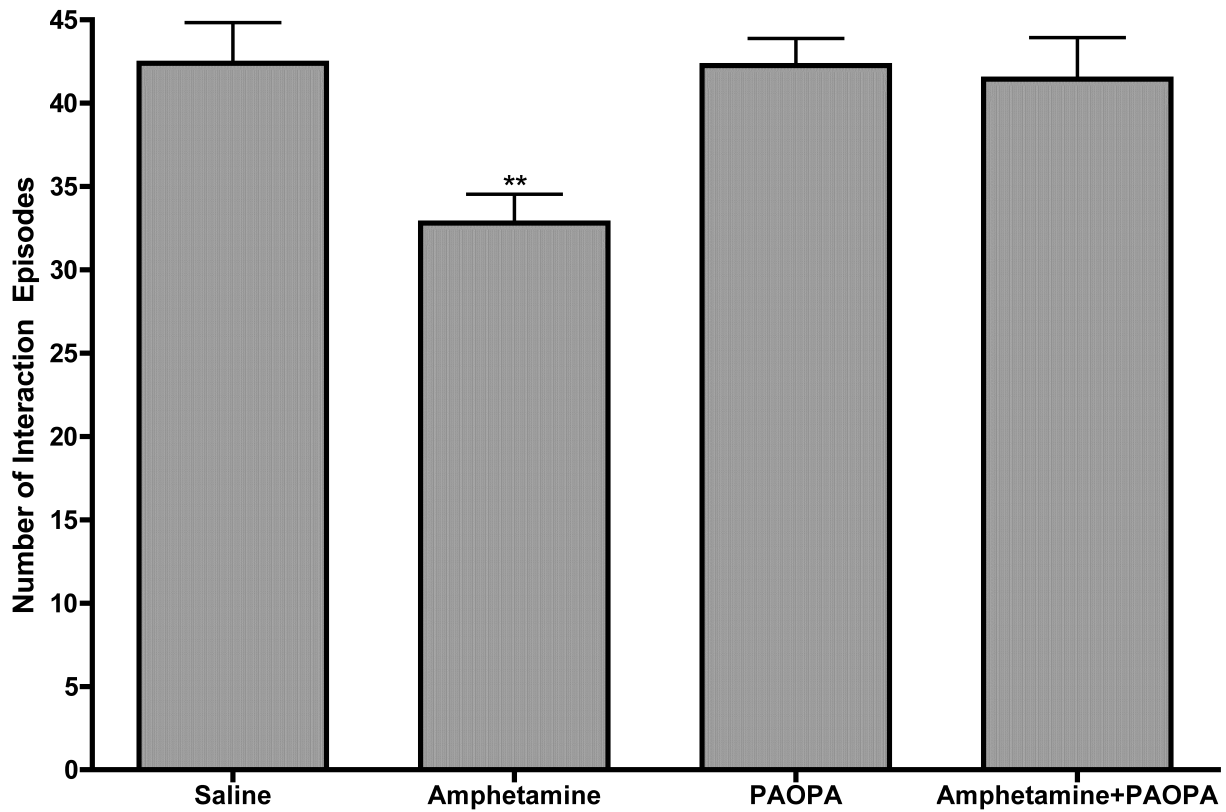
Figure 16. Effects of amphetamine and PAOPA on the number of interaction episodes. Graph depicts the number of interactions (mean ± SEM) that subjects of each treatment group engaged in during the 5-minute testing period. Amphetamine sensitized rats engaged in significantly fewer interactions than all other groups. PAOPA treatment alone had no effect on the number of interactions, and concurrent PAOPA treatment during amphetamine sensitization prevented deficits in this behaviour. $F_{(3,34)} = 5.195$; $**p = 0.0046$; Post-Hoc results: Saline vs. Amphetamine $p < 0.05$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.05$.

Figure 17. Effects of amphetamine and PAOPA on time spent interacting. Graph depicts the total time (mean ± SEM) that subjects of each treatment group spent interacting during the 5-minute testing period. Amphetamine-sensitized rats spent significantly less time interacting than all other treatment groups. PAOPA treatment alone had no effect on the time spent interacting, and concurrent PAOPA treatment during amphetamine sensitization prevented a deficit in this behaviour. $F_{(3,35)} =$ 9.595; ***$p < 0.0001$; Post-Hoc results: Saline vs. Amphetamine $p < 0.01$, Amphetamine vs. PAOPA $p < 0.01$, Amphetamine vs. Amphetamine+PAOPA $p <$ 0.001.

Figure 18. Effects of PAOPA treatment on the number of interactions among amphetamine-sensitized rats. Graph depicts the number of interactions (mean ± SEM) that amphetamine-sensitized rats engaged in during the 5-minute testing period during baseline testing and one hour following PAOPA administration (1mg/kg). PAOPA treatment caused a significant increase in the number of interaction episodes in amphetamine-sensitized rats compared to baseline levels. ***p < 0.0001.
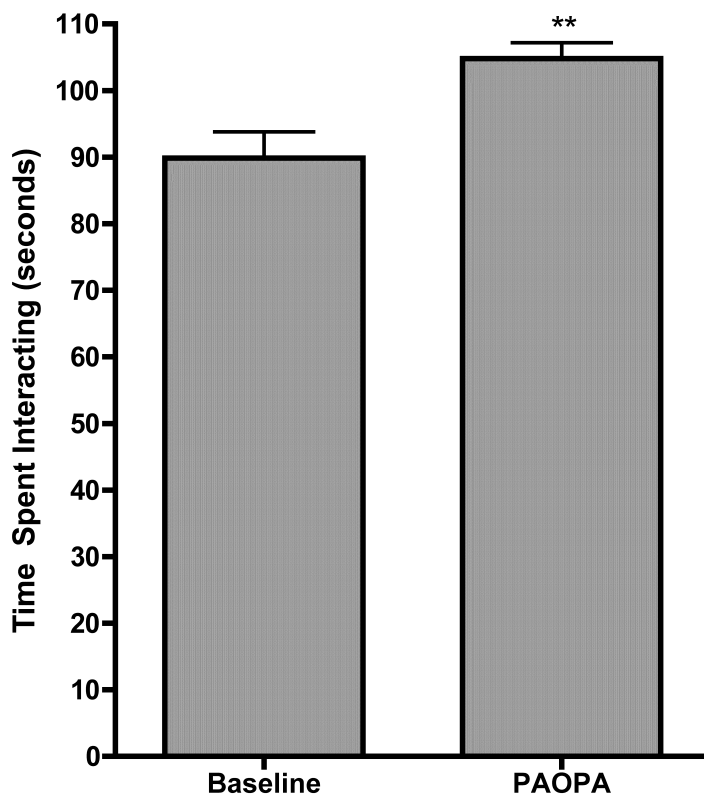
Figure 19. Effects of PAOPA treatment on time spent interacting among amphetamine rats. Graph depicts the total time (mean ± SEM) that amphetamine-sensitized rats spent interacting during the 5-minute testing period during baseline testing and one hour following PAOPA administration (1mg/kg). PAOPA treatment caused a significant increase in the time amphetamine-sensitized rats spent interacting compared to baseline levels. ***p < 0.0046.

4.3 Effects of Amphetamine Sensitization and PAOPA Treatment on Locomotor Activity

During the 1-hour Habituation period, amphetamine-sensitized rats had increased activity counts, indicating elevated locomotor activity (Figure 20), while rats treated concurrently with amphetamine and PAOPA had activity counts comparable to saline-treated rats (Figure 20). PAOPA treatment alone had no effect on locomotor activity (Figure 20). ANOVA results for the habituation period are as follows: $F(3,32) = 3.053$; $*p = 0.0425$; Post-Hoc results: Saline vs. Amphetamine p , 0.05.

During the 1-hour Saline Challenge (1 mL/kg saline) recording period, all four groups had comparable activity counts (Figure 20). ANOVA results for the Saline challenge period are as follows: $F(3,32) = 1.061$; $p = 0.3794$.

During the 1-hour Amphetamine Challenge (1mg/kg amphetamine) recording period, amphetamine rats had significantly increased activity counts when compared to saline rats (Figure 20), while the activity counts of rats treated concurrently with amphetamine and PAOPA were not significantly different from those of saline-treated rats (Figure 20). PAOPA treatment alone had no effect on locomotor activity (Figure 20). ANOVA results for the Amphetamine challenge period are as follows: $F(3,30) = 4.728$; $**p = 0.0081$; Post-Hoc results: Saline vs. Amphetamine $p < 0.01$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.05$.
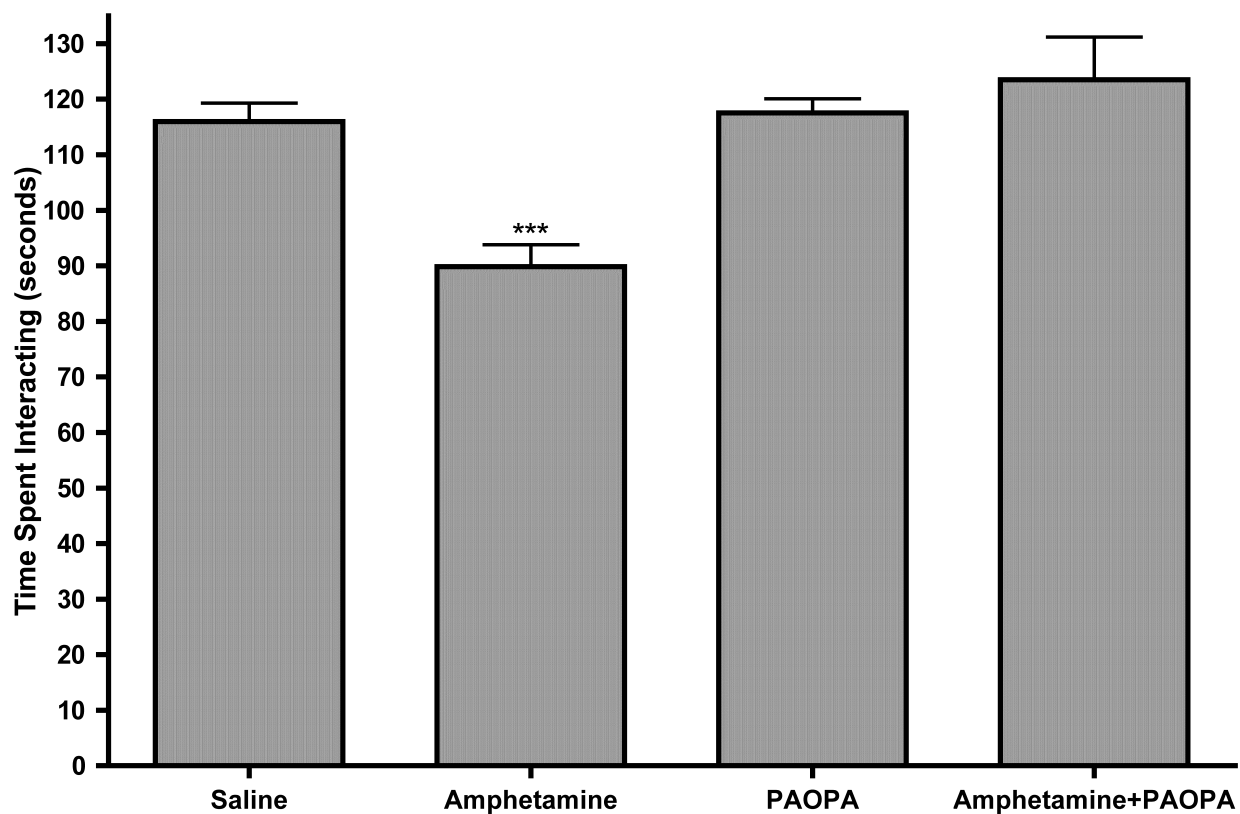
Figure 20. Effects of amphetamine and PAOPA on locomotor activity. Graph depicts distance traveled (mean ± SEM) by treatment groups during three testing periods. During the 1-hour habituation period, amphetamine-sensitized rats traveled significantly more than all treatment groups. PAOPA treatment alone had no effect on locomotion, and concurrent PAOPA treatment during amphetamine sensitization prevented the increase in distance traveled ($F(3,32) = 3.053$; *$p = 0.0425$; Post-Hoc results: Saline vs. Amphetamine p , 0.05). During the hour following a saline challenge, there was no significant difference in distance traveled across treatment groups ($F(3,32) = 1.061$; $p = 0.3794$). During the hour following an amphetamine challenge, amphetamine-sensitized rats traveled significantly more distance than all treatment groups. PAOPA treatment alone had no effect on locomotion, and concurrent PAOPA treatment during amphetamine sensitization prevented sensitivity to amphetamine challenge ($F(3,30) = 4.728$; **$p = 0.0081$; Post-Hoc results: Saline vs. Amphetamine p < 0.01, Amphetamine vs. PAOPA p < 0.05, Amphetamine vs. Amphetamine+PAOPA p < 0.05).
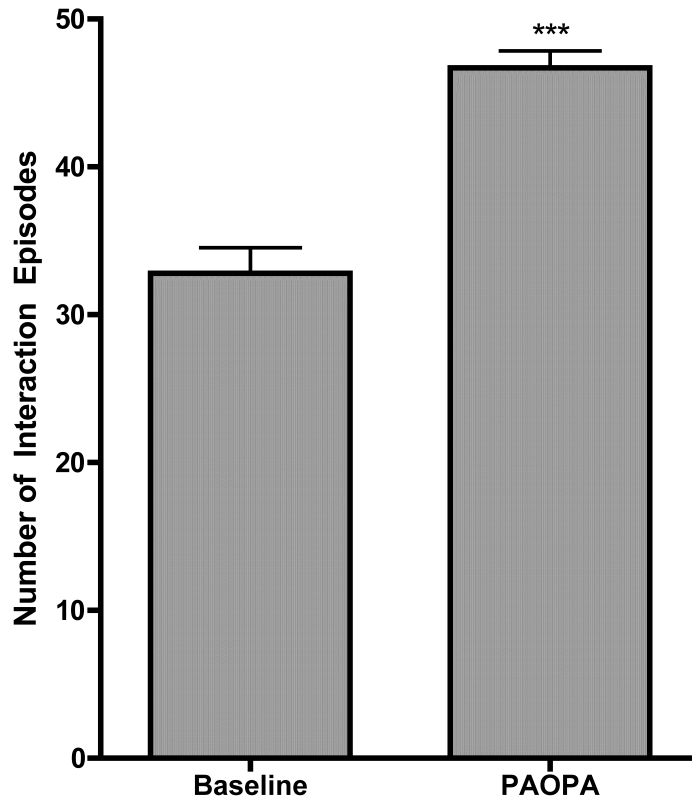
4.4 Effects of Amphetamine Sensitization on Post-Mortem Brain Dopamine Levels

Amphetamine-sensitized rats had significantly lower striatal dopamine than saline-treated rats (Figure 21). Rats treated concurrently with amphetamine and PAOPA had similar levels of striatal dopamine to saline-treated rats (Figure 21). PAOPA treatment had no effect on striatal dopamine (Figure 21). ANOVA results for post-mortem dopamine levels in the striatum are as follows: $F_{(3,33)} = 4.733$; $**p = 0.0074$; Post-Hoc results: Saline vs. Amphetamine $p < 0.01$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.05$.

Although the Amphetamine group had slightly elevated post-mortem dopamine levels in the NAc, the difference was not significant across treatment groups (Figure 22; $F_{(3,35)} = 0.3397$; $p = 0.7967$). Similarly, the Amphetamine group had slightly decreased dopamine levels in the mPFC, but the difference was not found to be significant (Figure 23; $F_{(3,31)} = 0.5532$; $p = 0.6499$).
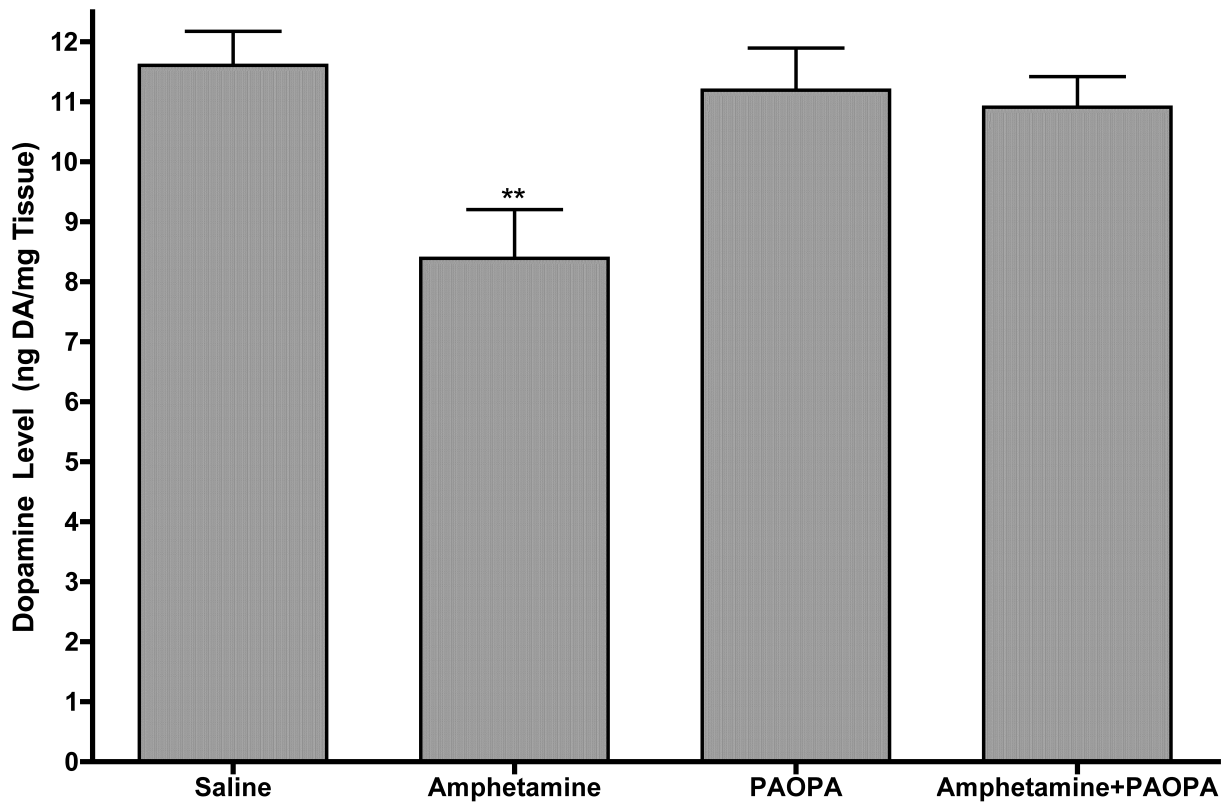
Figure 21. Effects of amphetamine and PAOPA on post-mortem dopamine levels in the striatum. Graph depicts post-mortem dopamine levels (mean ± SEM) in the striatum of each treatment group. Amphetamine sensitization caused a significant reduction in post-mortem dopamine levels in the striatum. PAOPA treatment alone had no effect on dopamine levels, and concurrent PAOPA administration during amphetamine sensitization prevented the reduction of dopamine levels. $F(3,33) = 4.733$; **p = 0.0074; Post-Hoc results: Saline vs. Amphetamine $p < 0.01$, Amphetamine vs. PAOPA $p < 0.05$, Amphetamine vs. Amphetamine+PAOPA $p < 0.05$.
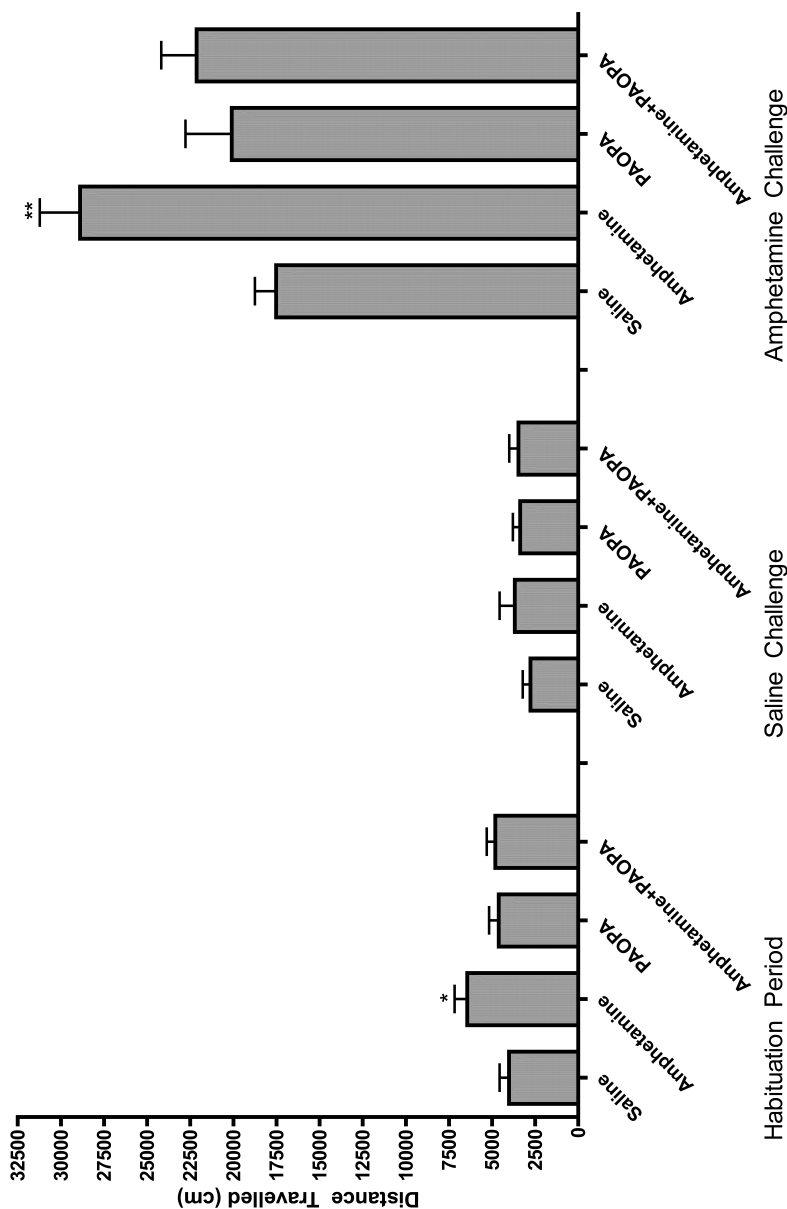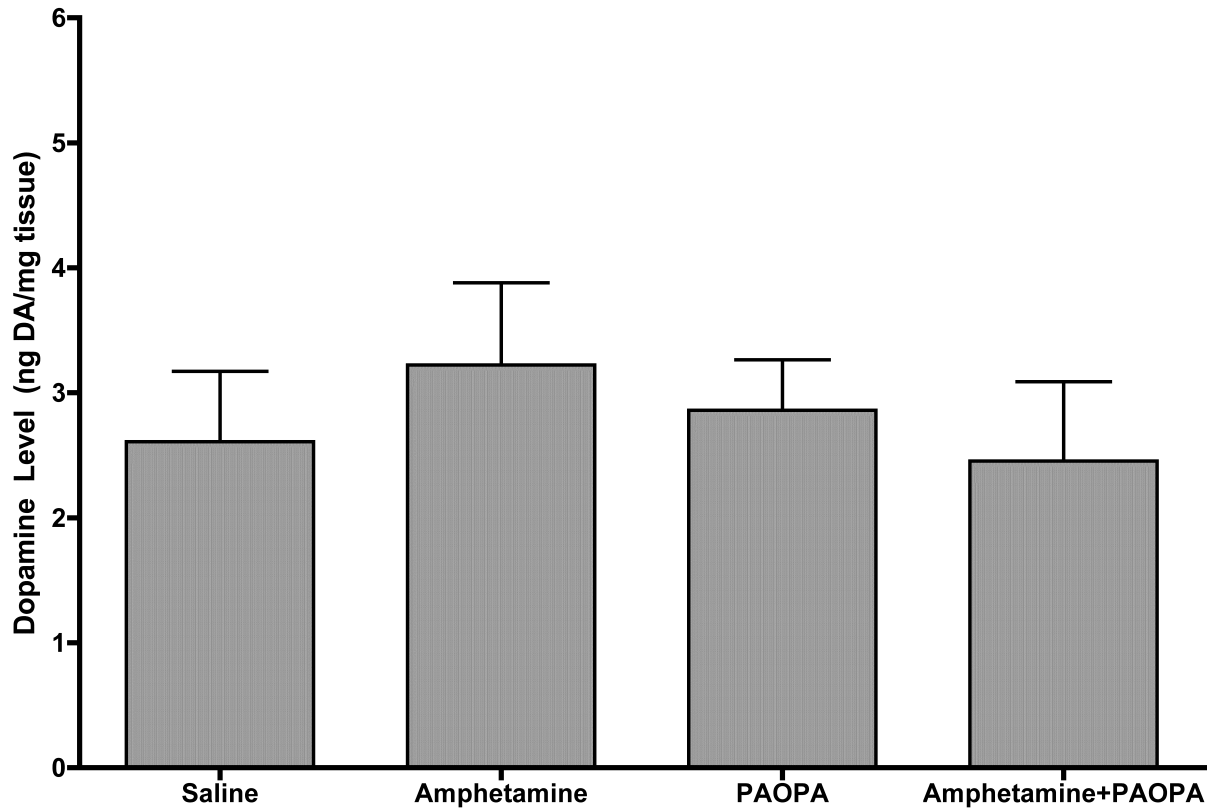
Figure 22. Effects of amphetamine and PAOPA on post-mortem dopamine levels in the nucleus accumbens. Graph depicts post-mortem dopamine levels (mean ± SEM) in the nucleus accumbens of each treatment group. There was no significant difference in dopamine levels in the nucleus accumbens across treatment groups. $F(3,35) = 0.3397$; $p = 0.7967$.
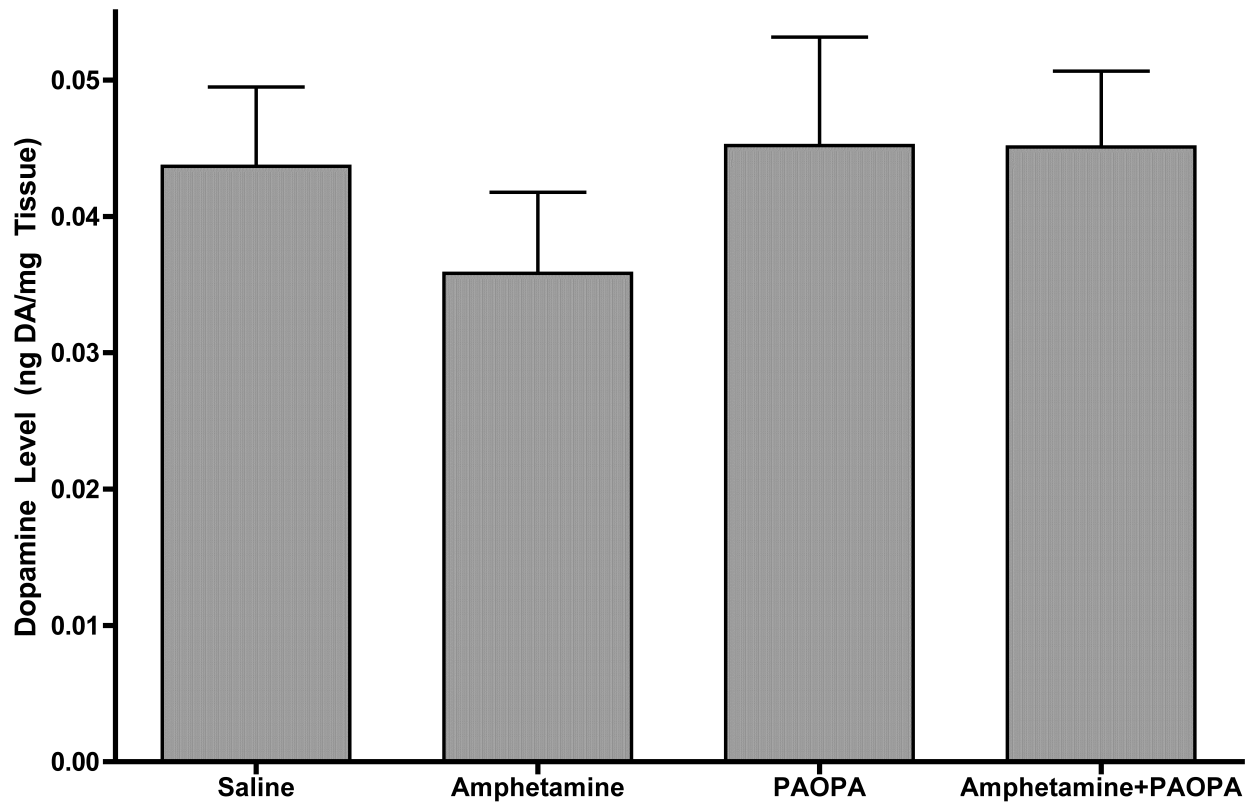
Figure 23. Effects of amphetamine and PAOPA on post-mortem dopamine levels in the medial prefrontal cortex. Graph depicts post-mortem dopamine levels (mean ± SEM) in the medial prefrontal cortex of each treatment group. There was no significant difference in dopamine levels in the medial prefrontal cortex across treatment groups. $F_{(3,31)} = 0.5532$; $p = 0.6499$.

5   DISCUSSION

5.1   PAOPA Prevents and Reverses Amphetamine-Induced Deficits in Prepulse Inhibition

Deficits in PPI have been observed in patients with schizophrenia (Grillon *et al.*, 1992; Geyer *et al.*, 2006) and in amphetamine-sensitized rats following intermittent amphetamine administration and withdrawal (Tenn *et al.*, 2003; Tenn *et al.*, 2005; Peleg-Raibstein *et al.*, 2006), Interestingly, PPI is not disrupted following continuous amphetamine dosing regimens (Peleg-Raibstein *et al.*, 2006b).

In the present study, amphetamine sensitization using the regimen described by Tenn *et al.* (2003) induced a deficit in %PPI following a 71dB prepulse but not following 68dB or 77dB prepulses. Following the same sensitization regimen, deficits in %PPI lasting up to 60 days have been reported following a 70dB prepulse but not following 75dB or 78 dB prepulse (Tenn *et al.*, 2003; Tenn *et al.*, 2005). Furthermore, the 68dB prepulse was only 3dB louder than the 65dB white noise, so the prepulse may not have been loud enough to prime animals for the much louder, 125dB stimulus pulse. Therefore it is reasonable that amphetamine sensitization reduced %PPI at the 71dB prepulse intensity but not at the 68dB or 77dB intensity. Rats treated concurrently with PAOPA during the amphetamine sensitization regimen showed no deficit in %PPI following a 71dB prepulse, indicating that PAOPA treatment was able to prevent the deficit in %PPI induced by amphetamine sensitization. Chronic PAOPA treatment had no effect on %PPI.

Because of similarities between amphetamine-induced deficits in %PPI at the previously-reported 70dB prepulse intensity (Tenn *et al.*, 2003; Tenn *et al.*, 2005) and the

71dB prepulse presented here, %PPI following a 71dB prepulse was used to determine whether PAOPA could reverse amphetamine-induced deficits in %PPI. Administration of a 1mg/kg dose of PAOPA increased %PPI in amphetamine-sensitized rats within one hour. Taken together, these results indicate that PAOPA administration can prevent and reverse deficits in sensorimotor gating caused by dopaminergic sensitization. These observations agree with the initial hypotheses. Furthermore, given the similarities between the amphetamine model and schizophrenia, PAOPA may be able to prevent and treat the development of sensorimotor gating deficits observed in patients with schizophrenia.

5.2   PAOPA Prevents and Reverses Amphetamine-Induced Deficits in Social Interaction

A more controversial measure of amphetamine sensitization is social withdrawal, which correlates in animal models to the negative symptoms of schizophrenia. Several investigators have reported deficits in social interaction following amphetamine treatment behaviour (Gambill and Kornetsky, 1976; Ellison *et al.*, 1978; Beatty *et al.*, 1984; Steinpreis *et al.*, 1994), while others have reported no change (Sams-Dodd, 1995; Sams-Dodd, 1998). These studies all differ in rat strains, doses, and injection schedules, which may account for the differences in behaviour. To date there have been no reports of amphetamine's effects on social interaction with the parameters measured here, following the sensitization regimen described by Tenn *et al.* (2003).

The most comparable study, which was performed decades ago, administered escalating doses of amphetamine daily, ranging from 1mg/kg to 8mg/kg. This study found a significant decrease in the social behaviour and an increase in aggressive

behaviour in amphetamine-sensitized Long-Evans rats over a 5.5-minute recording period, although interaction time was not measured (Gambill and Kornetsky, 1976). However, studies investigating escalating amphetamine infused continuously at low doses (Sams-Dodd, 1995) and high doses (Sams-Dodd-1998) found no changes in social behaviour using the same scoring parameters.

In the present study, amphetamine-sensitized rats showed a significant deficit in both the number of interaction episodes and time spent interacting over a 5-minute recording period when compared to saline-treated controls. This demonstrates that sensitization involving chronic, escalating doses of amphetamine followed by a three-week withdrawal period can induce social withdrawal in rats, although this phenomenon may be strain-specific. Furthermore, co-administration of PAOPA during sensitization can prevent the social deficits induced by amphetamine. PAOPA treatment alone had no effect on social behaviour.

Since the amphetamine-sensitization regimen used in the present study did induce deficits in social behaviour, the effect of PAOPA administration on social behaviour in amphetamine-sensitized rats was tested. Two rats were never paired together more than once, and the number of testing sessions was limited to reduce the effects of testing on social interaction. Therefore, a saline-challenge of amphetamine rats could not be performed as a control, and instead observations following PAOPA treatment were compared to baseline levels. One hour following administration of a 1mg/kg dose of PAOPA, amphetamine-sensitized rats demonstrated an increased number of interactions and an increase in the amount of time spent interacting when compared to baseline levels.

In agreement with the initial hypotheses, these results indicate that PAOPA can

prevent and reverse deficits in social behaviour induced by amphetamine. Although controversial, there may be a correlation between the deficits in social behaviour observed here and those seen in schizophrenia. Although negative symptoms of the disease are believed to be a result of hypofunctioning in the PFC, it has recently been proposed that striatal dysregulation could be the root cause of decreased cortical dopamine signaling (Simpson *et al.*, 2010). Therefore, the striatal effects of repeated, escalating amphetamine challenges could disrupt cortical circuitry and mimic the social aspects of schizophrenia. If this is the case, PAOPA may be able to prevent and treat the development of negative symptoms observed in patients with schizophrenia.

5.3   PAOPA Prevents Amphetamine-Induced Hyperlocomotion

The final behavioural test of PAOPA's effects on amphetamine sensitization was locomotor activity. Although patients with schizophrenia do not exhibit hyperactivity, increased locomotor activity is generally viewed as a marker of positive symptoms in animal models, as it is an indication of dopaminergic supersensitivity in the striatum.

Previous studies have investigated the effects of amphetamine sensitization on locomotor activity. It has been demonstrated that amphetamine induces hyperlocomotion in a dose-dependent manner following continuous infusion over 6-7 days (Sams-Dodd-1998). It has also been shown that rats are supersensitive to amphetamine following a regimen involving sensitization and withdrawal, as indicated by excessive locomotor activity when compared to saline-sensitized controls. A low dose of amphetamine to amphetamine-sensitized rats causes significant increases in locomotor activity (Tenn *et al.*, 2003; Tenn *et al.*, 2005), a phenomenon which lasts up to 180 days following sensitization (Paulson *et al.*, 1991; Paulson and Robinson, 1991).

In the present study, amphetamine-sensitized rats exhibited elevated locomotor activity during the 1-hour habituation period, but did not exhibit the same hyperlocomotion following a saline challenge. Therefore the increased locomotor activity of amphetamine-sensitized rats during habituation is likely a result of stimulation of a new environment. Furthermore, a challenge of 1mg/kg amphetamine induced increased locomotor behaviour in all groups, but the amphetamine-sensitized group of rats showed significantly higher locomotion following acute amphetamine challenge when compared to the other treatment groups. Co-administration of PAOPA during amphetamine-sensitization prevented the increase in locomotor activity during habituation and following amphetamine challenge. Chronic PAOPA administration alone had no effect on locomotor activity. In agreement with the initial hypotheses, these results indicate that PAOPA can prevent the development of amphetamine-induced hyperlocomotion and amphetamine sensitivity. Due to the similarities in mesolimbic sensitivity between amphetamine rats and patients with schizophrenia, amphetamine-induced locomotor activity is used to model positive symptoms of the disease. Therefore, PAOPA may be effective in preventing or improving the development of psychosis.

5.4   PAOPA Prevents Amphetamine-Induced Changes in Post-Mortem Striatal Dopamine Levels

To assess whether behaviour abnormalities observed in amphetamine-sensitized rats were due to altered dopaminergic transmission, high performance liquid chromatography was employed to determine levels of dopamine in the striatum, NAc, and mPFC of treatment groups. Previous reports have analyzed post-mortem tissue from chronic users of methamphetamine, which revealed decreased striatal dopamine (Wilson

et al., 1996). This phenomenon has been recreated in the rat by measuring dopamine

levels in the striatum *in vivo* using microdialysis and HPLC over the course of weeklong

amphetamine administration. During the daily dosing regimen, resting levels of striatal

dopamine before amphetamine challenge were shown to decrease each day. Although

resting dopamine levels decreased, rats became more sensitive to the effects of

amphetamine, indicated by more exaggerated increases in striatal dopamine (Cass *et al.*,

1989).

In the present study, amphetamine-sensitized rats were shown to have significantly

reduced post-mortem levels of dopamine in the striatum when compared to saline rats.

Co-administration of PAOPA during amphetamine sensitization prevented this reduction,

and chronic PAOPA administration alone had no effect on striatal dopamine. Despite a

slight increase, post-mortem dopamine levels were not significantly different in the NAc.

Similarly, there was no change in the mPFC.

These results contradict the intial hypotheses; it was hypothesized that the levels of

dopamine in the striatum and NAc would increase following amphetamine sensitization,

as there is increased dopamine synthesis in schizophrenia. It was also expected that

dopamine levels in the mPFC would decrease following amphetamine sensitization,

indicating a hypofrontality similar to the disease state. The observed results likely

occurred because the primary location that amphetamine induces dopamine release is the

striatum. Therefore, the effects would be very robust in this brain region, and perhaps

were not strong enough to alter dopamine levels in other brain regions, despite the

indication of social isolation among amphetamine-sensitized rats in this study.

The decrease in post-mortem striatal dopamine levels may be indicative of

mesolimbic dysregulation. Three possible mechanisms can be proposed to explain the

reduction of whole striatal dopamine levels following amphetamine sensitization. (1)

Decreases in striatal dopamine could be an indication of neurotoxicity in dopaminergic

neurons. Amphetamine is known to be neurotoxic during continous use. However, since

no change was observed in dopamine levels in the nucleus accumbens, dopaminergic

neurotoxicity is unlikely, and the reduction in striatal dopamine can be attributed to other

mechanisms. (2) Decreases in striatal dopamine could be a mechanism to compensate for

post-synaptic receptor hypersensitivity. Amphetamine sensitization causes an increase in

the in the proportion of dopamine receptors in a high affinity state (Seeman, 2009).   As

the proportion of high affinity receptors increases, less dopamine would be required for

neuronal stimulation and dopaminergic neurotransmission. Therefore, presynaptic

terminals might respond by decreasing levels of dopamine production to compensate for

the increased D2 receptor sensitivity. However it is unlikely that post-synaptic receptor

sensitivity would have such a large impact on presynaptic dopamine production. (3) The

final and most likely explanation is that decreased striatal dopamine could be indicative

of increased dopamine turnover. It is well established that there is increased dopamine

release in schizophrenia (Miyake *et al.* 2010), as well as in amphetamine-sensitized rats

(Paulson *et al.*, 1991). If dopamine release occurs at a higher rate than dopamine

synthesis, then increased dopamine turnover may be accompanied by decreased resting

levels of dopamine. However, confirming whether this occurs in schizophrenia would be

a difficult, as most patients undergo antipsychotic therapy. Accurately measuring the

levels of striatal dopamine would be hindered by APD use, which is known to alter

striatal dopamine levels (Kulkarni *et al.*, 2009; Wasti and Siddiqui, 2010).

Nevertheless, these data indicate that amphetamine-sensitized rats underwent some change in dopamine regulation during sensitization, and that this change was prevented by PAOPA. This change also correlates with the observed behavioural abnormalities in amphetamine-sensitized rats.A

5.5 Proposed Mechanism of Action for PAOPA in the Prevention and Reversal of Amphetamine-Induced Behavioural Sensitization

Despite several successful behavioural studies, the precise mechanism by which PAOPA improves schizophrenic-like behaviour in preclinical models is not yet known. However, based on the results presented here a potential mechanism can be proposed.

It has been previously demonstrated that PAOPA's affinities and potencies for the D2S autoreceptor and the D2L postsynaptic receptor are almost identical (Verma *et al.*, 2005). It is also known that the number of presynaptic autoreceptors, which are primarily D2S receptors, is much greater than the number of postsynaptic receptors (Meller *et al.*, 1987; Mailman and Murthy, 2010). Therefore at low to moderate doses, a ligand with great affinity and specificity for D2S and D2L receptors will most likely interact with presynaptic D2S autoreceptors. As a positive allosteric modulation, PAOPA increases the affinity of dopamine for these receptors (Mishra *et al.*, 1990) and would promote the their stimulation, thus activating pathways that reduce the synthesis and release of dopamine.

During amphetamine sensitization, the effects of PAOPA could counteract the actions of amphetamine, which stimulates the production of dopamine and its accumulation in the synapse (Sulzer, 2011). Therefore PAOPA may have prevented sensitization by counterbalancing the actions of amphetamine at every injection.

Following sensitization, PAOPA may be working in the same way. Amphetamine-sensitized rats have elevated levels of striatal dopamine release (Paulson *et al.*, 1991). By binding to presynaptic autoreceptors PAOPA can promote their stimulation by dopamine, resulting in decreased dopamine synthesis and subsequently decreased dopamine release while the drug is available to bind D2 receptors. Figure 24 depicts the proposed mechanism of action for PAOPA in preventing and reversing the effects of amphetamine.

However, PAOPA's mechanism of action is not likely this simple. Given what is known about receptor physiology, it is unlikely that dopamine D2 receptors exist *in vivo* only in a monomeric form. It has even been proposed that receptors may only exist as oligomers (George *et al.*, 2002), so PAOPA is likely interacting with D2 receptors that are involved in complex receptor-receptor interactions. The D1-D2 heterodimer, for example, exists in the striatum and is linked to Ca^{2+} signaling, a pathway not normally activated by either D1 or D2 receptors (Hasbi *et al.*, 2009). Whether PAOPA interacts with D2 receptors as a monomer or in complexes such as the D1-D2 dimer has yet to be studied. Furthermore, PAOPA has been shown to couple the D2 receptor to $G\alpha_i$ (Mishra *et al.*, 1999), but whether it can cause coupling to other downstream pathways has not yet been explored. Therefore, PAOPA may be acting as a functionally selective ligand by stimulating pathways that are not classically linked to dopamine D2 receptors.

To further elucidate PAOPA's mechanism, future studies should focus on PAOPA's effects on striatal dopamine synthesis and release *in vivo*. Radio-imaging studies and *in vivo* microdialysis would provide a much clearer picture of PAOPA's effects on dopaminergic neurotransmission, and how this compound is able to counteract the effects of amphetamine and exert its therapeutic effects to improve symptoms of

previously sensitized animals. *In vitro* studies should further investigate PAOPA's

effects on dopamine receptors and other receptors. It has yet to studied whether

PAOPA's interacts with dopamine D2 monomers, homodimers, or heterodimers formed

with other receptors. Furthermore, studies have focused on PAOPA's effects on adenylyl

cyclase activity, a classical D2-mediated pathway. However, given what is now known

about receptor physiology, downstream coupling to other pathways and signaling

molecules should be investigated. This includes G-protein dependent coupling via $G\alpha$

proteins other than $G\alpha_{i/o}$, as well as the effects on G-protein independent signaling.
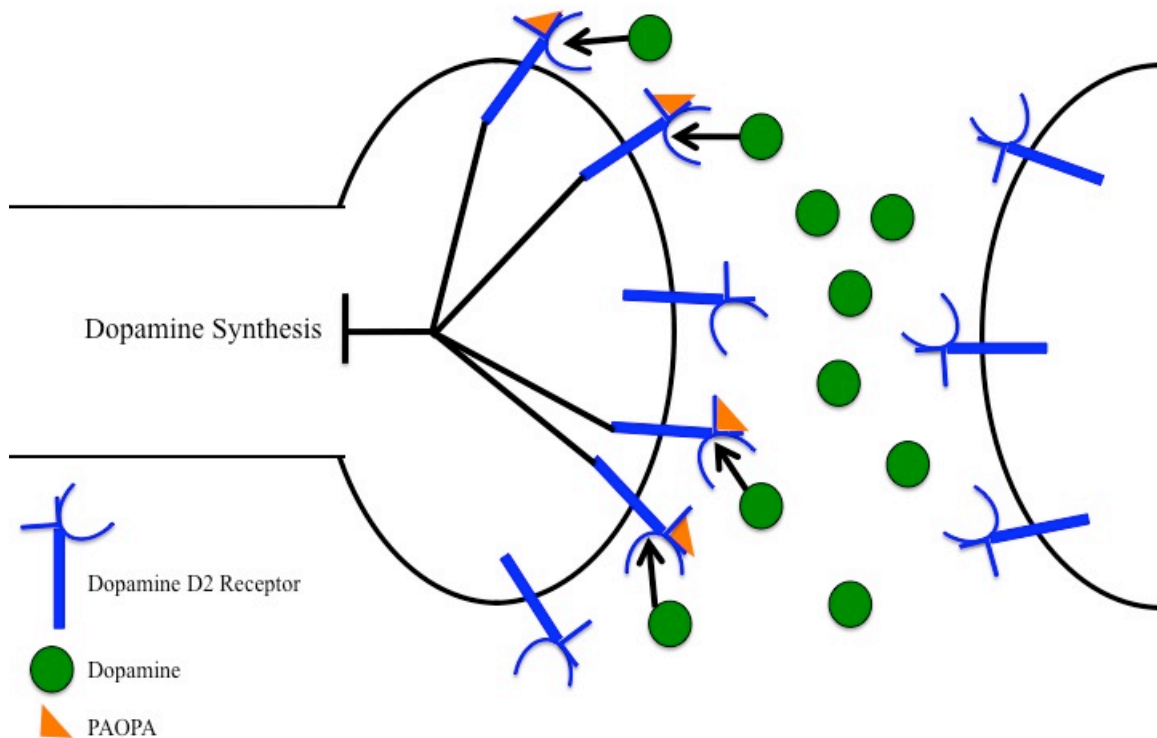
Figure 24. Proposed mechanism of PAOPA's action. Amphetamine causes an increase in dopamine synthesis synaptic dopamine levels. PAOPA interacts with dopamine D2 receptors and increases their affinity for dopamine. Since the presynaptic D2 receptor pool is greater than the postsynaptic D2 receptor pool, PAOPA likely interacts primarily with D2S autoreceptors on presynaptic terminals, increasing dopamine binding. This would inhibit dopamine synthesis in presynaptic neurons, and subsequently counteract the effects of amphetamine.

6   CONCLUSIONS

1.   Co-administration of PAOPA during an amphetamine sensitization regimen prevents the behavioural abnormalities induced by amphetamine. Amphetamine sensitization induced an array of behavioural abnormalities. Specifically, sensitized rats exhibited sensorimotor gating deficits, decreased social interaction, hyperactivity, and increased sensitivity to amphetamine. However, rats that received PAOPA concurrently with identical doses of amphetamine did not develop any of these behaviours.

2.   A single intraperitoneal dose of PAOPA is able to temporarily reverse behavioural abnormalities in rats previously sensitized with amphetamine. After baseline behaviours were measured, amphetamine-sensitized rats were subjected again to tests of sensorimotor gating and social interaction following a 1mg/kg dose of PAOPA. One hour after PAOPA treatment, the deficits in both %PPI and social interaction were reversed.

3.   Co-administration of PAOPA during amphetamine sensitization is able to prevent changes in striatal dopamine levels induced by amphetamine. Post-mortem tissue analysis revealed that amphetamine sensitization caused a reduction in total dopamine levels in the striatum, but not in the NAc or mPFC. Rats that received PAOPA concurrently with amphetamine had normal striatal dopamine levels.

4.   Chronic PAOPA treatment, at the dose tested, does not induce any abnormalities in any behavioural or biochemical parameter measured. Chronically treated PAOPA rats did not develop any abnormal behaviours in the tests performed. Furthermore, these rats had levels of striatal dopamine comparable to saline controls.

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