The Role of the Dopamine D5 Receptor and D1-D2 Receptor Heterooligomer Signalling in Rodent Prefrontal Cortex and Cognition

by

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Abstract

The dopamine D5 receptor is highly expressed in rodent prefrontal cortex (PFC) a region involved in cognition. We have previously shown that acute activation of the Gq-coupled dopamine D5 and D1-D2 receptor heteromer by the agonist SKF 83959 inhibited glycogen synthase kinase- 3 (GSK-3) activity in PFC, a protein that mediates cognitive performance in this region. Specifically, the dopamine D5 receptor suppressed GSK-3 activity through a mechanism dependent on brain-derived neurotrophic factor (BDNF) signaling, whereas inhibition of GSK-3 activity by D1-D2 heteromer appears to be independent of BDNF signaling in the PFC. We have demonstrated that SKF83959 improved cognitive performance in male rats using the egocentric learning task in the small Morris water maze. These studies potentially offer a novel therapeutic drug target to treat cognitive deficits in Alzheimer's disease and schizophrenia through activation of the dopamine D5 receptor signaling pathway involving increased BDNF signaling and inhibition of GSK-3 activity.

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Summary of Abbreviations

5-HT	5-hyroxytryptamine
Akt	protein kinase B
BDNF	brain derived neurotrophic factor
Bret	bioluminescence resonance energy resonance
cAMP	cyclic adenosine monophosphate
CaMKII	calmodulin-dependent protein kinase II
DAG	diacylglycerol
DHEA	dehydroepiandrosterone
FRET	fluorescent resonance energy transfer
GPCRs	G protein-coupled receptors
GSK-3	glycogen synthase kinase 3
IP3	inositol (1,4,5)-trisphophate
LTP	long term potentiation
MAM	mitochondrion-endoplasmic reticulum membrane
MeCP2	methyl CpG binding protein 2
mGluRs	metabotrophic glutamate receptors
PDK1	3-phosphoinositide-dependent protein kinase 1
PFC	Prefrontal Cortex
PI3K	phosphatidyl-inositol 3 kinase
PIP2	phosphatidylinositiol 4,5-biphosphate
PLC	phospholipase C
SNP	single nucleotide polymorphism
TrkB	tropomyosin related kinase B
WCST	Wisconsin Card Sorting Test

1 Introduction

1.1 G Protein-Coupled Receptors

The G protein-coupled receptors (GPCRs) are heptahelical receptors that make up the largest superfamily of receptors and that allow molecules to bind from outside of the cell to initiate signal transduction within the cell. There are around 800 different human genes that encode GPCRs in the human genome (Lagerstrom at al., 2008).

GPCRs are made up of seven transmembrane α helix domains, three intracellular and extracellular loops, an extracellular amino terminal, and an intracellular carboxyl terminal tail. The seven transmembrane domains are packed tightly together into a helical bundle to form a core which forms the fundamental structural basis of the receptors (Palczewski et al., 2000). Crystallography has validated several different GPCR structures after the initial crystallization of rhodopsin (Palczewski et al., 2000), with the first reported crystallized human GPCR being the β2 adrenergic receptor (Cherezov et al., 2007; Rasmussen et al., 2007) and others including the human muscarinic acetylcholine receptor (Haga et al., 2012), mouse serotonin 5-HT3 receptor (Hassaine et al., 2014) and the human D3 dopamine receptor (Chien et al., 2010).

The GPCRs are characterized into three major groups (A, B and C) by their protein sequence homology (Davies et al., 2007). The Class A rhodopsin-like family, which include dopamine receptors, is the largest of all classes and comprise about 85% of all GPCRs. Class B secretin-like receptors are a smaller family composed of approximately 53 receptors which bind to large peptides such as secretin, parathyroid hormone, glucagon, calcitonin, and growth hormone releasing hormone (Davies et al., 2007; Cardoso et al., 2006). Class C metabotrophic glutamate receptors (mGluRs) are a subtype of glutamate receptor that binds to glutamate, a neurotransmitter that induces neuronal excitatory activity (Davies et al., 2007).

The activation of GPCRs by the binding of an endogenous ligand to the receptor's binding domain results in a structural conformational change that allows the activation and release of the heterotrimeric G proteins, comprised of a Gα, Gβ, and Gγ subunit. Once the GPCR is activated, the associated inactive Gα subunit exchanges a GDP for GTP to allow the dissociation of the heterotrimeric G protein (Wess, 1998) leading to modifications in downstream intracellular effectors to elicit a cellular response. The Gα subunit can be further subdivided into subgroups which include Gαs, Gαi, Gα12 and Gαq. Gαs activates the membrane bound enzyme, adenylyl cyclase, to increase the production of cyclic adenosine monophosphate (cAMP) whereas Gαi inhibits adenylyl cyclase to decrease levels of cAMP. Of particular of interest to the present thesis is the Gαq proteins which induce the activation of the enzyme phospholipase C (PLC), leading to the production of diacylglycerol (DAG), inositol (1,4,5)-trisphophate (IP3) and increased calcium release from intracellular stores (reviewed, Beaulieu and Gainetdinov, 2011).

The Gβγ complex consists of tightly bound heteromeric G proteins composed of one Gβ and one Gγ subunit (Hurowitz et al., 2000). There are 5 Gβ and 11 Gγ subunits and the different combinations of proteins within the Gβγ complex allows for the initiation of different signalling cascades upon receptor activation and dissociation from the Gα subunit (Clapham and Neer, 1997). Downstream signalling by Gβγ is diverse, with the potential to activate and inhibit many targets including calcium channels (Ikeda et al., 1995) and adenylyl cyclase (Tang and Gilman, 1991). The Gβγ complex has also been shown to be involved in the inhibition of the Gα subunit (Khan et al., 2013; Calpham and Neer, 1997).

1.1.1 GPCR Oligomers

GPCRs were previously thought to exist as single monomeric entities, but it is now widely accepted that these receptors interact to form dimers, comprised of two receptors, or larger multi-receptor oligomers (George et al., 2002; Milligan, 2004; Terrillon et al., 2004). Various methods have been used to confirm the presence of oligomeric GPCR complexes which include co-immunoprecipitation (Kroeger et al., 2003; Park et al., 2004), fluorescent resonance energy transfer (FRET), bioluminescence resonance energy resonance (BRET) (Pfleger et al., 2005) and crystallography (Wu et al., 2010). The interaction between these receptors results from the formation of bonds within extracellular, intracellular and transmembrane domain regions of GPCRs (Kumar et al., 2002). For instance, the interaction between receptors within a homooligomer (comprised of receptors of the same subtype) has been shown to occur between the residues in the transmembrane domains, as has been shown with the dopamine D2 receptor (Guo et al., 2003; Lee et al., 2003), the α1b-adrenoceptor (Lopez-Gimenez et al., 2007), the serotonin 5HT1A (Gorinski et al., 2012) and the 5HT2C receptors (Mancia et al., 2008). In addition to forming homooligomers, GPCRs of different subtypes within the same family can also interact to form heterooligomers, such as has been shown to occur between different dopamine receptors (Rashid et al., 2007; Hasbi et al., 2010), serotonin (Xie et al., 1999) and opioid receptor subtypes (Fan et al., 2005; George et al., 2000) or between receptors from unrelated receptor families such as between dopamine receptors with somatostatin receptors (Rocheville et al., 2000) or with adenosine receptors (Gines et al., 2000). However, unlike that observed with homooligomers, the interaction between receptors within a heterooligomer does not appear to involve the transmembrane domains, and increasing evidence showing that the interaction involves non-covalent formation with arginine residues on one receptor with adjacent aspartic acids, glutamic acids or phosphorylated residues to form a stable complex (Jackson et al., 2006; Woods et al., 2005; O'Dowd et al., 2012).

The diversity in receptor binding as a result of oligomerization can have significant effects on receptor signalling in cells. Homodimerization of GPCRs can synergistically increase the effects of GPCRs, as the binding of a ligand can simultaneously activate multiple receptors within of the same oligomer leading to the amplification of the signal (George et al., 2002). Furthermore, the binding of a ligand to a receptor within an oligomer can induce changes to the other receptors binding domain, which may alter the affinity of a receptor for the ligand. It has been shown, for example, that the binding of an agonist to the leukotriene B4 receptor caused specific conformational changes to the receptor binding domain, which resulted in increased ligand affinity (Mesnier et al., 2004). In addition, evidence from X-ray crystallography has shown that the binding of an agonist to its receptor results in a conformational change resulting in a structure that promotes cooperative binding of the homooligomeric chemokine CXCR4 receptor complex (Wu et al., 2010).

Heteromerization allows the GPCR complexes to exhibit novel functions such as a switch in their downstream signalling pathways, as has been shown with the heteromerization between the μ and δ opioid receptor which results in Gz activation and to β-arrestin signaling, a pathway not activated when the μ-δ opioid receptor heteromer is destabilized (George et al, 2000; Rozenfeld et al., 2007). Similarly, the dopamine D1-D2 receptor heteromer was shown to couple to the Gq protein to induce PLC-dependent calcium signalling upon its activation (Lee et al., 2004, Rashid et al., 2007), a signalling pathway distinct from the ones activated by its constituent receptors. Other functions of heteromerization include the blockade of second messenger activation, such as is observed upon heteromerization of the histamine H3 and dopamine D1 receptors whereby D1 receptor coupling to the Gs protein is switched to Gi within the heteromer thus, resulting in the inhibition of cAMP production (Ferrada et al., 2009). Lastly, similar to homo-oligomerization, heteromerization can also result in an altered

ligand binding profile, where stimulation of one receptor in the heteromer can either increase or decrease the affinity of a receptor for an endogenous ligand (George et al., 2000; Ferre et al., 2007). In the adrenergic 2A-dopamine D2 receptor heteromer, the stimulation of the adrenergic receptor decreases the affinity of the dopamine D2 receptor for its agonist (Ferre et al., 1991; Hillion et al., 2002; Canals et al., 2003; Ciruela et al., 2004), whereas in the somatostatin SST5-dopamine D2 receptor heteromer, the stimulation of the dopamine D2 enhances the affinity of SST5R for its agonist and vice versa (Rocheville et al., 2000).

Aberrant heterooligomerization has also been implicated in neuropsychological disorders. For example, the dysregulation of the expression of the serotonin 5HT2A-metabotrophic glutamate receptor 2 (5HT2A-mGluR2) complex in cortical neurons of untreated patients with schizophrenia, indicating an abnormal signalling mechanism which may predispose them to psychosis (Gonzalez-Maeso et al., 2008). Moreover, evidence has suggested that dyskinesia induced by L-DOPA in patients with Parkinson's disease may be due to the increased in expression of the dopamine D1-D3 receptor heteromer leading to an altered signalling response (Fiorentini et al., 2008; Marcellino et al., 2008). In addition, patients with schizophrenia were shown to have an increased proportion of dopamine D1-D2 receptor heteromers in the high affinity state in the globus pallidus (Perreault et al., 2010), suggesting that this altered state of the receptor complex may have contributed to the pathophysiology of the disorder.

1.2 Dopamine and Receptors

1.2.1 Dopamine Receptors

The neurotransmitter dopamine is part of the catecholamine family of neurotransmitters with distinct and important functional roles in humans, such as the regulation of voluntary

motor activity, cognition, and reward pathways. The effects of dopamine are mediated through five subtypes of dopamine receptors, D1 to D5, which were initially characterized by similarities in structure and function. The dopamine D1-like receptor subtype includes the dopamine D1 and D5 receptors whereas the D2-like receptor subtype consists of dopamine D2, D3, and D4 receptors. Dopamine receptors are expressed in both the peripheral and central nervous system. For instance, in the brain, the D2R and D3R receptors, can function as autoreceptors presynaptically (Tepper et al., 1997; Usiello et al., 2000), occur postsynaptically in neurons (Sokoloff et al., 2006; Rankin et al., 2010; Rondou et al., 2010) and are also found in other non-neuronal locations such as the cardio-pulmonary and renal systems (Ricci et al., 2006; Hussain et al., 2003; Contreras et al., 2002). In the brain, the dopamine D1 and D2 receptors are more widely expressed, and are more abundant, than the dopamine D3, D4 and D5 receptors (Mansour and Watson, 1995; Hurley et al., 2006).

The D1-like dopamine receptors are composed of the dopamine D1 and the D5 receptor subtypes encoded by DRD1 and DRD5 genes respectively (Sunahara et al., 1990, Missale et al., 1998; Grandy et al., 1992). The dopamine D1 and D5 receptors have extensive sequence homology, sharing approximately 80% similarity in their transmembrane domains (Gingrich et al., 1993; Missale et al., 1998). The distribution and expression levels of the D1-like receptors are highly variable, with dopamine D1 receptor generally expressed at much higher levels than the dopamine D5 receptor in most regions of the brain such as dorsal striatum, nucleus accumbens, substantia nigra, amygdala, and frontal cortex (Hurley et al., 2006; Beaulieu et al., 2011), the exception being in the prefrontal cortex (PFC) (Luedtke et al., 1999). However, it has been shown that the D5 receptor has a 10 fold higher affinity for dopamine than the D1 receptor (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991).

The D2-like dopamine receptors are composed of the dopamine D2, D3 and D4 receptor subtypes encoded by the DRD2, DRD3, and DRD4 genes respectively (Missale et al., 1998). The dopamine D2 and D3 receptors share approximately 75% similarity, whereas the dopamine D2 and D4 receptors share approximately 53% similarity in their transmembrane domains (Missale et al., 1998). The dopamine D2 receptors are highly expressed in the striatum, nucleus accumbens, frontal cortex and the olfactory tubercle. The highest expression of the dopamine D3 receptors is found in the limbic areas, islands of Calleja and the olfactory tubercle (Sokoloff et al., 1992a,b; Sokoloff et al., 2006; Missale et al., 1998), whereas the highest expression of the dopamine D4 is found in the frontal cortex, amgydala, hippocampus, hypothalamus, thalamus (Missale et al., 1998; Rondou et al., 2010).

Dopamine D1-like receptors signal through Gαs to activate adenylyl cyclase to increase intracellular cAMP (Beaulieu et al., 2011; Kebabian and Greengard, 1971; Kebabian and Calne, 1979; Enjalbert et al., 1983; Missale et al., 1998). Dopamine D2-like receptors signal through Gαi to inhibit adenylyl cyclase (Sibley and Monsma, 1992; Missale et al., 1998; reviewed, Beaulieu and Gainetdinov, 2011).

1.2.2 The Dopamine D5 Receptor

The dopamine D5 receptor cloned here in the Department of Pharmacology (Sunahara et al., 1991) has 3 gene isoforms, only one of which is translated to an active-full length receptor and two nonfunctional pseudogenes (Nguyen et al, 1999; Tiberi et al., 1991; Grandy et al., 1991). The dopamine D5 receptor is expressed in the hippocampus, frontal cortex, thalamus, hypothalamus, amydala, and cerebellum of non-human primates (Bergson et al., 1995). Futhermore, the dopamine D5 receptor is more abundant than the dopamine D1 receptor in the prefrontal cortex (PFC) of rats, a region involved in cognitive functions and are

specifically localized in pyramidal neurons and GABAergic interneurons of the PFC (Oda et al., 2010). In contrast to the D1 receptor, which couples selectively to Gαs or Gαolf proteins, the dopamine D5 receptor has been shown to signal through both Gαs as well as Gαq to activate PLC, to induce the cleavage of phosphatidylinositol-4,5-biphosphate (PIP2) to produce IP3 and DAG (Berridge and Irvine, 1984, Berridge, 1984, Sahu et al., 2009; reviewed, Beaulieu and Gainetdinov, 2011). IP3 then binds to IP3 receptors (Mignery & Sudhof, 1990), leading to increased calcium release from intracellular stores (Michell et al., 1981), and results in increased CaMKII activity (Griffith, 2004). The administration of a Gq-coupled dopamine receptor agonist to dopamine D5 receptor knockout mice inhibited the production of IP3 and DAG in cortical, hippocampal and striatal tissues, suggesting that the activation of the dopamine D5 receptors leads to dopamine linked PLC/IP3 signalling in mammalian brain (Sahu et al., 2009). The calcium signalling from the dopamine D5 receptor activation arose from a different mechanism from that of the dopamine D1-D2 receptor heteromer in cell cultures as it required the influx of extracellular calcium through calcium channels (Figure. 1) (So et al., 2009, Hasbi et al., 2010).

SKF 83959 is a dopamine agonist shown to activate Gαq-coupled, but not Gαs-coupled or Gαi-coupled, dopamine receptors, and induced D5 receptor-mediated PLC and Ca^{+} signalling in HEK 293 cells stably expressing the dopamine D5 receptor (Figure. 1). Furthermore, SKF 83959 was shown to stimulate BDNF expression and signalling through its receptor TrkB, increase the activation of Akt and the inactivation of GSK-3 in rat PFC, mediated by the dopamine D5 receptor, effects that were not seen in dopamine D5 receptor gene deleted mice (Perreault et al., 2013). BDNF, TrkB, Akt and GSK-3 are proteins highly involved in cognition and its expression and activity in the hippocampus and PFC are implicated as potential mediators of cognitive disorders involved in schizophrenia and

Alzheimer's disease (Emamian et al., 2004; Beulieu, 2012; Weickert et al., 2003; Mattson et al., 2008; Zuccato et al., 2009; Corominas-Roso et al., 2013). The expression of the dopamine D5 receptor in the PFC, hippocampus and cerebral cortex may suggest a potential role in cognitive processes.

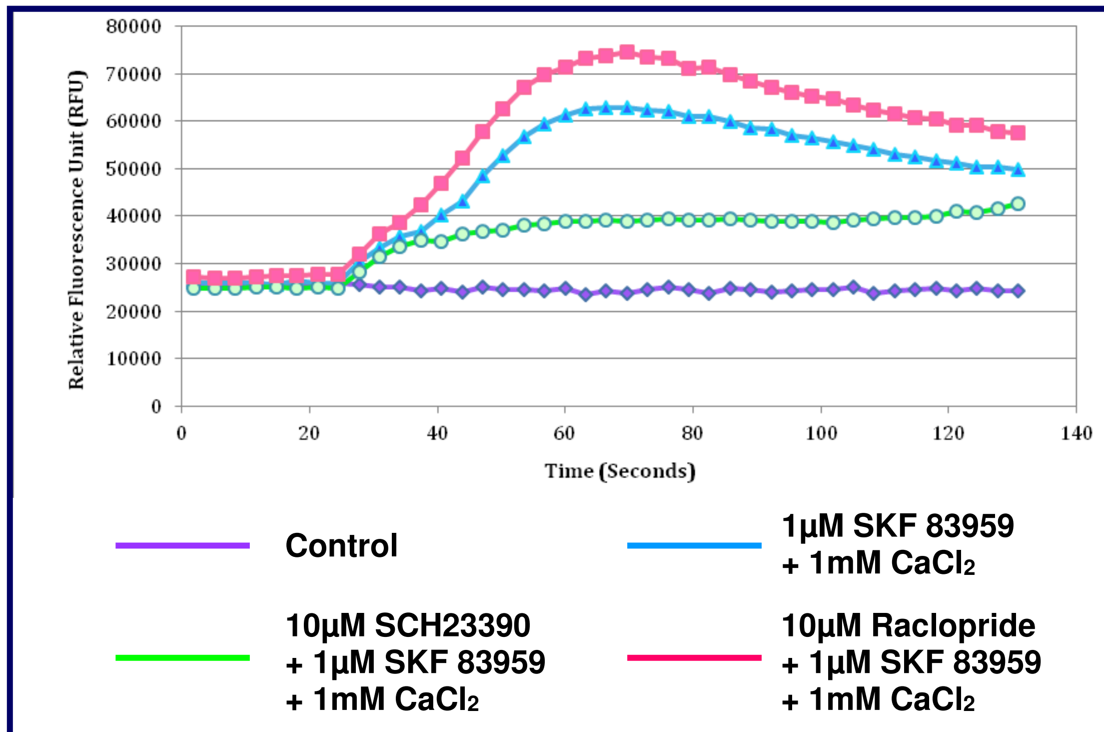


Figure 1: The calcium signal in a stable cell line (HEK cells) expressing the dopamine D5 receptor. Activation of the dopamine D5 receptor by SKF 83959 elicited an intracellular calcium release that was dependent on extracellular calcium ($CaCl_2$) in HEK cells. The dopamine D2 antagonist, raclopride had no effect on the SKF 83959 induced calcium signal but the dopamine D1-like antagonist, SCH23390 attenuated these effects. $CaCl_2$, Calcium Chloride.

1.2.3 Dopamine D1-D2 Receptor Heteromer

The first evidence to show that the dopamine D1 and D2 receptors interact to form a receptor complex in vivo was revealed in the rat striatum by coimmunoprecipitation (Lee et al., 2004). This finding was later supported utilizing quantitative confocal FRET in rat striatal neurons *in situ* to confirm the presence of the dopamine D1-D2 receptor heteromer in cultured neonatal striatal neurons and adult striatal neurons in brain sections *in situ* (Hasbi et al., 2009; Perreault et al., 2010). Heteromer formation through direct receptor-receptor interaction was shown as the distance between the native dopamine D1 and D2 receptors was less than 100Å. In the neurons that expressed the dopamine D1 receptor in rat nucleus accumbens core, shell and rat globus pallidus, approximately 27%, 36% and 60% respectively, also expressed the dopamine D2 receptor (Perreault et al., 2010). It has also been shown that there is a significant coexpression of dopamine D1 and D2 receptors in the pyramidal neurons in mouse PFC, ranging from about 20 to 25% (Zhang et al., 2010), suggesting the possibility of D1-D2 heteromer expression in this region.

Activation of the dopamine D1-D2 receptor heteromer results in a novel physiological function (Hasbi et al., 2009; Lee et al., 2004; Rashid et al., 2007; So et al., 2009; Verma et al., 2010). For instance, at the molecular level, the dopamine D1 and D2 receptors have opposing effects on the production of the second messenger cAMP (Missale et al., 1998; Sibley and Monsma, 1992). However, co-activation of both receptors within the dopamine D1-D2 heteromer leads to the activation of a Gq-linked and PLC-dependent calcium signal (Lee et al., 2004; Rashid et al., 2007; Perreault et al., 2012). A peptide that disrupts the interaction between the dopamine D1-D2 receptor complex inhibited calcium signalling in cells and striatal neurons expressing the D1 and D2 receptor, indicating that the heteromer must be involved in this signalling pathway (Hasbi et al., 2014).

The activation of the dopamine D1-D2 heteromer was demonstrated to induce the phosphorylation and activation of CaMKIIα both in neonatal rat striatal neurons and in rat nucleus accumbens *in vivo* (Rashid et al., 2007; Hasbi et al., 2009; Ng et al., 2010). Furthermore, the expression of BDNF, a neurotrophin regulated by CaMKIIα, was also demonstrated to be increased in ventral tegmental area (Perreault et al., 2012) and in the nucleus accumbens (Hasbi et al., 2009; Perreault et al., 2012).

Patients with schizophrenia are known to have hyperdopaminergic signalling in subcortical regions, but it has also been suggested that altered calcium signalling may play a role (Lidow et al., 2003). It has been demonstrated that a dopamine D2 receptor antagonist can disrupt the dopamine D1-D2 receptor heteromer signalling (Hasbi et al., 2009; Rashid et al., 2007), thus anti-psychotic drugs would also inhibit D1-D2 heteromer function. In addition, it has been reported that high levels of dopamine stimulation enhances dopamine D1-D2 receptor heteromer expression and/or high affinity agonist binding in rat striatum (Perreault et al., 2010), in cells (Dziedzicka-Wasylewska et al., 2006) and in post-mortem globus pallidus samples of patients with schizophrenia (Perreault et al., 2010). These findings suggest that anti-psychotic drugs may play a role in propagation of cognitive deficits, symptoms which already exist in patients with schizophrenia by inadvertently targeting the D1-D2 receptor heteromer. It has been reported that schizophrenia patients have increased cortical GSK-3β activity (Emamian et al., 2004) and that increased GSK-3β activity has been linked to cognitive dysfunction (Freyberg et al., 2010; Karam et al., 2010). Furthermore, SKF 83959 which activates the dopamine D5 receptor and D1-D2 receptor heteromer has been shown to increase BDNF signalling and inhibit GSK-3β activity in the PFC of rodents (Perreault et al., 2013), suggesting that this compound may potentially identify therapeutic targets for cognitive improvement in patients with schizophrenia.

1.2.4 The Prefontal Cortex (PFC), Dopamine and Cognition

One of the most essential functions of the brain is the ability to collect information about the environment and store them as memory. Memory is the initial process and is necessary to achieve other higher cognitive processes. The process of generating memory consists of different stages, which includes acquisition (encoding), consolidation (formation), storage and retrieval (Bekinschtein et al., 2008). Spatial learning and memory is the process involved in encoding and recording information about the environment and spatial orientation, which is important for navigation and the formation of episodic memory (Pilly and Grossberg, 2012). Spatial learning and memory are highly dependent on hippocampal processes in rats (O'Keefe et al., 1971; O'Keefe et al., 1978; Broadbent et al., 2004). However, there is an abundance of evidence to suggest that other regions of the brain, such as the PFC, are also involved in spatial processes (Yong Sang Jo et al., 2007; Slotnick and Moo, 2006; Lacroix, 2002; Lee and Kesner, 2003).

The PFC is part of the frontal lobe in the brain and is involved in higher cognitive functioning including rational thinking, reasoning, decision making, and social behaviour (Yang et al., 2009). The PFC has connections with many different regions of the brain, such hippocampus, associated with memory (Wood and Grafman, 2003; Doyere et al., 1993). Glutamatergic afferents from regions of the brain such as hippocampus and other cortical regions have been shown to innervate and regulate the PFC (Gigg et al., 1994). The PFC plays a critical role in memory such as recent memory (Nelson et al., 2011; Corcoran and Quirk, 2007), remote memory (Frankland et al., 2004; Takashima et al., 2006), and short term memory (Seamans et al., 1995; Narayanan et al., 2006) and deficits in PFC functions are associated with impulsiveness, distractibility, hyperactivity, forgetfulness, poor organization and planning (Pennington et al., 1996). The rat medial PFC is involved attentional processes,

working memory, behavioural flexibility, and short term spatial memory (Heidbreder et al., 2003; Gabbott et al., 2005; Briand et al., 2007; Lee et al., 2003; Pratt et al., 2001). Evidence has shown that the impairment of learning and memory in rats may be linked to dysfunction in the prefrontal cortex and hippocampus (Winocur, 1992; Yoon et al., 2008; Preston et al., 2013). Lesions in the PFC of male rats have been shown to impair spatial learning and memory (Morris et al., 1982; Sutherland et al., 1982; Whishaw and Kolb et al., 1984; Winocur and Moscovitch, 1990: Squire et al., 1993; James et al., 1993). Moreover, medial PFC lesions impaired the processing of spatial navigation in the egocentric (self to object: represents the location of an object relative to self) test using the Morris water maze (Ethier et al., 2001; Sutherland et al., 1982; Whishaw and Klob, 1984), effects not seen in the allocentric learning (object to object: represents the location of an object with respect to other objects) test (Ethier et al., 2001; Lacroix et al., 2002; de Bruin et al., 1994) and also, in rats previously trained in the radial arm maze (Becker et al., 1980), suggesting that the mPFC plays a role in spatial processes. Its function in the short term memory processing of spatial information allows for the planning of movement and motivation in searching behaviour (Lee et al., 2003; Pratt et al., 2001). Moreover, in rodent studies, it has been suggested that rats have the capabilities to use their spatial memory to locate previously hidden food, using the radial maze (Bird et al., 2003). In humans, patients with PFC damage demonstrate impaired cognitive performance in the Wisconsin Card Sorting Test (WCST), a test widely use to assess working memory (Alvarez et al., 2006).

Dopamine transmission in the PFC has been shown to play a significant role in cognitive functioning. Dopamine works through four different distinct dopaminergic pathways in the brain, which consists of the nigrostriatal, mesolimbic, tuberoinfundibular and the mesocortical neurons. The mesocortical pathway is the pathway that begins from the ventral

tegmental area to the different regions in cortex, including the PFC, where dopaminergic neuron terminals in the cortex synapse with pyramidal neurons, non-pyramidal neurons or GABAergic neurons in the PFC (Vincent et al, 1995). This mesocortical dopaminergic pathway to the PFC is crucial for specific cognitive functioning such as learning and memory (Morris et al., 1982; Sutherland et al., 1982; Whishaw and Kolb, 1984; Winocur and Moscovitch, 1990: Squire et al., 1993; James et al., 1993) and disruption of mesocorticolimbic dopaminergic transmission by lesions in both rats and primates result in cognitive deficits (Nieoullon et al., 2002).

The first evidence that showed dopamine was linked to cognitive processes in animals was shown using rhesus monkeys whereby the depletion of dopamine in the dorsolateral PFC resulted in the impairment of spatial working memory, characteristics that also resembled results from removing the PFC itself (Brozoski et al., 1979). Furthermore, the disruption of dopaminergic signalling in the PFC showed it to be essential for the acquisition of spatial working memory tasks (Collins et al., 1998) and the dysregulation of D1 receptor signalling in this region has also been shown to impair spatial working memory in monkeys (Vijayraghavan et al., 2007). In rodent studies, systemic and intra-accumbens injections of haloperidol, a non-selective D2 dopamine receptor antagonist impaired spatial learning and memory in the Morris water maze (Ploeger et al., 1992; Ploeger et al., 1994). It has been reported that the decrease of dopamine in the medial frontal cortex is associated with a decrease in the performance in the Morris water maze (Lee et al., 1994). Intraperitoneal injection or systemic administration of a D1-like dopamine receptor agonist was shown to reverse spatial learning and memory in aged male rats (Bach et al., 1999; Hersi et al., 1995). Furthermore, dopamine D1 receptor knockout mice were severely impaired in the Morris water maze (Smith et al., 1998; El-Ghundi et al., 1999; Karasinska et al., 2000; Granado et al., 2008). Moreover, injections of dopamine D1 and

D2 receptor antagonists into the PFC of mice was shown to selectively impair spatial learning in the Morris water maze (Rinaldi et al., 2007). Other studies support these findings where intra-PFC injections of the dopamine D1 receptor antagonist, SCH 23390 or lesions to this area, caused an impairment in spatial learning in the radial arm maze (Seamans et al., 1995; Seamans et al., 1998; Floresco et al., 1997).

High levels of dopamine have been documented to have unfavourable effects on working memory function, which suggests that there is an optimum level for dopamine in cognitive functioning (Williams et al., 2006). This relationship between dopamine levels and cognitive performance is best described by an inverted U or a bell curve (Vijayraghavan et al., 2007). It is hypothesized that this bell curved relationship may be due to dopamine's concentration dependent effects on the different receptor subtypes that dopamine binds to (Dopamine D1-like vs. D2 receptors) (Savitz et al., 2006). It has been shown that low levels of dopamine D1 receptor activity in the PFC leads to executive functioning impairments, while high levels of dopamine D1 receptor activity caused cognitive inflexibility (Savitz et al., 2006), findings that suggest small changes in dopamine levels can have significant and sometimes negative effects on cognitive functioning.

Various lines of evidence have shown an involvement for dopamine in cognitive processes in humans, as has been seen in patients with Parkinson's disease, a neurodegenerative disorder that results in the death of dopaminergic neurons in the substantia nigra pars compacta causing motor impairments, autonomic dysfunction and cognitive impairments such as dementia (Beitz et al., 2014). It was observed that these patients exhibited cognitive deficits in visuospatial abilities and executive functions, which included planning and working memory (Azuma et al., 2003), the latter dependent on frontal cortical functioning (Alvarez et al., 2006). Evidence has shown that L-DOPA administered to patients with Parkinson's disease resulted in

the improvement of working memory (Mattay et al., 2002), behavioural flexibility (Cools et al., 2001) and planning (Cools et al., 2002). Dopamine has also been implicated in other neuropsychiatric disorders affecting cognition such as schizophrenia (O'Carroll et al., 2000, Goldman-Rakic et al., 2004; Tamminga, 2006) and it is abnormal signalling of the mesocortical pathway that is thought to be associated with cognitive disorders in schizophrenia (Masana et al., 2011).

1.3 Downstream Effectors Associated with Dopamine D5 and D1-D2 Heteromer Receptor Signalling that are Involved in Cognition

1.3.1 Calcium Calmodulin-Dependent Protein Kinase II

The dopamine D5 receptor and D1-D2 receptor heteromer have been shown to regulate CaMKII activity and expression through Gq-coupled-PLC linked calcium signalling in the rat brain (Hasbi et al., 2009; reviewed, Hasbi et al., 2011). Specifically, CaMKII is a serine/threonine protein kinase involved in an array of signalling cascades, and has been shown to be associated with learning and memory (Silva et al., 1992; Lisman et al., 2012; Coultrap et al., 2012; Yamauchi et al., 2005). In the brain, CaMKII is responsible for neuronal regulation, neurotransmitter synthesis and release, and synaptic plasticity (Yamauchi et al., 2005). CaMKII consists of four isoforms (α, β, γ, and δ), with CaMKIIα and β being expressed mainly in the brain (Yamauchi et al., 2005) with CaMKIIα highly abundant in the forebrain, and CaMKIIβ expressed more in the cerebellum (Sugiura et al., 1992). The activation of CaMKII is dependent on intracellular Ca^{2+} and calmodulin, a protein involved in the displacement of the auto-inhibitory domain of CaMKII (Griffith, 2004). Once activated, CaMKII prolongs Ca^{2+} signalling and is involved with the induction of long-term potentiation (LTP), an increase in signal transmission and strengthening of synaptic efficacy between neurons known to be

responsible for learning and memory (Lisman et al., 1994; Lledo et al., 1995; Otmakhov et al., 1997; Makinson et al., 1999). The activity of CaMKII was shown to increase during memory formation (Cammarota et al., 1998), whereas memory formation was impaired when the activity of CaMKII was blocked (Lisman et al., 2002; Elgersma et al., 2004; Irvine et al., 2006; Wayman et al., 2008; Lucchesi et al., 2011; Coultrap et al., 2012), indicative of a role for CaMKII in memory formation. Genetically mutated mice, which exhibit characteristics to prevent autophosphorylation of CaMKII at Thr286, showed impairments in cognitive performance in the Morris water maze, suggesting that autophosphorylation of CaMKII was involved in spatial learning (Giese et al., 1998). CaMKII has also been shown to be necessary for the induction of BDNF promoter activity (Zheng et al., 2012), a neurotrophin repeatedly shown to be involved in learning and memory (Yamada et al., 2002; Cunha, 2010).

1.3.2 Brain-Derived Neurotrophic Factor and Tropomyosin Related Kinase B

Dopamine has been shown to play a role in the regulation of BDNF in different regions of the brain. For example, activation of the dopamine D5 receptor by the administration of SKF 83959 has been linked to an increase in BDNF expression and signalling in PFC (Perreault et al., 2013). It has also been demonstrated that the activation of the dopamine D1-D2 receptor heteromer increased BDNF expression and signalling in striatal neurons and nucleus accumbens (Hasbi et al., 2009). BDNF is encoded by the BDNF gene (in humans on chromosome 11p) and is secreted throughout the brain, and its functions are to regulate neuronal growth, survival and differentiation (Patappoutian and Reichardt, 2001; Pang et al., 2004). BDNF protein and its receptor, tropomyosin related kinase B (TrKB) are highly expressed in the frontal cortex of primates including humans (Huntley et al., 1992; Hayashi et al., 2000; Weickert et al., 2003; Weickert et al., 2005) and in rodents (Hofer et al., 1990;,

Phillips et al., 1990; Connor et al., 1997; Yan et al., 1997; Lipska et al., 2001). The induction of BDNF transcription (Tao et al., 1998; Tabuchi et al., 2000; Zheng et al., 2011) and expression (Hasbi et al., 2009) has been linked to calcium signalling, whether through activation of CaMKII and the consequent inactivation of the transcriptional repressor MeCP2 (Zheng et al., 2012), or through phosphorylation of the transcription factor CREB following extracellular calcium influx (Tao et al., 1998). BDNF transcription can also be induced following D1-like receptor mediated PKA activation, which also results in the phosphorylation of CREB (Fang et al., 2003).

BDNF has been widely implicated in structural and synaptic plasticity, neuroprotection, anxiety, addiction, and learning and memory (Arancio and Chao, 2007; Bekinschtein et al., 2008; Vargas-Perez et al., 2009; Pang et al., 2004; Rattiner et al., 2005; Tyler et al., 2002; Yamada et al., 2003). The dysregulation of brain BDNF signalling in heterozygous BDNF knock-out mice has been shown to result in impairments in the acquisition of spatial memory (Linnarsson et al., 1997). In rats, the deprivation of endogenous BDNF using i.c.v. administration of anti-BDNF antibodies or an antisense oligonucleotide to BDNF was shown to impair spatial learning and memory in adult rats (Mu et al., 1999; Mizuno et al., 2000). It has been demonstrated that the induction of LTP in dentate gyrus of rat hippocampus showed increased BDNF expression and mRNA (Dragunow et al., 1993; Castren et al., 1993). BDNF has since been shown to be highly critical for the process of LTP (Cooke and Bliss, 2006; Bliss and Collingridge, 1993; Malenka and Bear 2004) and therefore plays an important role in long term memory (Bekinschtein et al., 2008, Alondo et al., 2005). Mature BDNF from the cleavage of proBDNF is necessary for LTP in the hippocampus and exogenous BDNF was also shown to enhance and even rescue synaptic transmission, whereas LTP was blocked when BDNF expression was inhibited at the CA1 synapses of the hippocampus, indicating that

BDNF alone was adequate for synaptic transmission in this region (Pang et al., 2004; Kang and Schuman, 1995). This finding was supported in homozygous BDNF knockout mice, which exhibited LTP impairments at the CA1 synapses of the hippocampus (Korte et al., 1995; Patterson et al., 1996). Heterozygous BDNF knockout mice also demonstrated deficits in LTP, effects similar to homozygous mice, suggesting that two copies of the BDNF gene are necessary for LTP (Bartoletti et al., 2002). Interestingly, these synaptic deficits were reverted back to normal with acute administration of BDNF or with viral-mediated increases in BDNF expression (Patterson et al., 1996; Korte et al., 1996). Furthermore, rats that were trained to locate a hidden platform in the water maze exhibited increased BDNF mRNA expression in the hippocampus, suggesting that learning may be linked to BDNF expression (Kesslak et al., 1998). BDNF gene deletion in the hippocampus of mice resulted in cognitive impairment in the novel object recognition task and spatial navigation task in the hidden-platform Morris water maze (Heldt et al., 2007). Similarly, the infusion of an anti-BDNF antibody in rat hippocampus impaired avoidance learning tasks (Alonso et al., 2002) and the injection of an antisense RNA to BDNF in the hippocampus of rats hindered contextual fear learning (Barns et al., 2008; Lee et al., 2004). The dysregulation of BDNF signalling in the brain has been shown to impair the formation of spatial memory (Mizuno et al., 2000; Saarelainen et al., 2000) and the regulation of hippocamal LTP (Minichiello et al., 2009). BDNF expression in rat hippocampus is essential in the consolidation and persistent storage of long term fear memory demonstrated in the inhibitory avoidance task and contextual fear conditioning (Bekinschtein et al., 2007). Furthermore, mature BDNF expression in rat hippocampus was shown to be essential for the acquisition and extinction of fear memory (Peters et al., 2010).

In addition to the hippocampus, BDNF expression and signalling through its receptor TrkB has been implicated in mediating neuronal activity and regulating PFC functioning

(Lewis et al., 2005; Savitz et al., 2006; Woo and Lu, 2006). Dopamine transporter knockout mice showed impaired spatial working memory in the spontaneous alternation task in the Y-maze, together with a significant decrease in BDNF protein in the frontal cortex (Li et al., 2010), suggesting that the alteration in dopamine regulation may play a role in cognitive functioning. It has been shown that Ts65Dn mice (trisomy of chromosome 16), a model of Down's syndrome had significantly decreased levels of BDNF protein in the frontal cortex, and these mice exhibited working memory impairments in the water-radial arm maze (Bimonte-Nelson et al., 2003). Moreover, mutant mice with selective BDNF knockout in the forebrain exhibited spatial learning and memory deficits as demonstrated in the Morris water maze (Gorski et al., 2003). TrkB knockouts in the forebrain of mice demonstrated LTP impairments at the CA1 hippocampal region and impaired spatial learning in the Morris water maze (Minichiello et al., 1999). In addition, transgenic mice, which overexpressed the truncated non-functional TrkB receptor in cortical neurons were shown to have impaired long-term spatial memory, assessed using the Morris water maze task (Saarelainen et al., 2000).

BDNF signalling has been linked to the etiology of cognitive disorders such as in schizophrenia and Alzheimer's disease (Zhang et al., 2012; Zuccato and Cattaneo, 2009; Xiu et al., 2009; Arancio et al., 2007). The dysregulation in cognitive function associated with schizophrenia has been associated with decreased BDNF and TrkB expression in the dorsolateral PFC (Weickert et al., 2003; Weickhert et al., 2005; Hashimoto et al., 2005). Studies in normal humans have revealed that a single nucleotide polymorphism (SNP), from valine to methionine at position 66 of the BDNF gene is highly correlated with impairments of episodic memory (Egan et al., 2003; Hariri et al., 2003). The frontal cortex and hippocampus of persons with Alzheimer's disease also exhibit decreased BDNF and full length TrkB

expression, along with an increased expression of the truncated non-signalling TrkB isoform (Gupta et al., 2013; Ferrer et al., 1999).

1.3.3 Akt (Protein Kinase B)

Once BDNF binds to TrkB, it initiates a number of signalling pathways including the phosphatidyl-inositol 3 kinase (PI3K) cascade (Huang et al., 2001; Cross er al., 1995). The PI3K pathway is an intracellular signalling pathway involved in the activation of Akt by its phosphorylation at Ser473, a process mediated by the enzyme 3-phosphoinositide-dependent protein kinase 1 (PDPK1 or PDK1) (Minichiello et al., 2009; Hart and Vogt, 2011; Hemmings and Restuccia, 2012). Akt is a serine/threonine kinase protein which plays an important role in cellular processes involved in cell survival, growth, proliferation, metabolism, angiogenesis and migration (Manning and Cantley, 2007) as well as in synaptic plasticity, working memory and fear conditioning (Lin et al., 2001; Lai et al., 2006; Niizuma et al., 2009; Freyberg et al., 2010). The activation of the Akt signalling pathway is crucial for LTP (Sanna et al.; 2002; Karpova et al., 2006) and the blockade of this pathway using PI3K inhibitors (LY294002 and wortmannin) was shown to impair long-term memory consolidation, recognition memory (Horwood et al., 2006), and spatial learning in rats (Mizuno et al., 2003). There is reported evidence that the levels and activity of Akt are decreased in the PFC of patients with schizophrenia (Emamian, 2012; Lai et al., 2006; Thiselton et al., 2008). Moreover, in post-mortem human brain samples from schizophrenic patients, the levels of Akt expression were reduced in the hippocampus and frontal cortex, when compared to healthy control subjects (Emamian et al., 2004). Also, the decrease in AKT activity has been implicated in other neuropsychiatric disorders, such as shown in post-mortem brain samples of patients with Alzheimer's disease (Lee et al., 2009). In the APP/PS1 mouse model of Alzheimer's disease,

the increase in AKT activity induced by pyrrolidine dithiocarbamate (an inhibitor of nuclear factor- κB) was shown to improve spatial learning (Malm et al., 2007).

A mechanism by which Akt may regulate cognitive function is through the inhibition of GSK-3 (Figure. 1). Akt has been shown to regulate GSK-3 activity through its phosphorylation in response to incubation with BDNF, demonstrated in cerebellar granule neurons (Smillie, 2013), and in gliomas (Atkins, 2012).

1.3.4 Glycogen Synthase Kinase-3

Glycogen synthase kinase 3 is a serine/threonine kinase that plays a significant role in apoptosis, cell proliferation, and differentiation. There are two isoforms of GSK-3, GSK-3α and GSK-3β, with GSK-3β more widely studied as a result of its role in the pathophysiology of neurodegenerative and neuropsychiatric disease (Li et al., 2014). The most significant known role for GSK-3 is in Alzheimer's disease (Phujan et al., 2010; Peineau et al., 2008) with Alzheimer's disease patients exhibiting increased activation of GSK-3 in the frontal cortex and hippocampus which is linked to the production of hyperphosphorylated tau protein, a pathophysiologic characteristic of this disorder (Blalock et al., 2004; Leroy et al., 2007; Hooper et al., 2008; Lei et al., 2011). It has been reported that patients with schizophrenia also exhibit decreased levels of Akt and increased activity of GSK-3 in post-mortem hippocampus and frontal cortex (Emamian et al., 2004; Koros et al., 2007). Furthermore, the mood-stabilizing medication lithium, which is used in the treatment of patients with schizophrenia has been shown to increase the inactivation of GSK-3 (Beaulieu et al., 2011; Emamian et al., 2012). Specifically, patients with schizophrenia have significantly reduced levels of phosphorylated GSK-3β in the frontal cortex (Emamian et al., 2004; Karege et al., 2007). In rodents, it has been shown that the overexpression of GSK-3 in the forebrains of rats induced

spatial learning deficits which was ameliorated following the normalization of GSK-3 activity (Hernandez et al., 2002). The overexpression of GSK-3β has also been shown to prevent the induction of LTP in mouse hippocampus (Hooper et al., 2007), rat hippocampus (Zhu et al., 2007) and its effects mediated through the enhancement of long-term depression (Peineau et al., 2007), suggesting that it is important in the induction of memory formation. The effects of LTP suppression were ameliorated with the administration of lithium, a GSK-3 inhibitor, in rat hippocampus (Zhu et al., 2007), suggesting that overactivation of GSK-3β may be linked to the working memory impairment observed in patients with schizophrenia. Interestingly, previous work from our laboratory have shown that the activation of the dopamine D5 receptor and the dopamine D1-D2 receptor heteromer suppressed the activity of GSK-3, whereby the dopamine D5 receptor mediates this effect through BDNF signalling (Perreault et al., 2013), suggesting that these dopamine receptors may play an important role in cognition.

1.4 SKF 83959: A Methodological Tool to Study Gq-Coupled Dopamine Receptor Function: Advantages and Limitations

SKF 83959 (3-methyl-6-chloro-7,8-hydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine), an atypical dopamine D1-like receptor agonist was shown to activate both the dopamine D5 and D1-D2 receptor heteromer, leading to an increase in intracellular calcium levels, mediated by the PLC pathway (Lee et al., 2004; Rashid et al., 2007; Sahu et al., 2009; Perreault et al., 2013). SKF 83959 was shown to have none or minimal agonist effect at the dopamine D1 receptor homooligomer coupled to Gs-mediated adenylyl cyclase activity, or function as a partial antagonist as evidenced by the decrease of cAMP production induced by the dopamine D1 receptor agonist, SKF 81297 (Jin et al., 2003; Makihara et al., 2007; Fujita et al., 2010). SKF 83959 also exhibits affinity for other receptors including the PLC-coupled

serotonin 5HT-2C receptor (Chun et al., 2013), the α-adrenergic receptor 2C (Chun et al., 2013) and may act as an allosteric modulator for the sigma-1 receptor (Guo et al., 2013). Currently, there are no available drugs that specifically target and activate the Gq-coupled dopamine receptors, with the exception of SKF 83959. However, SKF 83959 as stated before, can have cross-reactivities for other receptors, which may or may not activate other pathways and produce unwanted effects (Table 1), since other than the affinity shown by radioligand binding, the effects of SKF 83959 on the activation state of these receptors has not been shown.

Basic Properties of Receptors that Bind to SKF 83959

Receptors Bound by SKF 83959	Dopamine D1-D2 Receptor Heteromer (activation)	Dopamine D5 Receptor (activation)	Serotonin 5-HT 2C Receptor (binding)	α-Adrenergic Receptor 2C (binding)	Sigma-1 Receptor (Allosteric Modulator)
Gene Symbol	DRD1, DRD2	DRD5	HTR2C	ADRA2C	SIGMAR1
G-Protein Coupling	Gαq	Gαs, Gαq	Gαq	Gαi	N/A; modulate IP3 receptor
Affinities for SKF 83959 (nM)	D1R - K_i = 1.7 D2R - K_i = 567 D1R-D2R K_i = 10	K_i = 4.0	K_i =32.8	K_i = 31.1	N/A

Table 1. Signalling properties of the receptors modulated by SKF 83959.

D1R, D1 dopamine receptor; D2R, D2 dopamine receptor; IP3 receptor, inositol triphosphate receptor.

1.4.1 Serotonin 5HT-2C Receptor

SKF 83959 has been demonstrated to have high affinity radioligand binding for serotonin 5HT-2C receptor, with a Ki value of 32.8 nM (Chun et al., 2013), however, the activation of this receptor by SKF 83959 has not been shown. The serotonin receptor (5-hyroxytryptamine, 5-HT) receptor family are class A GPCRs that are localized in the central and peripheral nervous systems (Nichols et al., 2008; Hoyer et al., 1994; Frazer et al., 1999). Serotonin is a monoamine neurotransmitter that plays a role in endocrine functions, depression, cognition, memory, sleep, sex, aggression and appetite (Frank et al., 2002; Popova et al., 2002; Millan, 2005; Millian et al., 2008; Nichols et al., 2008).

The 5-HT2 family of receptors are Gαq-coupled and include 5-HT2A, 5-HT2B and 5-HT2C receptors (Sah et al., 2000; Nichols et al., 2008). In the brain, the 5-HT2C receptors are present widely distributed in the cortex, amygdala, basal ganglia, hippocampus and thalamus (Clemett et al., 2000; Pasqualetti et al., 1999) and are expressed in GABAergic, glutamatergic and dopaminergic neurons (Jensen et al., 2010). As with the Gq-coupled dopamine receptors, the Gq-coupled 5-HT2C receptor is also linked to PLC activation and intracellular calcium release (Jensen et al., 2010). The 5-HT2C receptors are implicated in cognitive processes such as memory (Tecott et al., 1998; Berg et al., 2008), spatial memory (Du et al., 2007) and learning (Berg et al., 2008). In rodents, a 5-HT2C receptor agonist was demonstrated to improve cognitive performance in the novel object recognition test (Siuciak et al., 2007). Also, SB-242084, a selective 5-HT2C receptor antagonist was shown to impair the performance in the 5-choice serial reaction time task (Robinson et al., 2008; Fletcher et al. 2007). Other findings have contradicted these studies, where the intraperitoneal administration of 5-HT2C receptor antagonist (SB 243213A) to rodent models of schizophrenia was shown to improve reversal learning (McLean et al., 2009). Studies have shown that chronic

intraperitoneal administration of a 5-HT2C receptor antagonist (S32006) resulted in increased BDNF mRNA expression in rat dentate gyrus (Dekeyne et al., 2008) and increased BDNF protein expression in the mPFC when locally infused into the ventral tegmental area (Opal et al., 2013). Furthermore, 5-HT2C receptor knockout mice were demonstrated to have a significant increase of BDNF in the hippocampus (Hill et al., 2011), suggesting that the 5-HT2C receptors play a potential role in the regulation of BDNF.

1.4.2 α-Adrenergic 2C Receptor

SKF 83959 has been demonstrated to have high affinity for the α-adrenergic 2C receptor, with a Ki value of 31.1 nM (Chun et al., 2013), however the effects of SKF 83959 on the activation state of the receptor has not been discerned. The adrenergic receptors are a class of GPCRs that bind to norepinephrine and epinephrine and their role in brain processes include attention, arousal, mood, learning, memory and mediation of the stress response (Sved et al., 2001). The receptors are divided into two subgroups, α and β adrenergic receptors. The α adrenergic receptors consist of α1 and α2 subtypes. The α1 receptors are coupled to Gq linked PLC activated IP3-calcium signalling and the α2 receptors are coupled to the Gi protein and inhibition of adenylyl cyclase activity. The α2 receptors are present on presynaptic neurons, exerting inhibitory neuronal effects and are further divided into 2A, 2B and 2C subtypes (Newcorn et al., 2003). The α adrenergic 2A receptors are highly expressed in the PFC, amygdala, and hippocampus (Aoki et al., 1994), the α adrenergic 2B receptors are highly expressed in the thalamus, and the α adrenergic 2C receptors are highly expressed in the PFC, amygdala, substantia nigra, ventral tegmentum, striatum and hippocampus (Scheinin et al., 1994; Saunders et al., 1999), although they are expressed at low abundance on the cell surface (Olli-Lahdesmaki et al., 1999). Evidence suggests that the physiological/pharmacological

effects of α2 receptor agonists on working memory are not likely mediated by the adrenergic 2C receptors subtypes (Newcorn et al., 2003) but may be due to the other α adrenergic receptor subtypes (MacMillan et al., 1996; Avery et al., 2000; Wang et al., 2007; Levy, 2008). Studies have shown that attention and working memory in rats and monkeys were improved by administration of an α-2 adrenergic receptor agonist, guanfacine (Ramos et al., 2006; Sagvolden et al., 2006; Rama et al., 1996). Furthermore, these improvements in monkeys were observed in a dose-dependent manner (Arnstern et al., 1988; Franowicz and Arnsten, 1999). The beneficial effects of guanfacine on cognition were lost in α adrenergic 2A receptor knockout mice but not in α adrenergic 2C receptor knockout mice, (Tanila et al., 1999; Franowicz et al., 2002), supporting that these cognitive effects were not mediated by the α adrenergic 2C receptors. Moreover, an α-adrenergic 2A receptor antagonist was shown to impair spatial working memory (Li and Mei, 1994). Together these studies indicate that while SKF 83959 may exhibit affinity for the α-adrenergic 2C receptor *in vitro*, the coupling of the receptor to the Gi protein, its intracellular localization, and its lack of contribution to cognitive functioning suggests that there is unlikely to be any potential effect of SKF 83959 on cognitive performance which would be mediated through this receptor.

1.4.3 Sigma-1 Receptors

SKF 83959 has been demonstrated to be a potent allosteric modulator of the binding of ^{3}H(+)-pentazocine, a selective sigma-1 receptor agonist, to the sigma-1 receptor (Guo et al., 2013). SKF 83959 as a positive allosteric modulator would promote the binding of a ligand to its receptors and reduce the dissociation rate between ligand and its receptor (Cobos et al., 2006). The sigma receptor is comprised of sigma-1 and sigma-2 receptor subtypes, and known exogenous ligands that bind to these receptors include, fluvoxamine, methamphetamine,

dextromethorphan (Guitart et al., 2004; Hindmarch and Hashimoto, 2010; Shin et al., 2007; Kaushal and Matsumoto, 2011). Recent studies suggest that N,N- dimethyltryptamine or dehydroepiandrosterone (DHEA) are now considered the endogenous ligands (Fontanilla et al., 2009; Li et al., 2009). The sigma-1 receptors are intracellular chaperone proteins that reside in the mitochondrion-associated endoplasmic reticulum membrane (MAM) (Kourrich et al., 2012), and are expressed highly in neurons (Hayashi and Su, 2005). The sigma-1 receptors are localized in the cerebral cortex, hippocampus, and substantia nigra (Walker et al., 1990; Bouchard and Quirion, 1997; Alonso et al., 2000; Langa et al., 2003). It has been demonstrated that the sigma-1 receptors translocate from the mitochondria-associated endoplasmic reticulum membrane (MAM) to interact and bind to IP3 receptors (Su et al., 2010; Maurice et al., 2001). Through the regulation of IP3 receptors, the sigma-1 receptors can modulate calcium signalling (Hyashi et al., 2000; Maurice and Su, 2009; Su et al., 2010; Fishback et al., 2010). Evidence suggests that the sigma-1 receptors are not directly coupled to G-proteins (Hong and Werling., 2000). Furthermore, rats treated chronically with intraperitoneal injections of a selective sigma-1 receptor agonist, SA4503, were shown to have enhanced BDNF expression levels in the hippocampus (Kikuchi-Utsumi and Nakaki, 2008), a process due to the increase in post-translational modification of converting pro-BDNF into mature BDNF, demonstrated using a rat neuroblastoma cell line (Fujimoto et al., 2012). This suggests that BDNF regulation may be mediated by the activation of sigma-1 receptors. Studies using the selective sigma-1 receptor agonist, 2-(4-morpholinoethyl)-1-phenylcyclohexane-1-carboxylate hydrochloride (PRE-084), have shown that subcutaneous administration of this compound to aged rats showed significant improvement in spatial learning in the water maze (Maurice et al., 2001).

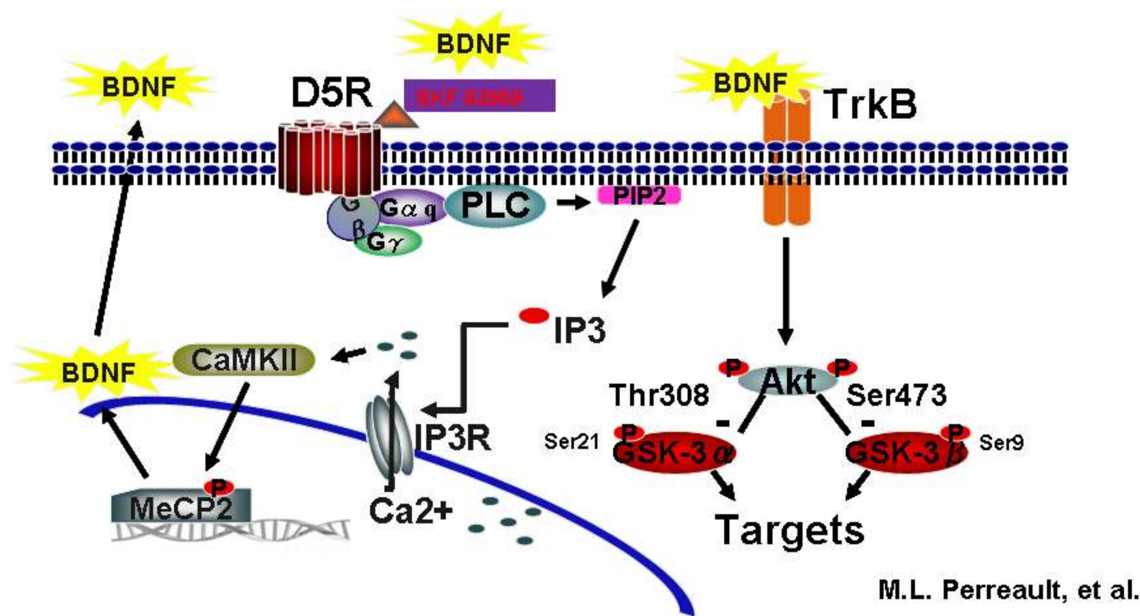


Figure 2: A proposed schematic diagram of the Gq coupled signaling mediated by the dopamine D5 receptor. The activation of the Gq-coupled D5 receptor results in the activation of PLC. PLC cleaves PIP2 to DAG and IP3 from the intracellular membrane. IP3 binds to IP3 receptors to increase intracellular $Ca2^{+}$ which results in the activation of CaMKII. CaMKII increases the transcription of BDNF through the phosphorylation of a transcriptional regulator MeCP2. BDNF is released and binds to its receptor TrkB to initiate a downstream signalling pathway to activate Akt and inactive GSK-3. PLC, phospholipase C; PIP2, phosphatidylinositiol 4,5-biphosphate; DAG, diacyl-glycerol; IP3, inositol triphosphate; CaMKII, calcium calmodulin-dependent protein kinase II; BDNF, brain-derived neurotrophic factor; MeCP2, methyl CpG binding protein 2; TrkB, tropomyosin receptor kinase B; Akt, protein kinase B; GSK-3, glycogen synthase kinase-3. (Perreault, 2013).

1.5 Thesis Hypothesis, Objectives and Rationale

Since the discovery of dopamine by the Nobel prize winner Arvid Carlsson (Carlsson et al., 1957), the role of dopamine in many important physiological functions has been established and it has also been implicated in many neuropsychiatric disorders such as schizophrenia, attention deficit hyperactive disorder, drug addiction, neurodegenerative diseases as well as cognition in general (Pivonello et al., 2007, De Keyser et al., 1990; McMahon and Bradley, 1990; Goldberg et al., 1989; Berman and Weinberger, 1990; Park et al., 1992; Oktubo et al., 1997; Russell et al., 1995; Nieoullon, 2002). Cognitive disorders are a serious problem as most cognitive impairments, which are linked to genetic disorders, injury, aging or neuronal degeneration, progressively worsen and do not have any treatments available at the present time (Torpy et al., 2010).

Studies have shown that the mesocortical dopaminergic signaling pathway is highly implicated in cognitive processes (Morris et al., 1982; Sutherland et al., 1982; Whishaw and Kolb, 1984; Winocur and Moscovitch, 1990: Squire et al., 1993; James et al., 1993). The dopamine D5 receptor (Luedtke et al., 1999) and D1/D2 receptor coexpression (Zhang et al., 2010) are highly abundant in the PFC, a region dominantly involved in learning and memory. This suggests a potential functional role of these receptors in the regulation of cognitive processes, a finding supported by our previous work showing the regulation of several proteins in this region known to be involved in cognition. Specifically, evidence has shown that acute activation of the Gq-coupled dopamine D5 receptor increased BDNF-TrkB and Akt signalling in the PFC that coincided with inactivation of GSK-3. Similarly, dopamine D1-D2 receptor heteromer activation suppressed GSK-3 activity through a BDNF-independent mechanism (Perreault et al., 2013). As a result of these findings we hypothesize that the activation of Gq-coupled dopamine receptors may lead to enhanced specific cognitive performance, through

proposed mechanisms that we have identified in our lab (Figure 2). The agonist SKF 83959 will be used to investigate the role of Gq-coupled dopamine receptors in specific cognitive tests involving learning and recognition memory in rats. As the effect of longer SKF 83959 administration on protein expression in PFC has not been evaluated the effects of repeated receptor activation on expression and activation of BDNF-TrkB, Akt, and GSK-3 in this region will be also be deteremined.

1.5.1 Overall Purpose and Objectives:

The overall purpose of this investigation was to elucidate a physiological role for the Gq-coupled dopamine receptors in regulating cognition in rats. This investigation will be carried out with three experiments. Utilizing the agonist SKF 83959 we will first evaluate the effects of activating the Gq-coupled dopamine D5 receptor and D1-D2 receptor heteromer on the expression and activation of proteins linked to cognitive performance in the PFC of rats. Moreover, we will assess the effects of SKF 83959 on certain types of PFC related memory functions. Spatial learning and memory in rats will be evaluated using the egocentric learning task in the Morris water maze. Finally, recent, location and recognition memory will be analyzed using three tests of object recognition memory.

1.5.2 Hypotheses and Rationale:

Hypothesis 1: Activation of the dopamine D5 receptor and D1-D2 receptor heteromer, through acute and repeated agonist administration will increase BDNF expression and signaling through TrkB, activate Akt and inhibit GSK-3 in the PFC.

Rationale: Evidence indicates that the acute activation of dopamine D5 receptor will result in the increased the expression of CaMKIIα, BDNF, TrkB, and Akt and phosphorylation of Akt (Ser473), GSK-3α, and GSK-3β in the PFC. Acute activation of the dopamine D1-D2 receptor heteromer was shown to increase the expression of CaMKIIα and phosphorylation of GSK-3α, and GSK-3β in the PFC.

Hypothesis 2: Activation of the dopamine D5 receptor and D1-D2 receptor heteromer in rats will result in improved spatial learning and memory and object recognition memory

Rationale: The activation of the dopamine D5 receptor and D1-D2 receptor heteromer using SKF 83959 increased the expression and activation of proteins in the PFC that are highly involved in cognition. The PFC is also involved in various forms of memory, such as working memory and spatial memory. Together these findings suggest a potential role for these receptors in spatial learning and memory and object recognition memory.

Objectives:

Objective 1: To examine the effects of Gq-coupled dopamine receptor activation in rats on the expression levels of proteins in the PFC known to be linked with cognition.

Objective 2: To examine the effects of Gq-coupled dopamine receptor activation on spatial learning and memory using the Morris water maze.

Objective 3: To examine the effects of Gq-coupled dopamine receptor activation using SKF 83959 on recency memory, objection location memory and recognition.

2 Methods and Materials

2.1 Animals

Male Sprague Dawley rats (Charles River, Quebec, Canada) aged 3-4 months were housed (two to three per cage) in the Division of Comparative Medicine at the University of Toronto. Each rat weighed approximately 250-300 g at the start of the experiments and was maintained in a standard 12h light/dark cycle in a temperature and humidity controlled room, with free access to standard rodent chow and water. Testing was performed during the light cycle of the day. The animal protocols were approved by the University of Toronto Animal Committee, in accordance with the guidelines set by the Canadian Council on Animal Care.

2.2 Drugs

SKF 83959 hydrobromide (Tocris Bioscience), was dissolved in saline with 5% DMSO and vehicle was comprised of saline with 5% DMSO. Various Doses of SKF 83959 (0.4, 1.5 and 2.5 mg/kg) were utilized throughout this experiment. Repeated administration of 0.4 mg/kg s.c. SKF 83959 to rats has been shown to increase BDNF expression in the nucleus accumbens (Hasbi et al., 2009), whereas an acute injection of 1.5 mg/kg s.c. SKF 83959 has been shown to increase BDNF signalling in the PFC of rats (Perreault et al., 2013). 2.5 mg/kg s.c. SKF 83959 was used in the high dose SKF 83959 treatment group to make certain that this dose would elicit an effect in rats after a single injection.

2.3 Tissue Processing Preparation

Animals were administered a single injection of 1.5mg/kg s.c. SKF 83959 or vehicle for the acute treatment group. The rats from the repeated SKF 83959 treatment group were

administered SKF 83959 0.4 mg/kg s.c. daily for 3 days. The control groups were administered vehicle (5% DMSO in saline) daily for 3 days. All injections were administered at a volume of 1 ml/kg. Ninety minutes after the final injection, rats were decapitated, the brains removed, and the PFC dissected and frozen on dry ice. At his time-point in previous studies it has been shown that maximal increases in BDNF expression following SKF 83959 treatment occurs in striatal neurons (Hasbi et al., 2009) and inactivation of GSK-3 occurs in cortical neurons (Yu et al., 2008). Samples were stored at -70°C for subsequent Western Blot analysis. Tissue was thawed on ice and homogenized by sonication in a hypotonic lysis buffer solution containing protease and phosphatase inhibitors and 30 micrograms of the protein was aliquoted and lysis buffer was added to make up a total volume of 15 μl. An equal volume of sample buffer containing 0.5M Tris HCl (pH 6.8), 200 μl glycerol, 10% SDS, 1% bromophenol blue, 50 μl β-mercaptoethanol, and distilled water was added to each sample followed by incubation of the samples for 3 minutes at 95˚C.

2.4 Western Blot Analysis

Tissue samples were separated by SDS-polyacrylamide gel electrophoresis using pre-cast 10% Tris-Glycine gels (Invitrogen) and then electroblotted onto a polyvinylidene difluoride membrane (PVDF) for 2 hours and 15 minutes (Fan et al., 2005). Membranes were blocked with Tris buffered saline with Tween-20 (TBS-T) containing 5% milk (skim milk powder in Tris-buffered-Tween) at room temperature for an hour and then incubated with primary antibodies at 4°C to CaMKIIα, Akt, pAkt Ser473, GSK -3α/β, pGSK -3α/β (Cell Signaling), pCaMKIIα (Pierce Antibodies), TrkB (BD Bioscience), BDNF, GAD67, and GAPDH (Abcam) for 12 hours at 4°C. The dilutions of the antibodies were CaMKIIα (1:1500), Akt (1:1000), pAkt Ser473 (1:1000), GSK-3α/β (1:5000), pGSK-3α/β (1:2500),

pCaMKIIα (1:5000), TrkB (1:2500), BDNF (1:10000), GAD67 (1:5000), and GAPDH (1:10000). Membranes were then washed in TBS-Tween and incubated at room temperature for 2 hours with species-specific secondary antibodies (Bio-Rad Laboratories, Hercules, CA, USA). The detection of proteins conjugated with antibodies was performed with chemiluminescence (Mandel Scientific). Blots were exposed to film, and signal intensity was quantified relative to the loading control GAPDH, using Image J software.

2.5 Morris Water Maze Test (Egocentric Learning)

The Morris water maze is a traditional method frequently utilized in behavioural neuroscience to investigate cognitive functions (Morris et al., 1981). Spatial learning and memory are required for navigation around an environment and are dependent on both allocentric and egocentric learning strategies (Morris et al., 1981; Warburton et al., 1997). Allocentric navigation strategies in the Morris water maze are dependent on the utilization of external cues and involves hippocampal processing (Maaswinkel et al., 1999; Morris et al., 1981; Save and Moghaddam, 1996; O'keefe et al., 1978; Eichenbaum et al., 1990), whereas the egocentric learning strategies in the Morris water maze are dependent on the spatial relationship between objects and the position of the subject and involves PFC functioning (Ma et al., 2003; Ethier et al., 2001; Save et al., 1996). The Morris water maze uses the motivation to escape a stressful situation (water) as an aversive stimulus to condition the rats to spatially learn (Xing et al., 2012), whereas the radial arm maze, which also measures spatial learning and memory uses food as a motivational reward (Olton et al., 1976). The egocentric learning Morris water maze test consisted of two groups of male rats, SKF 83959 (n = 8) and vehicle (n = 8) treatment groups. Rats were administered a single injection of 0.4 mg/kg SKF 83959 or vehicle s.c. 5 minutes before the start of the experiment each day. Two circular tanks (4 feet

and 6 feet diameter) were filled with room temperature water (20˚C). A hidden circular glass platform was submerged an inch under the water in one of the four quadrants within the tank. The Morris water maze was located in a temperature controlled, sound isolated room and lights were dimmed during the experiments.

The experiment consisted of 4 consecutive trials per day over 4 days, where in each trial the hidden platform was continually relocated to the designated quadrant of the tank (quadrant #1 to quadrant #4). The rats were released in specific starting areas whereby they always needed to swim to the right to find the hidden platform to escape the water. Each inter-trial interval was 10-30 seconds in length. During this time the pool was cleaned. Rats that failed to find the hidden platform within 2 minutes were guided to the platform to rest on it for 30 seconds. After all 4 trials, the rats were placed in a cage under a heating lamp to dry. The whole experiment was recorded by a video recording camera located directly above the water tank. The latency time to escape the Morris water maze was recorded for each trial.

2.6 Tests of Object Recognition Memory

The experiments were conducted in an opaque open field (1 meter x 1 meter) which was surrounded by a plastic wall that measured 60 cm high. The open field apparatus was placed in a temperature controlled, sound isolated and a dimly-lit room. A camera placed above the open field was used to record the time each rat spent on exploring the objects and the animal's behaviour. Three different paired sets of objects were used, which consisted of a white sphere with a conical base, a glass bottle filled with water, and a white and black cube attached to a cuboid. The different objects varied in shape, size, colour, and were heavy enough so that they could not be displaced by the animal. The pairs of objects were placed in the open field and the whole apparatus cleaned with 10% virox before each trial to eliminate

odor. Animals were placed near the sides of the wall in the open field at the start of the exploratory phase and test phase. The time spent exploring each object was recorded when the nose of the rat touched the object. Before the start of the experiment, vehicle group 1 and high dose SKF 83959 treatment group were habituated for 30 minutes, whereas vehicle group 2 and low dose SKF 83959 treatment group were habituated for 5 minutes. The three tests of recognition memory, which consisted of a recency task, object location recognition test and the novel object recognition test requires judgements about recency, object location, and object identity. Recognition memory is the ability to discriminate a previously encountered stimulus, from a novel stimulus. These tests take advantage of a rodent's natural behaviour to explore novel objects, used in many studies to examine recognition memory (Dere et al., 2007; Winters et al., 2008; Warburton et al., 2010).

2.6.1 Recency Task

The recency task is a behavioural test used to assess recent or short term memory. In this study, four groups of adult male rats were used consisting of two separate vehicle 1 mL/kg s.c. (5% DMSO in saline) (n = 10-11) groups, high dose SKF 83959 2.5mg/kg s.c. (n = 12) and low dose SKF 83959 0.4 mg/kg s.c. (n = 10) treatment groups. Injections were made 5 minutes before the start of the experiment.

The experiment consisted of 3 phases (exploratory phase 1, 2 and test phase). Each rat was allowed to explore the two identical objects for 5 minutes in each phase. Exploratory phase 1 consisted of two identical objects A, exploratory phase 2 consisted of two identical objects B and the test phase consisted of an object A and an object B. There was a delay of an hour between the two exploratory phases and a 15 minute interval between exploratory phase 2 and the start of the test phase. During the test phase, the time spent exploring each object was

recorded. If the rat has an intact recency memory, it would prefer to explore the least recently seen object (object A) (Nelson et al., 2011).

2.6.2 Object Location Task

The object location task is a behavioural test used to assess object location memory. In this study, four groups of adult male rats were used consisting of two distinct vehicle 1 mL/kg s.c. (5% DMSO in saline) (n = 10-11) groups, high dose SKF 83959 2.5mg/kg s.c. (n = 12) and low dose SKF 83959 0.4 mg/kg s.c. (n = 10) treatment groups. Injections were made 5 minutes before the start of the experiment.

The experiment consisted of 2 phases, an exploratory phase and a test phase. Each rat was allowed to explore two identical objects for 5 minutes in each phase. The exploratory phase consisted of 2 identical objects that the animals could explore. During the test phase, one of the two objects was displaced to a novel spatial location. There was an interval of 10 minutes between the exploratory phase and the test phase. During the test phase, the time spent exploring each object was recorded. If the rat has an intact object location memory, it would prefer to explore the displaced object (Nelson et al., 2011).

2.6.3 Novel Object Recognition Task

The novel object recognition task is a behavioural test used to assess recognition memory. In this study, four groups of adult male rats were used consisting of two distinct vehicle 1 mL/kg s.c. (5% DMSO in saline) (n = 10-11) groups, high dose SKF 83959 2.5mg/kg s.c. (n = 12) and low dose SKF 83959 0.4 mg/kg s.c. (n = 10) treatment groups. Injections were made 5 minutes before the start of the experiement.

The experiment consisted of 2 phases, an exploratory phase and a test phase. During the exploratory phase each rat was allowed to explore the two identical objects A for 5 minutes after which the rat was removed from the arena, and one of the objects replaced with a novel object B. Ten minutes later, the rat was placed back into the arena and the time spent exploring each object was recorded. If the rat has an intact object recognition memory, it would prefer to explore the newly replaced object (object B) (Nelson et al., 2011).

2.7 Statistical Analysis

Statistical analysis and graph generation were made using SPSS and GraphPad Prism software. All results were reported as mean ± s.e.m. For western blot analysis, the data was analyzed by densitometry (optical density of the band x area of the band / optical density of loading control, GAPDH). The graphs were normalized and expressed as a percent relative to the vehicle-treated groups. Comparisons of means for protein expression in the PFC of rats were performed using the Student's t-test (two-tailed, unpaired; statistical significance was set to $P<0.05$). For the Morris water maze, a repeated measures ANOVA was used to analyze the difference in escape latency between groups for all four days using treatment as the between subject factor and the days as the within subject factor, followed by a post-hoc analysis (Bonferroni). The Student's t-test (two-tailed, unpaired; statistical significance was set to $P<0.05$) was used to analyze the difference in escape latency between groups on each day. Data analysis from the object recognition memory tests was performed by Student's t-test (two-tailed, unpaired; statistical significance was set to $P<0.05$) to compare the difference between the vehicle and SKF 83959-treated groups for the time spent exploring each object during the test phases.

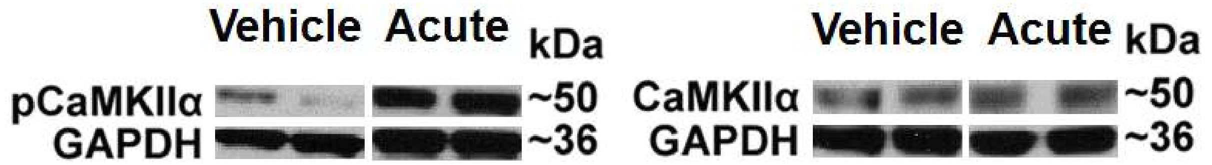
3 Results

The effects of SKF 83959 on the activation of BDNF-TrkB signalling in PFC

A single injection of SKF 83959 administered to rats or mice has previously been shown to increase CaMKIIα and BDNF expression in the PFC (Perreault et al., 2013) and in the nucleus accumbens (Rashid et al., 2007; Hasbi et al., 2009), whereas its repeated daily administration reduced CaMKII expression levels in NAc (Perreault et al., 2010). As the activation (phosphorylation) of CaMKIIα has been implicated in the expression BDNF (Zhou et al., 2006), the effects of acute and repeated administration of SKF 83959 on CaMKIIα activation and BDNF-TrkB signalling in rat PFC were compared in rats. Acute or repeated treatment with SKF 83959 did not have any effects on the total levels of CaMKIIα expression in rat PFC compared to the vehicle (Fig. 3A). However, increased CaMKIIα activation was observed in PFC following acute ($T(10)= 2.773$, $P=0.020$; Fig. 3B), but not repeated treatment with SKF 83959 as evidenced by a significant increase in the phosphorylation of CaMKIIα at Thr 286. A trend towards increased expression of BDNF in the PFC of the repeated treatment group ($T(9)= 2.055$, $P=0.07$; Fig. 4) was observed, an effect that may have shown significance with a larger sample size.

The effects of BDNF are mediated through its receptor, TrkB, which exists as two isoforms, a full length functional TrkB (fTrkB) and a truncated non-functional receptor (tTrkB). Both acute and repeated treatment with SKF 83959 significantly increased the expression of fTrkB in the PFC (Acute: $T(10)= 2.881$, $P=0.016$; Repeated: $T(10)= 2.567$, $P=0.028$; Fig. 5A). These effects were associated with a corresponding increase in the expression of tTrkB although statistical significance was only achieved following acute SKF 83959 treatment (Acute: $T(10)= 2.834$, $P=0.018$; Repeated: $T(10)= 2.061$, $P=0.066$; Fig. 5B). As BDNF has been previously linked to increasing the GABA synthesizing enzyme glutamate

decarboxylase, GAD67 expression levels in PFC of rats following a single administration of SKF 83959 (Perreault et al., 2013), we also evaluated the effect of acute or repeated SKF 83959 treatment on GAD67 levels. Whereas acute treatment with SKF 83959 in rats significantly increased the expression levels of GAD67 in rat PFC ($T(10) = 3.246$, $P = 0.009$, Fig. 6), no changes were evident following repeated SKF 83959 treatment.



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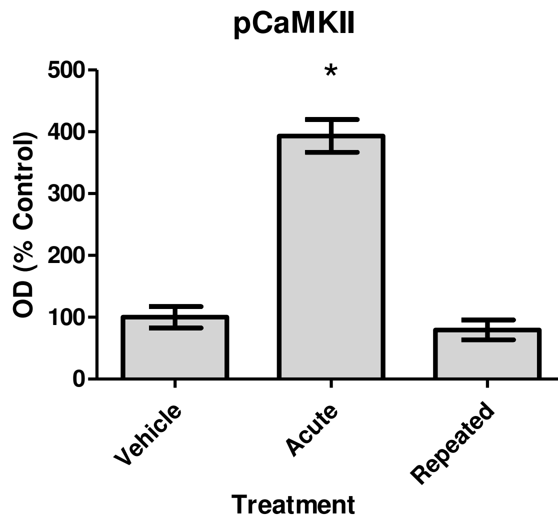


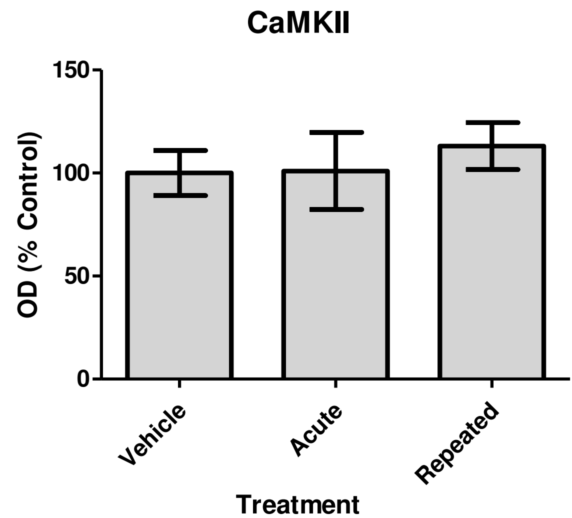


Figure 3. Increased phosphorylation of CaMKII expression by SKF 83959 in rat PFC. (A) SKF 83959 increased the phosphorylation of CaMKIIα at Thr286 in the acute treatment group. (n=6/group) (B) SKF 83959 did not increase the total levels of CaMKIIα in either the acute (1.5mg/kg) or repeated (0.4mg/kg) treatment group. N=6 rats/group. Values shown are the means ± S.E.M.
* $p<0.05$.

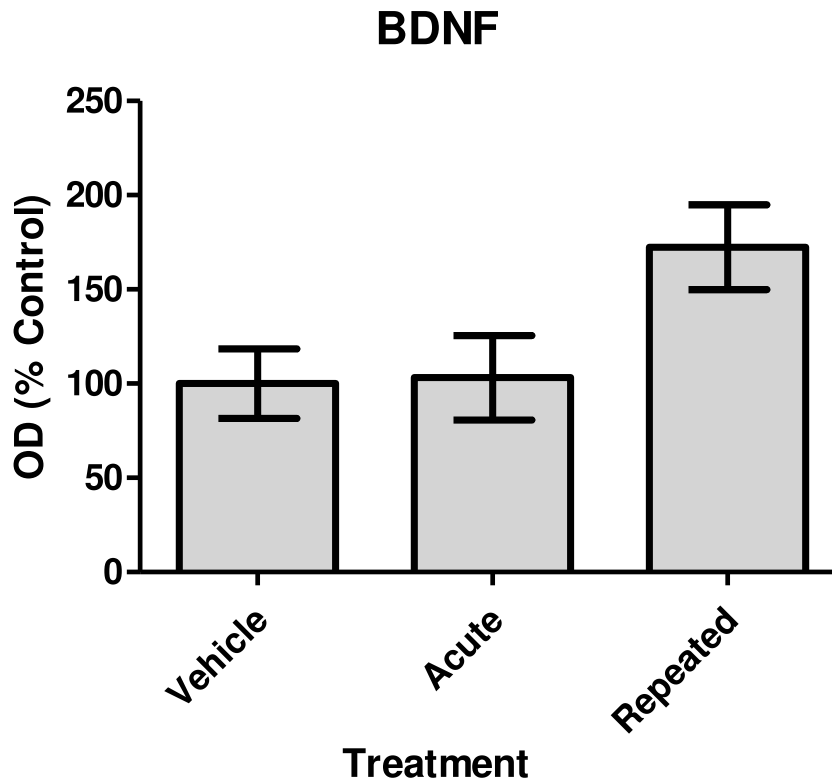


Figure 4. Effect of SKF 83959 on BDNF expression in rat PFC. SKF 83959 did not increase the total levels of BDNF in the acute treatment group (1.5mg/kg; n=6) but a trend towards increased BDNF expression in the PFC in the repeated (0.4mg/kg) treatment group. N=5-6 rats/group. Values shown are the means ± S.E.M.

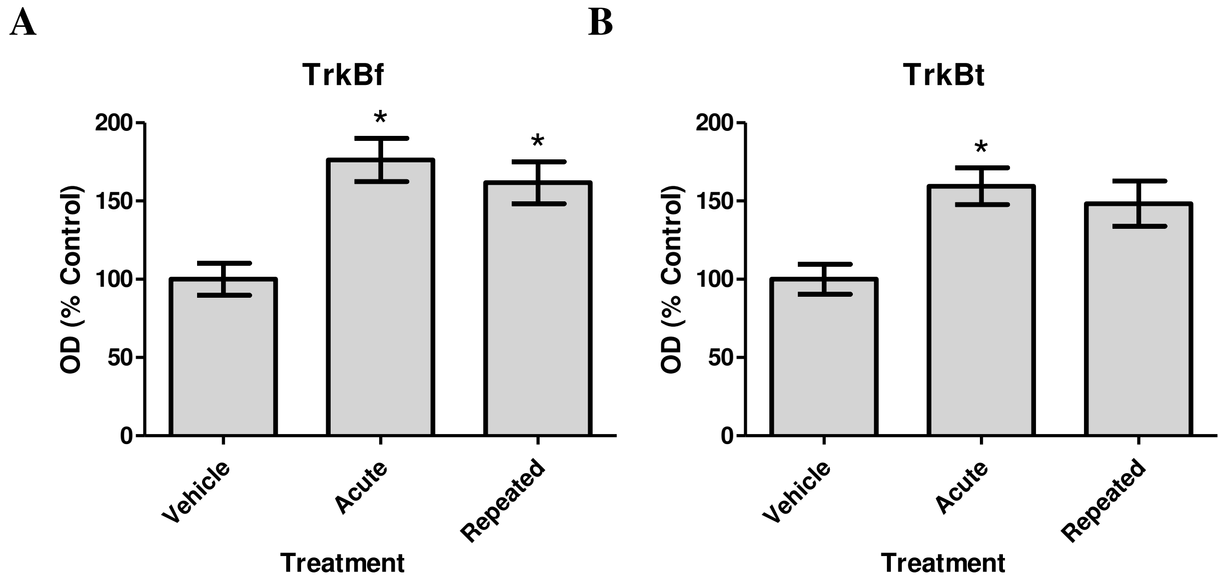
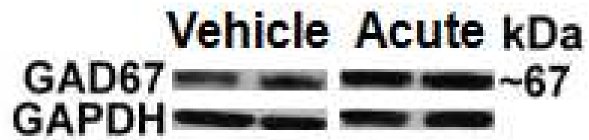


Figure 5. Increased full length and truncated TrkB expression by SKF 83959 in rat PFC. (A) SKF 83959 increased full length TrkB expression in both the acute (1.5mg/kg) and repeated (0.4mg/kg) treatment groups. (B) SKF 83959 increased truncated TrkB expression in the acute treatment group with a trend towards increased expression in the repeated treatment group. N=6 rats/group. Values shown are the means ± S.E.M. * $p<0.05$.



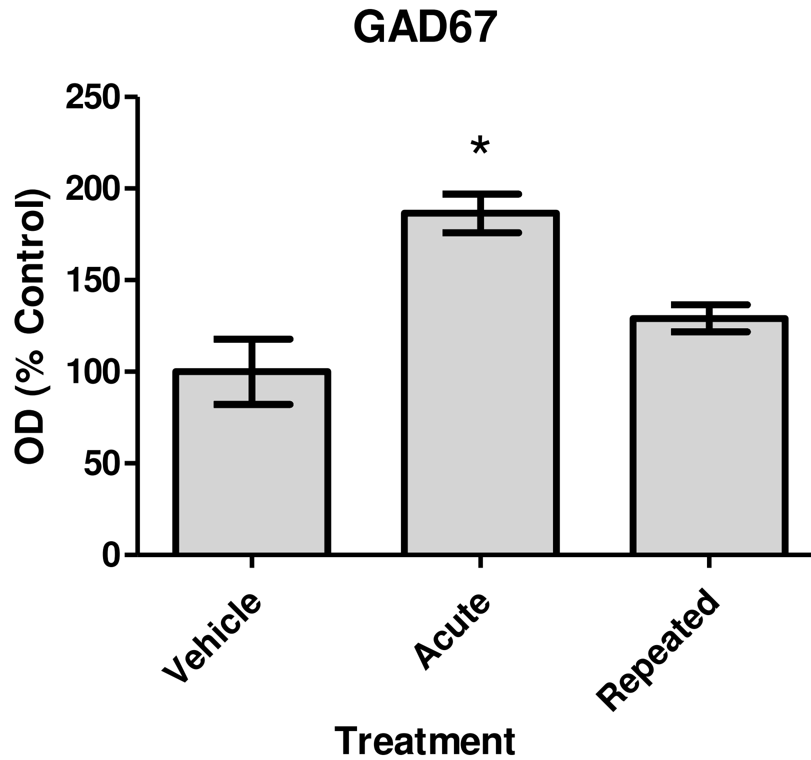
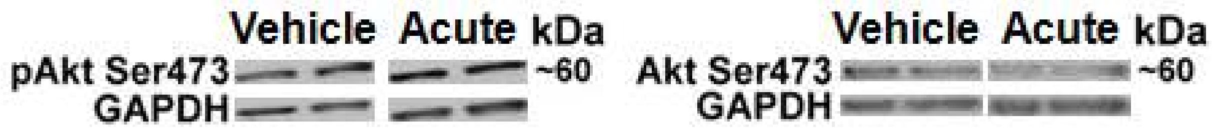


Figure 6. Increased GAD67 expression by SKF 83959 in rat PFC. SKF 83959 increased the total level of GAD67 in the acute (1.5mg/kg) but not in the repeated (0.4mg/kg) treatment group. N=6 rats/group. Values shown are the means ± S.E.M. * $p<0.05$.

The effects of SKF 83959 on the activation of Akt signalling in PFC

SKF 83959 has previously been shown to increase Akt signalling in cultured cortical neurons (Yu et al., 2008) and in rat PFC following a single dose administration (Perreault et al., 2013). The effect of an acute administration of SKF 83959 to rats on Akt signalling in PFC *in vivo* was shown to occur in tandem with increased expression and signalling of BDNF (Perreault et al., 2013). To compare the effects of acute and repeated administration of SKF 83959 on Akt signalling, we evaluated the expression levels of total and phosphorylated Akt, as well as the expression and activation state of downstream effectors of Akt namely GSK-3α and GSK-3β (Figs. 7-9). Both the acute and repeated administration of SKF 83959 to rats resulted in a significant increase in the phosphorylation of Akt at Ser 473 in PFC ($T(10)= 6.035$, $P<0.001$; $T(10)= 4.559$, $P=0.001$; Fig. 7B), with no effects on the total levels of Akt expression (Fig. 8A). There was a significant increase in the phosphorylated GSK-3α expression in the acute treatment group ($T(10)= 2.321$, $P=0.043$; Fig. 8B) and in the phosphorylated GSK-3β expression of the repeated treatment group in rat PFC $(10)= 4.386$, $P=0.001$), with no effects on the total levels of either GSK-3α and GSK-3β expression when compared to the vehicle group (Fig. 8A & 9A).



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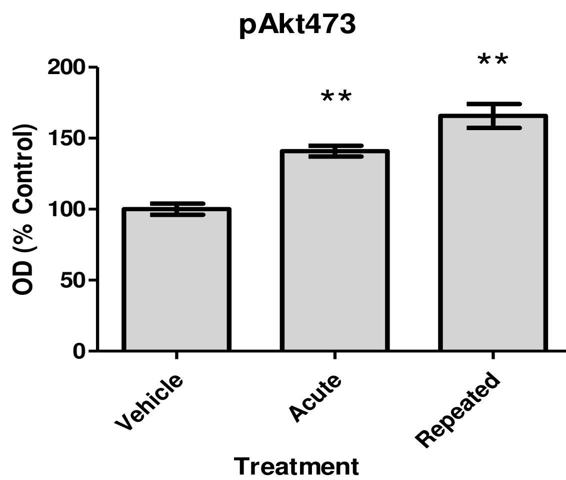


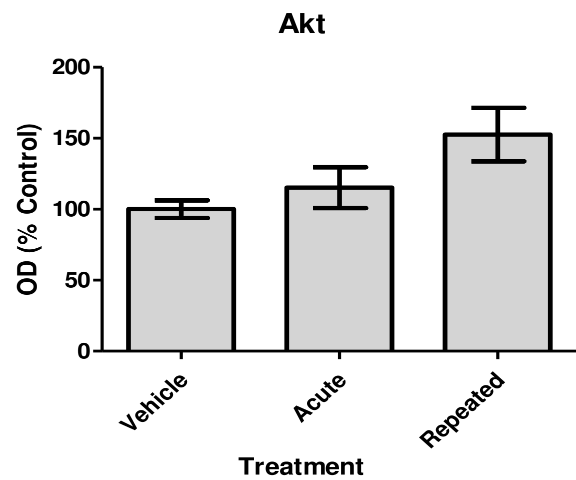
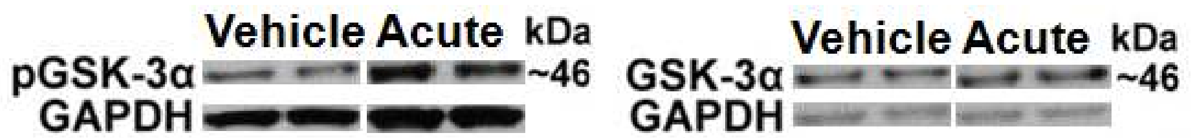


Figure 7. Increased pAkt473 expression by SKF 83959 in rat PFC. (A) SKF 83959 increased the phosphorylation of Akt at Ser473 in both the acute and repeated treatment groups. (B) SKF 83959 did not increase the total levels of Akt in both the acute (1.5mg/kg) and repeated (0.4mg/kg) treatment groups. N=6 rats/group. Values shown are the means ± S.E.M. * $p<0.01$.



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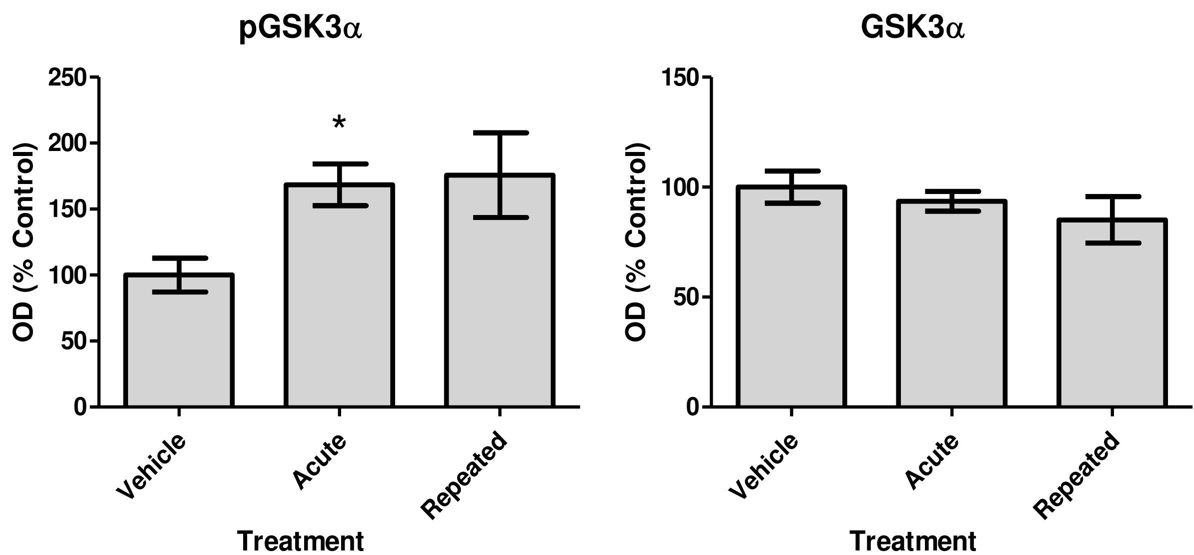
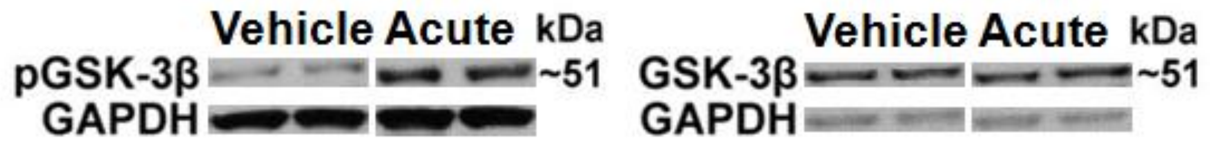


Figure 8.
Increased pGSK-3α expression by SKF 83959 in rat PFC. (A) SKF 83959 increased the phosphorylation of GSK-3α in the acute treatment group. (B) SKF 83959 did not alter the total levels of GSK-3α in both the acute (1.5mg/kg) and repeated (0.4mg/kg) treatment groups. N=6 rats/group. Values shown are the means ± S.E.M. * $p<0.05$.



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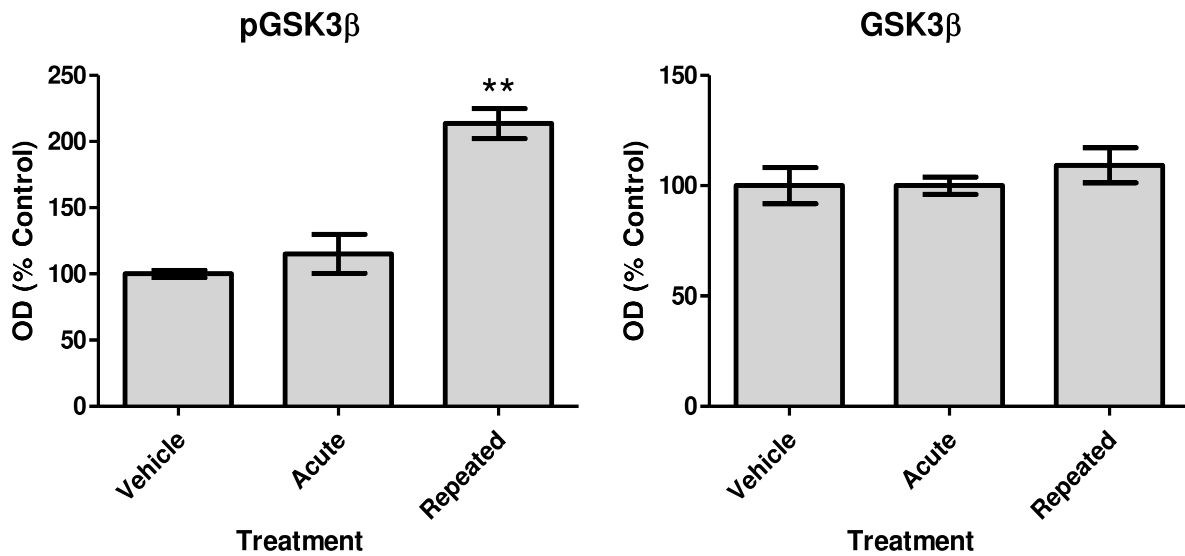


Figure 9.
Increased pGSK-3β expression by SKF 83959 in rat PFC. (A) SKF 83959 increased the phosphorylation of GSK-3β in the chronic treatment group. (B) SKF 83959 did not alter the total levels of GSK-3β in either the acute (1.5mg/kg) or repeated (0.4mg/kg) treatment groups. N=6 rats/group. Values shown are the means ± S.E.M. * p<0.01.

The effects of SKF 83959 on spatial learning and memory

BDNF, Akt and GSK-3 signalling have all been linked to cognitive performance in both rodents (Lipska et al., 2001; Sanna et al., 2002; Hernandez et al., 2002) and in humans (Weickert et al., 2005; Karpova et al., 2006; Caballol et al., 2007). Our next goal was therefore to evaluate the effects of SKF 83959 on cognitive performance in rats using an egocentric learning task in the Morris water maze. In the large Morris water maze, a repeated measures ANOVA indicated that there was a significant difference in the performance between the vehicle and SKF 83959 treatment groups across the four days of testing ($F(1,13)= 5.486$, $p=0.036$; Fig. 10A). Whereas the vehicle-treated rats exhibited learning over the course of the experiment, as evidenced by the significant reduction in the latency to escape to the hidden platform across the 4 days (within subjects effect: $F(1,13)= 5.595$, $p=0.034$; Fig. 10A), the SKF 83959-treated group did not learn the task and therefore the vehicle-treated rats exhibited significantly reduced latency compared to the SKF 83959-treated rats on day 4 ($T(13)= 2.926$, $P=0.012$). Further analysis of the rats' swimming behaviour pattern in the larger Morris water maze using their swimming path tracings revealed that the vehicle group displayed greater exploratory behaviour, swimming often into the centre of the maze, resulting in their ability to locate the hidden platform and escape the water (Fig. 11A). Conversely, the SKF 83959-treated group swam predominantly around the edge of the pool with little exploration into the centre, thus hindering their ability to locate the hidden platform (Fig. 11B).

Preliminary evidence from our laboratory suggested that SKF 83959 may have anxiogenic properties, as rats exposed to the drug spent significantly more time in the closed arms of an elevated plus maze (Shen, et al., in revision). We therefore repeated the water maze experiment using a smaller pool in an attempt to reduce or eliminate the anxiety-promoting effects of SKF 83959 that were evident in a larger open environment. We were able to

demonstrate that, in the smaller pool SKF 83959-treated rats not only learned the task (Within Subjects Effects: $F(1,24)= 41.382$, $p<0.001$; Fig, 10B) but did so at a faster rate than their vehicle-treated counterparts ($F(1,24)= 6.082$, $p=0.021$; Fig. 10B). The SKF 83959-treated group performed better than the vehicle-treated rats shown by the significantly reduced latency to locate the hidden platform on day 2 ($T(24)= 2.259$, $P=0.033$).

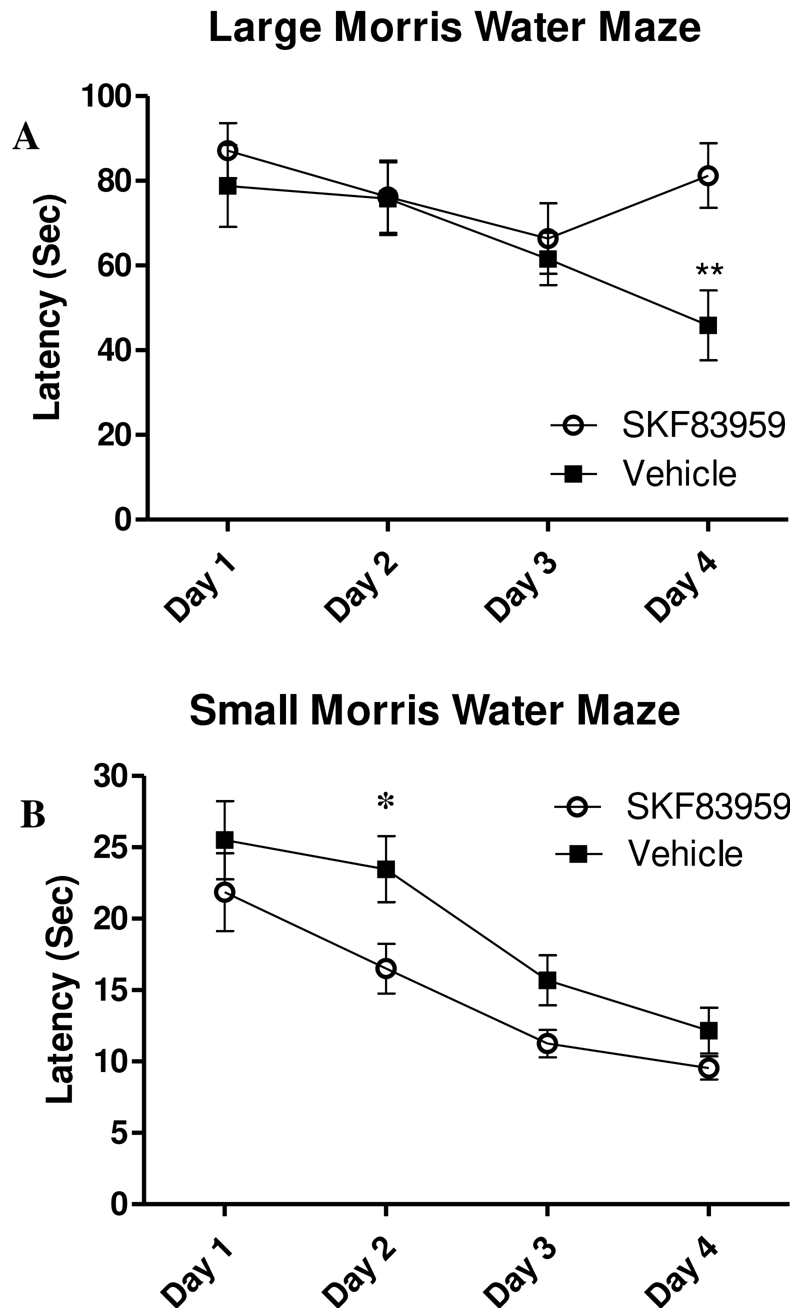


Figure 10. SKF 83959 improves PFC-based cognitive performance. (A) Administration of SKF 83959 (0.4mg/kg, s.c./day) inhibited learning and increased the latency to escape in the egocentric learning task in the large Morris water maze task of PFC function. N=16 rats/group. (B) SKF 83959 (0.4mg/kg, s.c.) decreased the latency to escape in the small egocentric learning Morris water maze task of PFC function. N=11-15 rats/group. Values shown are the means ± S.E.M. * $p<0.05$, ** $p<0.01$.

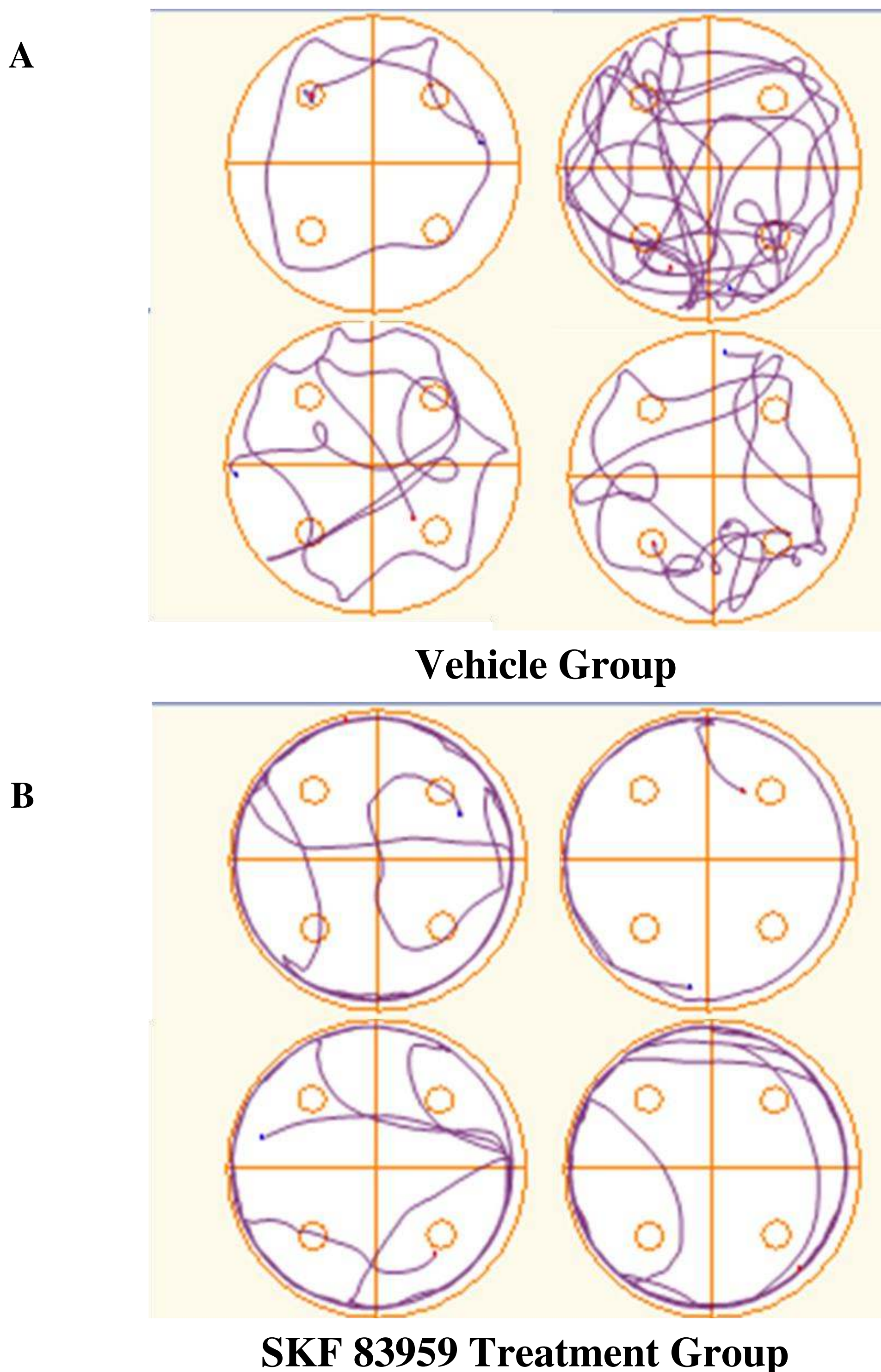


Figure 11.
Representative images of the swimming patterns recorded in the large Morris water maze of vehicle and SKF 83959 treated rats. (A) Vehicle-treated rats demonstrated greater exploratory behaviour which enabled them to venture out into the maze to locate the hidden platform. (B) SKF 83959-treated rats swam around the edge of the pool and did not venture into the center of the maze, thus inhibiting them from locating the hidden platform.

The effects of SKF 83959 on three tests of object recognition memory

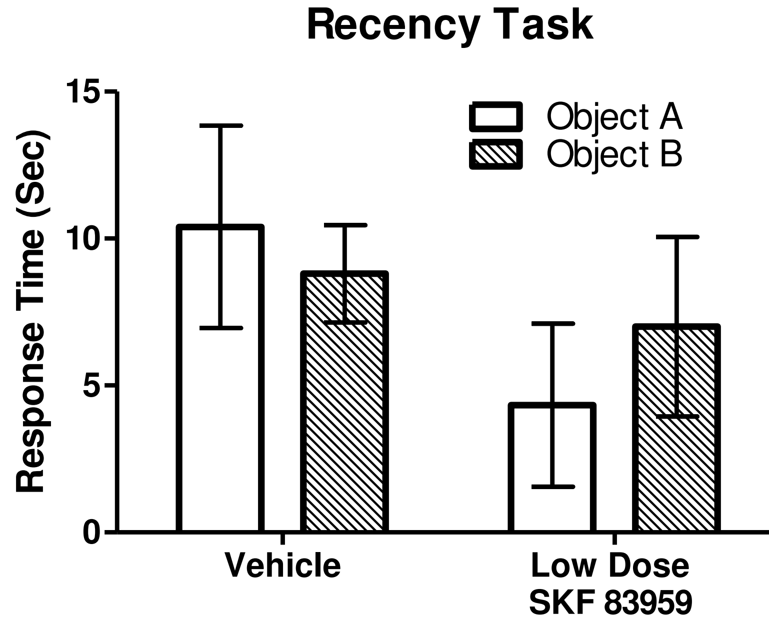
To further evaluate the effects of SKF 83959 on cognition, we assessed whether SKF 83959 could influence various aspects of object recognition memory by performing three distinct tests that evaluated recency memory, object location memory and object identity memory. In the recency task, animals were exposed to two different objects and the amount of time spent interacting with the most recently seen, versus the previously seen object was evaluated. There was no difference in time spent interacting with the previously seen object or the more recent object for both the vehicle group 1 or the high dose SKF 83959 treated group ($T(10)= 1.619$, $P=0.137$; $T(11)= 0.628$, $P=0.543$; Fig. 12B). Also there was no difference in time spent interacting with the previously seen object or the more recent object for both the vehicle group 2 or the low dose SKF 83959 treated group ($T(9)= 0.423$, $P=0.682$; $T(9)= -1.187$, $P=0.266$; Fig. 12A). Overall, there was no evidence that SKF 83959 had any effect on the rats' ability to discriminate a recent from a previously seen object (Fig. 12).

In the object location test, rats were exposed to two objects, following which one of the objects was displaced to a new location and the time spent exploring each object was evaluated. There was no difference in time spent interacting with objects that were moved to a novel spatial location and a static object for both the vehicle group 1 or the high dose SKF 83959 treated group ($T(10)= -0.951$, $P=0.364$; $T(11)= 0.919$, $P=0.378$; Fig. 13A). Also there was no difference in time spent interacting with the object that was moved to a novel spatial location and a static object for both the vehicle group 2 or the low dose SKF 83959 treated group ($T(9)= -0.471$, $P=0.649$; $T(9)= 0.805$, $P=0.442$; Fig. 13B). Overall, there was no evidence that SKF 83959 had any effect on the rats' ability to discriminate an object displaced to a novel spatial location (Fig. 13).

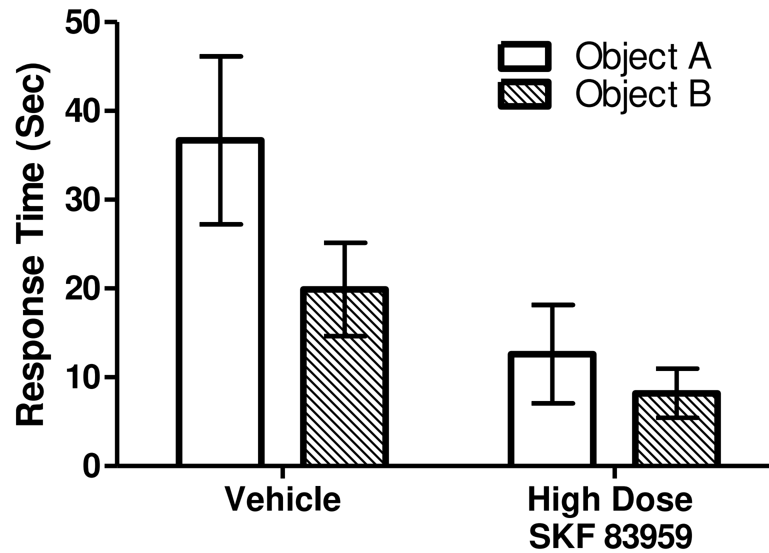
In the novel object recognition test rats were exposed to two similar objects following which one of the objects was replaced with a novel object in the same location. There was no difference in time spent interacting with a novel object and a familiar object for both the low and high dose SKF 83959 treated groups (T(9)= -1.626, P=0.138; T(11)= 0.161, P=0.875; Fig. 14). However, as expected there was a significant difference in the time spent interacting with a novel object over the familiar object for both the vehicle group 1 and the vehicle group 2 (T(10)= -3.485, P=0.006; T(9)= -4.022, P=0.003; Fig. 14). The vehicle-treated rats spent significantly more time interacting with the novel object compared to the previously encountered object. Overall, there was no evidence that SKF 83959 had any effect on the rats' ability to discriminate a novel object from a familiar object but the results demonstrate that the vehicle-treated rats have intact novel object recognition memory (Fig. 14).

Consistent with previous findings, SKF 83959 treated rats demonstrated anxiogenic behaviour characterized by a decrease in locomotion, thigmotaxis, lack of exploration, and much time spent motionless. The results showed that SKF 83959 treated rats had a significant decrease in the time exploring the objects in five of the six tests of recognition memory (Table 2).

A



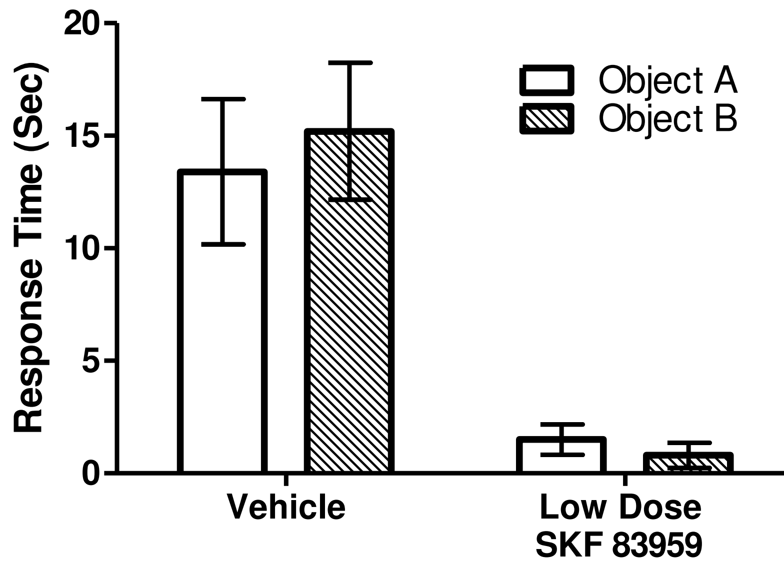
B



Treatment Groups

Figure 12.
The effects of low (0.4mg/kg, s.c.; n=9) and high dose SKF 83959 (2.5mg/kg, s.c.; n=11) on recency memory. Test performance for Vehicle and SKF 83959 treatment groups are presented as the time spent exploring each object. The white bar represents the preferred and least recently seen object. (Vehicle 1: n=10; Vehicle 2: n=9). (A-B) There was no difference in time spent interacting with the previously seen object or the more recent object for both the vehicle group or the SKF 83959 treated groups. Values shown are the means ± S.E.M.

A



B

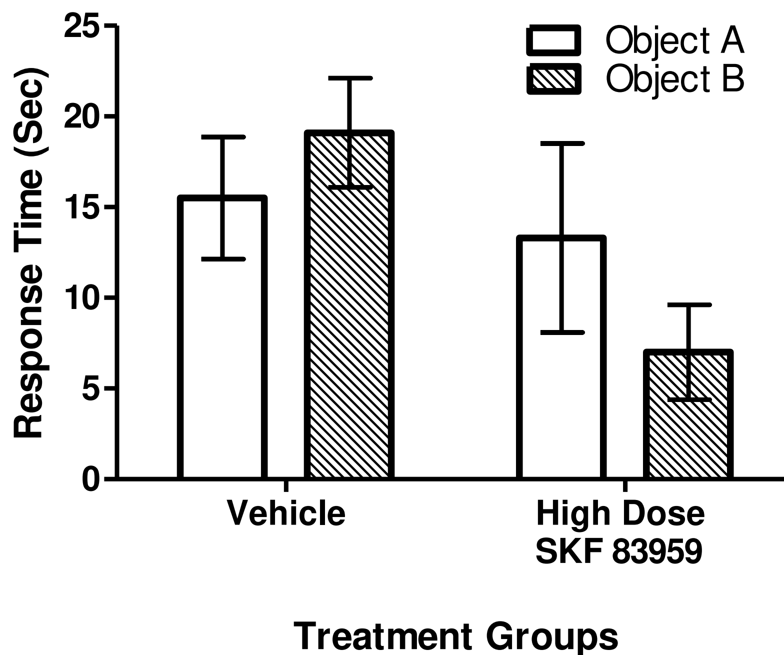
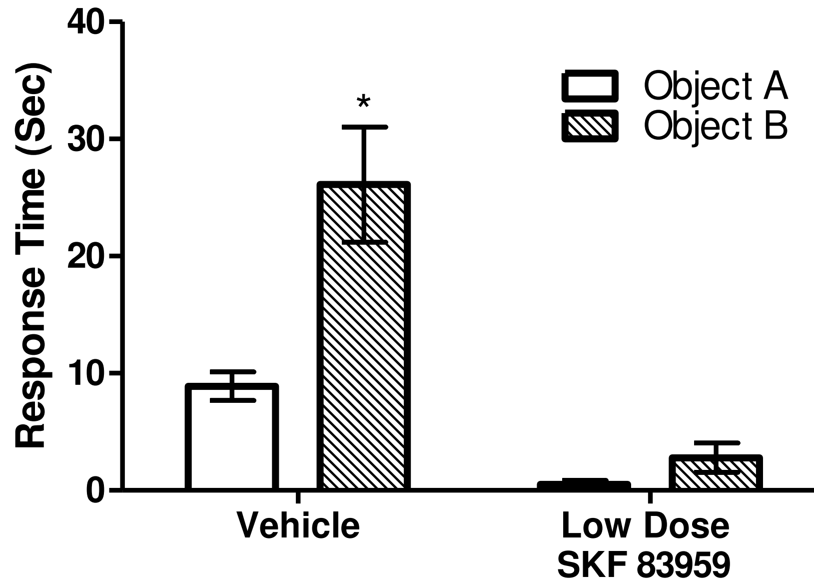
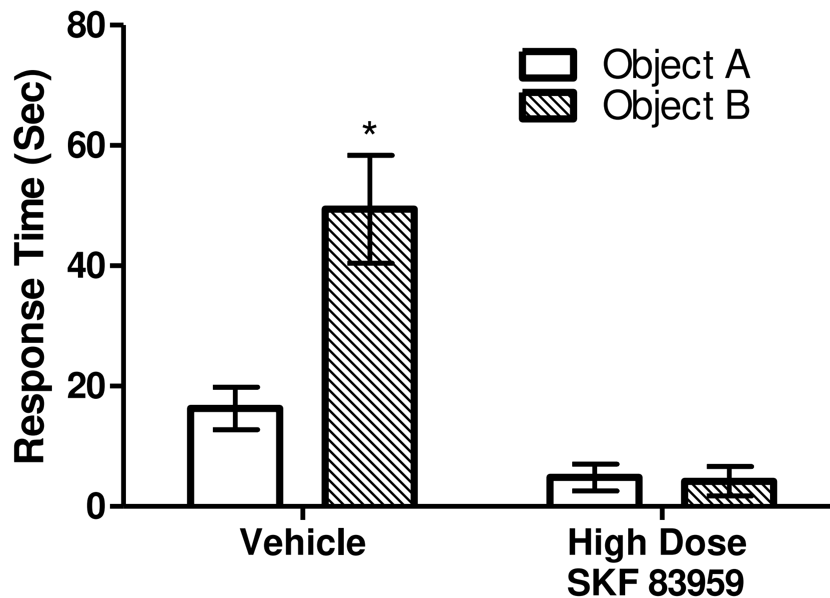


Figure 13.
The effects of low (0.4mg/kg, s.c.; n=9) and high dose SKF 83959 (2.5mg/kg, s.c.;n=11) on location recognition memory. Test performance for Vehicle and SKF 83959 treatment groups are presented as the time spent exploring each object. The shaded bar represents the preferred and displaced object. (Vehicle 1: n=10; Vehicle 2: n=9). (A-B) There was no difference in time spent interacting with objects that were moved to a novel spatial location and a static object for both the vehicle group or the SKF 83959 treated group. Values shown are the means ± S.E.M.

A



B



Treatment Groups

Figure 14.
The effects of low (0.4mg/kg, s.c.; n=9) and high dose SKF 83959 (2.5mg/kg, s.c.; n=11) on novel object recognition memory. Test performance for Vehicle and SKF 83959 treatment groups are presented as the time spent exploring each object. The shaded bar represents the preferred and novel object. (Vehicle 1: n=10; Vehicle 2: n=9) (A-B) There was no difference in time spent interacting with a novel object and a familiar object for both SKF 83959 treated groups, however, there was a significant difference in the time spent interacting with a novel object over the familiar object for both the vehicle groups. Values shown are the means ± S.E.M. * $p<0.05$.

Table 2. Time Spent Exploring the Objects for each of the Treatment Groups

A

Test	Recency Task		Spatial Location		Novel Object Recognition	
Treatment Groups	Low Dose SKF 83959	Vehicle 2	Low Dose SKF 83959	Vehicle 2	Low Dose SKF 83959	Vehicle 2
Mean	17.4	19.2	2.3	28.6	3.3	35
S.E.M.	6.85	3.86	0.87	4.97	1.17	5.74
Rats/Group	10	10	10	10	10	10
Independent T-Test	T(18)=0.2289, P=0.8215		T(18)=5.2087, P<0.001**		T(18)=5.4135, P<0.001**	

B

Test	Recency Task		Spatial Location		Novel Object Recognition	
Treatment Groups	High Dose SKF 83959	Vehicle 1	High Dose SKF 83959	Vehicle 1	High Dose SKF 83959	Vehicle 1
Mean	20.75	56.64	20.33	33.55	8.92	65.64
S.E.M.	5.24	11.23	4.52	4.11	2.98	9.75
Rats/Group	12	11	12	11	12	11
Independent T-Test	T(21)=2.9781, P<0.001**		T(21)=2.1476, P=0.0436*		T(21)=5.7761, P<0.001**	

(A-B) Mean, S.E.M. and T-test results of the total time spent exploring the objects for Vehicle and SKF 83959 treated rats. S.E.M., standard error of mean.

4 Discussion:

In this study, we showed that SKF 83959 plays a role in the regulation of CamKIIα BDNF, Akt and GSK-3 signalling in the PFC of rats. Acute injection of SKF 83959 administered to rats has been shown to increase CaMKIIα activation in the PFC which has also been previously shown in rats and mice (Perreault et al., 2013). However, repeated SKF 83959 treatment to rats had no effects on CaMKIIα activation, as these effects may be due to the downregulation of the acute responses seen after the first SKF 83959 administration (Verma et al., 2010). Specifically, there was an increased trend in the expression of BDNF after repeated administration of SKF 83959, enhanced expression of both the TrkB receptor isoforms (full length and truncated) after a single injection of SKF 83959, and increased fTrkB expression following repeated administration of SKF 83959. These results indicate that after SKF 83959 administration, there was an increase in BDNF and its signalling via its receptor in PFC. The acute changes were also concurrent with an increase in the expression of GAD67 which is the primary enzyme responsible for the synthesis of the neurotransmitter GABA. The downregulation of GABA would be involved in mediating the dysfunction of inhibitory neurons of the PFC, characteristics that may contribute to cognitive deficits. Single and repeated administration of SKF 83959 enhanced the phosphorylation and activation of Akt at Ser473 in the PFC. In line with this, SKF 83959 increased phosphorylation and inactivation of the Akt substrate, GSK-3α, after a single injection of SKF 83959, and GSK-3β following repeated administration of SKF 83959. Our findings showed that the administration of SKF 83959 to rats impaired their cognitive performance in the large Morris water maze, effects which may be due to the anxiogenic properties of the drug. Unlike the vehicle group, which continually improved in performance as evidenced by the reduction in latency to escape the water across the four days, during the course of the experiment. Therefore, a smaller Morris

water maze was implemented in the egocentric learning task to reduce the anxiogenic effects of SKF 83959. Interestingly, this strategy did work as it eliminated the thigmotactic behaviour observed previously in SKF 83959 treated rats in the large Morris water maze. In the smaller water maze, the SKF 83959 treated rats performed significantly better than the vehicle treated rats by learning to locate the hidden platform in a shorter period of time.

In the recency task, we revealed that both SKF 83959 treated rats (low and high dose) and the vehicle groups were unable to significantly discriminate among the objects, based on the recency of interaction. That the data from the vehicle treated rats were unable to reach statistical significance in two of the three object recognition tests may be due to methodological differences in our studies compared to what has been described previously, such as different animal strains and size of the open field (Nelson et al., 2011). In the object location recognition test, both groups of SKF 83959 treated rats (low and high dose) and the vehicle groups were unable to significantly detect spatial changes when an object was moved to a novel location. In the novel object recognition test, we have shown that the vehicle groups could significantly differentiate a novel object from a familiar object, whereas the administration of both low and high dose of SKF 83959 impaired the rats' ability to differentiate a novel object from a familiar object. These results from the vehicle groups were consistent with previous findings, demonstrating that the rats have an intact recognition memory to discriminate novel objects over familiar objects (Ennaceur et al., 1988).

The administration of SKF 83959 to rats enhanced the expression of BDNF and TrkB in the PFC and we have previously shown using mice gene-deleted for the D1R or the D5R, that this occurs through a D5R-mediated mechanism (Perreault et al., 2013). This finding potentially implicates this receptor in the regulation of cognition via alterations in BDNF signalling in PFC. For example, the levels of BDNF in the frontal cortex in Ts65Dn mice

(trisomy of chromosome 16), an animal model of Down syndrome has been shown to be negatively correlated with working memory errors, demonstrated using the water radial-arm maze (Bimonte-Nelson et al., 2003). Dopamine transporter knockout mice have been shown to have significantly decreased levels of BDNF proteins in the frontal cortex and were severely impaired in spatial working memory, assessed by using the spontaneous alternation in the Y-maze (Li et al., 2010). Similarly, BDNF knockout in the forebrain of mice resulted in spatial memory deficits in the Morris water maze (Gorski et al., 2003). Therefore the D5 receptor mediated increase in BDNF in PFC may have potential to improve cognitive dysfunction in rodent models.

Studies have shown that the upregulation of BDNF in the hippocampus plays a significant role in long term potentiation, a major mechanism in learning and memory (Cooke et al., 2006; Bliss et al., 1993; Malenka et al., 2004). The increase in BDNF in the hippocampus was shown to enhance LTP and rescue synaptic transmission in rodents, whereas the inhibition of BDNF synthesis was shown to impair LTP (Pang et al., 2004, Kang et al., 1995). Moreover, heterozygous and homozygous BDNF knockout mice both showed impairments in LTP in hippocampal slices, indicating the importance of hippocampal BDNF in the process of LTP (Korte et al., 1995; Patterson et al., 1996; Bartoletti et al., 2002). Furthermore, the LTP impairments were shown to be ameliorated with hippocampal administration of recombinant BDNF or through viral-mediated increases in BDNF expression (Patterson et al., 1996; Korte et al., 1996). This shows that the upregulation of BDNF in other areas of the brain such as the hippocampus can also play a widespread role in cognition and interestingly our preliminary evidence showed increased BDNF expression in hippocampus following a single injection of SKF 83959 at the same dose used in the present study. As the administration of SKF 83959 to rats was shown to improve spatial learning and memory in the

small Morris water maze, further studies examining a relationship between SKF 83959-mediated hippocampal BDNF signalling and its role in hippocampus-based learning and memory may be a worthwhile endeavor. Furthermore, although we have previously shown that SKF 83959-induced increases in BDNF signalling in PFC were mediated through the dopamine D5 receptor (Perreault et al., 2013), the mechanism by which SKF 83959 increased hippocampal BDNF remains unknown.

Reduced BDNF signalling in the forebrain has been implicated in the etiology of neuropsychiatric disorders including drug addiction and schizophrenia (Zhang et al., 2012; Zuccato et al., 2009; Xiu et al., 2009; Arancio et al., 2007) and increasing evidence suggests that elevating BDNF signalling in the PFC may have significant therapeutic benefits in disorders associated with cognitive dysfunction. Patients with schizophrenia exhibit deficits in working memory. It has been revealed that in post-mortem PFC samples from patients with schizophrenia, there is a significant decline in BDNF and TrkB mRNA transcripts and BDNF protein expression when compared to healthy subjects (Thompson Ray et al., 2011; Weickert et al., 2003; Weickert et al., 2005), and the expression levels of GAD67 mRNA were significantly correlated with both BDNF and TrkB mRNA expression levels in post mortem samples of both healthy subjects and patients with schizophrenia (Hashimoto et al., 2005). This could suggest that the upregulation of BDNF in the PFC could be associated with improved cognition. Furthermore, heterozygous and homozygous fBZ mice which exhibit decreased TrkB mRNA levels in the PFC also exhibited reduced levels of GAD67 mRNA expression, evidence that supports the involvement of TrkB in the regulation of GAD67 in the PFC (Hashimoto et al., 2005). Furthermore, it has been demonstrated that using heterozygous reeler mice, which exhibit similar deficits in the protein reelin such as are observed in schizophrenia, are also characterized by decreased GAD67, BDNF and fTrkB expression levels

in the frontal cortex and hippocampus (Kutiyanawalla et al., 2011). Interestingly, the administration of cysteamine, a neuroprotective drug, increased GAD67, BDNF and fTrkB expression to normal levels and improved cognitive functioning using the Y-maze spatial recognition task (Kutiyanawalla et al., 2011). Similar to that observed with schizophrenia, levels of BDNF and fTrkB are decreased in the frontal cortex and hippocampus of post mortem samples from patients with Alzheimer's disease (Ferrer et al., 1999; Allen, 1999), and indeed studies showed that increasing BDNF signalling in the hippocampus by administering L-3-n-butylphthalide orally in Alzheimer's disease mouse models (APP/PS1) improved spatial learning and memory deficits in the Morris water maze (Xiang et al., 2014). The decrease in BDNF signalling by overexpressing the truncated TrkB receptor in the neurons of the Alzheimer model APP/PS1 mice further accelerated spatial memory impairments, whereby the overexpression of the full length TrkB receptor ameliorated spatial memory impairments (Kemppainen et al., 2012).

We showed an inhibition of GSK-3 activity was apparent in the PFC of rats following administration of SKF 83959, and we have shown previously that both dopamine D5 receptor and D1-D2 receptor heteromer contribute to this effect albeit by different mechanisms. Specifically, whereas dopamine D5 receptor induced suppression of GSK-3 activity likely occurred through a BDNF/Akt-dependent mechanism, the reduction in activity of this kinase by the dopamine D1-D2 receptor heteromer appears to be independent of BDNF signalling in the PFC (Perreault et al., 2013). Evidence has been provided that GSK-3 signalling is highly involved in schizophrenia (Beaulieu et al., 2012; Emamian et al., 2004). For example, lithium, a conventional mood stabilizer for mood disorders is known to regulate the PI3K/Akt/GSK-3 pathway (Kitagishi et al., 2012), resulting in the phosphorylation and inhibition of GSK-3 activity (Alimohamad et al., 2005; Sutton and Rushlow, 2011). The overexpression of GSK-3

in the forebrains of rats was shown to induce spatial learning deficits, but normalizing the levels of GSK-3 activity ameliorated the cognitive deficits in the same rats (Hernandez et al., 2002). In addition, overexpression of GSK-3β was shown to impair LTP induction in the hippocampus of rats, whereas the suppression of LTP was removed (Zhu et al., 2007), suggesting that the inactivation of GSK-3β is important in memory formation and may be associated with working memory deficits in patients with schizophrenia. Cognitive deficits in patients with schizophrenia are thought to be linked to hypodopaminergic neurotransmission through the dopamine D1-like receptors in the PFC (Goldman-Rakic et al., 2004), suggesting that hypoactivity of dopamine D1 and D5 receptors may play a role in the cognitive impairments associated with this disorder. It has been shown that the atypical antipsychotics which also target the dopamine D1-like receptors improves cognitive functioning in patients with schizophrenia (Meltzer, 1999; Purdon et al., 2000). This occurs as these antipsychotics have been shown to regulate Akt signalling in rat PFC, by increased phosphorylation of Akt and GSK-3 (Sutton et al., 2011). In Alzheimer's disease, GSK-3 is known to phosphorylate tau proteins which aggregate into neurofibrillary tangles, a pathological hallmark of the disorder (Hooper et al., 2008). Studies have shown that there is an increase in GSK-3 activity, by measuring the phosphorylation at residue Tyr216 in the frontal cortex of patients with Alzheimer's disease (Blalock et al., 2004; Leroy et al., 2007; Hooper et al., 2008; Lei et al.; 2011). Furthermore, it has been reported that the phosphorylated GSK-3 was highly co-localized with phosphorylated tau proteins in the frontal cortex of Alzheimer's patients (Leroy et al., 2007). In rodent models of Alzheimer's disease, lithium administered to mice that overexpress tau proteins resulted in an increase in phosphorylated GSK-3β (indicative of decreased activity) in the brain and attenuated the degradation of axons (Macritchie and Young, 2004; Muyllaert et al., 2008). In rat models of Alzheimer's disease,

intracerebroventricular infusion of amyloid β induces hyperactivation of GSK-3 and the administration of a GSK-3 inhibitor, SB216763 leads to neuronal protection from amyloid β induced damage, neuroinflammation and improved behavioural deficits in the Morris water maze (Hu et al., 2009). In APP/PS1 mice models of Alzheimer's disease, which elevates amyloid β production, lithium was shown to reduce amyloid β deposition and improved cognitive functioning in the Morris water maze (Toledo and Inestrosa, 2010; Zhang et al., 2011). In addition, lithium has also been shown to decrease tau hyperphosphorylation, amyloid β deposition in the cerebral cortex of APP mice and reverted spatial memory impairments in the Morris water maze (Rockenstein et al., 2007).

Given the positive effects of SKF 83959 on the expression of proteins involved in improving cognitive performance, it was surprising to observe significantly longer latencies to escape in the Morris water maze in rats treated with the drug. At first glance, it would appear to simply reflect a worsening in spatial memory, however this may not be completely accurate as SKF 83959 appeared to have other behavioural effects that likely attributed to the apparent reduction in cognitive performance. Specifically, rats that were treated with SKF 83959 exhibited reduced exploratory behaviour, as these animals preferred to swim around the edge of the pool, which drastically inhibited them from locating the hidden platform. Previous studies have termed this behaviour as thigmotaxis and, in the absence of drug-induced sensorimotor deficits, can be attributed to increased anxiety (Inostroza et al., 2011; Wolfer et al., 1998). There is evidence of an association between performance in the Morris water maze and anxiety, where spatial learning is inversely correlated with corticosterone levels in the blood, a measure of stress response (Harrison et al., 2009). Similarly, thigmotactic behaviour in the Morris water maze was positively correlated to the time spent in the open arms in the elevated plus maze (Inostroza et al., 2011). These findings indicate that SKF 83959 may have

had anxiogenic effects, an idea also supported by the findings from the recognition memory test in the open field whereby the administration of SKF 83959 robustly inhibited locomotor activity and exploration, effects that could not be attributed to motor deficits induced by the drug as SKF 83959 has been shown to significantly increase locomotion in smaller activity chambers (Perreault et al., 2010). Indeed, new evidence from our laboratory showed that SKF 83959 does induce strong anxiogenic responses in both mice and rats, as indexed by more time spent in the closed arms of an elevated plus maze and increased latency to approach a sweetened drinking solution in a novel (anxiety-promoting) environment (Shen et al., in revision). In line with this, when a smaller pool was used, the SKF 83959-treated rats did not exhibit any signs of thigmotactic behaviour and were able to learn the task at a faster rate than the vehicle-treated controls.

There could have been a number of contributors to the anxiety response that was observed in the present study following the administration of SKF 83959 to rats. For example, using a highly selective disrupting peptide we developed for the D1-D2 receptor heteromer (Hasbi et al., 2014), we showed that the mechanism by which SKF 83959 induces anxiety is, at least in large part, through the D1-D2 heteromer (Perreault et al., 2015). Although we cannot conclusively identify the brain region through which the D1-D2 heteromer is mediating these anxiogenic effects (since the disrupting peptide was administered i.c.v), we have shown high expression of the D1-D2 heteromer in medium spiny neurons of the nucleus accumbens (NAc) (Hasbi et al., 2009; Perreault et al., 2010), a region highly involved in reward and motivation (Wise, 2004). It has been demonstrated that dopamine D1 receptor knockout mice when compared to wild-type mice, displayed a loss in motivation to obtaining rewards such as food, sucrose (El-Ghundi et al., 2003; Young and Geyer, 2010), revealing that dopamine does play an important role in the regulation of motivation to work for rewards. Thus it is possible that

the D1-D2 heteromer in NAc may function to suppress reward, so that activation of the heteromer would result in a depression-like state, a state that is often comorbid with anxiety. This idea is given credence with our findings that showed activation of the D1-D2 heteromer by SKF 83959 reduced the latency to float in the forced swim test indicative of depressive-like behaviour (Hasbi et al., 2014; Shen et al., in revision), induced conditioned place aversion, reduced sucrose consumption, and abolished the motivation for animals to work for a palatable treat (Hasbi et al., in preparation), behaviours that are derived by the inhibition of the reward pathways of the brain.

Although we have identified a role for the D1-D2 heteromer in inducing anxiogenic behaviour in rats, other receptors could have contributed to the ability of SKF 83959 to induce the observed anxiety-like responses. Although SKF 83959 has high affinity for the dopamine D5 receptor, a recent report using transgenic rats expressing the human dopamine D5 receptor (hD5R) indicated that SKF 83959 did not affect the amount of time spent in the open arms of the elevated plus maze in wildtype or heterozygous transgenic hD5R rats, but did so in rats expressing a hD5R with a mutated C-terminal tail (Xu et al., 2012). Although the discrepancy between this study and our previous study showing reduced time spent in the open arms in rats following SKF 83959 may have been attributed to simple timing differences post-injection, this study in the hD5R rats would nonetheless support an anxiolytic, or anxiety-reducing, role for the dopamine D5 receptor. Another possibility is that SKF 83959 acted through the serotonin 5HT2C receptor. Although activation of this receptor by SKF 83959 has not been shown, previous findings have shown that SKF 83959 binds to this receptor with relatively high affinity (Chun et al., 2013). Similar to SKF 83959, 1-(m-chlorophenyl)-piperazine (mCPP), a 5HT2C receptor agonist has been shown to induce anxiety (Cornelio and Nunes, 2007; Hackler et al., 2007; Bagdy et al., 2001) and inhibit locomotor activity (Stiedl et al., 2007). Moreover,

there is increasing evidence which suggests that 5HT2C receptor antagonists can reduce anxiety-like behaviour. For instance, the 5HT2C receptor antagonist, SB-242084 was shown to reduce anxiety in rats, demonstrated in the social interaction test (Bagdy et al., 2001) and preclinical studies have shown that agomelatine, a combined melatonin receptor agonist and 5HT2C receptor antagonist was an effective treatment in anxiety disorder (Stein et al., 2008; Millan et al., 2005). There is currently no evidence which suggests that the α-adrenergic 2C receptor or the sigma-1 receptor play a role in inducing anxiety.

Limitations

Western blot is a common technique used in laboratories to detect the presence of a protein, but there are limitations of western blotting. For instance the expression levels of BDNF were measured throughout the whole PFC whereas the changes in BDNF expression levels in individual neurons (pyramidal and GABAergic interneurons) expressing the dopamine D5 receptors can not be measured. (immunohistochemistry)2 Also, western blots are semi-quantitative, and quantifies the expression levels of the protein relative to control. Furthermore, the sample sizes for each group in the western blot experiment was relatively small (n=6), which may explain the large variances in some of the results. With a larger sample size for each group we may have shown significance in some of the results such as the expression levels of BDNF in the repeated treatment group. Moreover, animals testing has its own disadvantages, such as differences in anatomy, physiology, and pharmacology when compared to humans and these genetic variances makes it very difficult to directly extrapolate data from animals to humans.

Significance and Conclusions

In the present study we have demonstrated that acute and/or repeated SKF 83959 treatment to rats increased CaMKII, BDNF and Akt signalling and inhibited GSK-3 activity in the PFC, an effect we attributed to activation of the dopamine D5 receptor and/or the dopamine D1-D2 receptor heteromer (Perreault, 2013). Increase in BDNF signalling and GSK-3 inactivation in the PFC has been previously linked to an improvement in cognitive deficits associated with LTP, working memory, and spatial learning and memory suggesting that selective activation of these dopamine receptors would improve cognitive performance. However, despite showing a significant improvement in spatial learning and memory in a small diameter Morris water maze, SKF 83959 induced anxiety-like behaviour in both the larger diameter water maze and the open field, findings that suggest SKF 83959 may not be a suitable compound for cognitive enhancement due to unwanted side effects. Nonetheless, as we have been able to now attribute the anxiety-promoting effects of SKF 83959 in large part to the D1-D2 heteromer and not the D5 receptor, future studies examining a selective role for the dopamine D5 receptor in cognition seem worthwhile given its positive involvement in increasing BDNF signaling and mediating GSK-3 suppression in PFC.

Future Studies:

Although selective pharmacological agents that target the dopamine D5 receptor are not presently available, these studies could be performed using pharmacological and genetic techniques to alter dopamine D5 receptor expression in select brain regions such as PFC, to ascertain whether the dopamine D5 receptor positively contributes to cognition and determine whether the receptor could represent a novel therapeutic target for drug discovery for disorders of cognitive dysfunction. The following future studies will elucidate the role of the dopamine D5 receptor expression or activation in the PFC and its effects on cognition.

1. Increase BDNF signalling via the dopamine D5 receptor in the PFC and examine its effects on cognition.

This investigation can be carried out with two different experimental approaches:

A). Activation of the dopamine D5 receptor by administering SKF 83959 and TAT-D1 peptide directly into the PFC of rats. SKF 83959 is an agonist for both the dopamine D5 receptor and D1-D2 receptor heteromer and the administration of the TAT-D1 peptide will block the D1-D2 heteromer signalling and function. Therefore, this will result in dopamine D5 receptor activation to increase BDNF signaling in normal and in animal models of cognitive dysfunction and examine its effects on cognition.

B). Increase the dopamine D5 receptor gene expression by administering viral contructs containing the cDNA of the dopamine D5 receptor directly into the PFC of rats to increase BDNF signaling in normal and in animal models of cognitive dysfunction and examine its effects on cognition.

2. Decrease BDNF signalling via the dopamine D5 receptor in the PFC and examine its effects on cognition.

A). Silence the dopamine D5 receptor gene expression by administering viral contructs containing shRNA directed against the dopamine D5 receptor gene directly into the PFC of rats to decrease the dopamine D5 mediated signalling in normal and examine its effects on cognition.

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