# GENETIC DISSECTION OF THE BEHAVIORAL EFFECTS OF THE DOPAMINE RECEPTOR AGONIST SKF83959 DOES NOT SUPPORT THE D1/D2 RECEPTOR HETEROMER MODEL

By

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## DEDICATION

This work is dedicated to Nilaja

who has been my biggest fan and cheerleader,

and to the entire Frederick/Murphy family

for their endless support and encouragement.

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## ABBREVIATIONS

**6-OHDA:** 6-hydroxydopamine

**$\alpha_2$ receptors:** $\alpha_2$ adrenergic receptors

**AC:** adenylyl cyclase

**ACC:** anterior cingulate cortex

**ADHD**: attention-deficit hyperactivity disorder

**AMPA**: $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

**AMPAR:** AMPA-type glutamate receptor

**ANOVA:** analysis of variance

**BAC:** bacterial artificial chromosome

**BDNF:** brain-derived neurotrophic factor

**BRET:** bioluminescence resonance energy transfer

**$Ca^{2+}$:** calcium

**CaM:** calmodulin

**CaMKII$\alpha$:** $Ca^{2+}$/calmodulin-dependent protein kinase II $\alpha$

**cAMP:** cyclic adenosine monophosphate

**CNS**: central nervous system

**COMT:** catechol-o-methyl transferase

**CREB:** cAMP-response element binding protein

**$D_1$ receptors:** dopamine $D_1$ receptors

**$D_2$ receptors:** dopamine $D_2$ receptors

**$D_2$ L: dopamine:** $D_2$ receptor long isoform

**$D_2$S: dopamine:** $D_2$ receptor short isoform

**$D_3$ receptors:** dopamine $D_3$ receptors

**$D_4$ receptors:** dopamine $D_4$ receptors

**$D_5$ receptors:** dopamine $D_5$ receptors

**DA:** dopamine

**DARPP-32:** cAMP-regulated phosphoprotein of 32kDa

**DAT:** dopamine transporter

**DSM-IV:** diagnostic and statistical manual of mental disorders, fourth edition

**DRD4:** gene that encodes the dopamine $D_4$ receptor

**ERK2:** extracellular signal-regulated kinase 2

**FGF:** fibroblast growth factor

**FRET:** fluorescence resonance energy transfer

**G-protein:** guanine nucleotide binding protein

**GABA:** $\gamma$-aminobutyric acid

**$G_{\alpha i/o}$:** inhibitory G-proteins

**$G_{\alpha q}$ :** G-proteins that stimulate PLC

**$G_{\alpha s/olf}$:** stimulatory G-proteins

**GDP:** guanosine diphosphate

**GluR1:** AMPAR subunit

**Gna11:** gene that encodes for $G_{\alpha 11}$

**Gnaq:** gene that encodes for $G_{\alpha q}$

**Gnas*:*** gene that encodes for $G_{\alpha s}$

**GPCR:** G-protein coupled receptor

**GTP:** guanosine-5'-triphosphate

**GTPase:** hydrolyzes guanosine triphosphate

**(i.p.):** intraperitoneal

**$IP_3$:** inositol triphosphate

**mGluR5:** metabotropic glutamate receptor subtype 5

**MOA:** monoamine oxidase

**MSN:** medium spiny neuron

**NET:** norepinephrine transporter

**NMDA:** n-methyl-d-aspartate

**PCR:** polymerase chain reaction

**PFC:** prefrontal cortex

**PI:** phosphatidylinositol

**PKA:** cAMP-dependent protein kinase A

**PLC:** phospholipase C

**PP1:** protein phosphatase 1

**PSD:** postsynaptic density

**SN:** substantia nigra

**SSRI:** selective serotonin reuptake inhibitor

**STAR*D:** sequenced treatment alternatives to relieve depression

**VTA:** ventral tegmental area

## DRUG REFERENCE LIST

**Atipamezole:** (5-(2-Ethyl-2,3-dihydro-1*H*-indene-2-yl)-1*H*-imidazole hydrochloride; $\alpha_2$ **receptor antagonist**

**Cocaine HCl; DAT blocker**

**Desipramine:** (3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-*N*-methylpropan-1-amine; **tricyclic antidepressant**

**Fluoxetine:** ((±)-*N*-Methyl-γ-[4-(trifluoromethyl)phenoxy] benzenepropanamine hydrochloride; **SSRI**

**MK-801:** ([5R,10S]-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; **NMDA receptor antagonist**

**MTEP:** ([(2-methyl-1, 3-thiazol-4-yl) ethynyl]pyridine; **mGluR5 receptor antagonist**

**PG 01037:** (*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-*trans*-but-2-enyl)-4-(pyridine-2-yl)benzamide hydrochloride; **$D_3$ receptor antagonist**

**Raclopride:** (3,5-Dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide (+)-tartrate salt; **$D_2$ receptor antagonist**

**SCH23390:** (R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; **$D_1$ receptor antagonist**

**SKF83822:** ([*R/S*]-6-chloro-7, 8-dihydroxy-3-allyl-1-[3-methyl-phenyl]-2,3,4,5-tetrahydro-1*H*-3-benzazepine; **$D_1$ receptor agonist**

**SKF83959:** 3-methyl-6-chloro-7,8-dihydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine; **$D_1$ receptor agonist**

# CHAPTER I

# INTRODUCTION

## Dopamine and Disorders of Dopamine Dysregulation:

The neurochemical dopamine (DA) is an important modulator in the central nervous system (CNS) implicated in a wide range of behavioral, cognitive, and homeostatic functions. Due to the fact that dopamine systems are involved in a variety of regulatory functions, dysregulation of dopaminergic neurotransmission often results in clinical phenotypes.

Imbalances in dopamine signaling appear to contribute to a variety of disorders including those of movement (ex: Parkinson's disease), executive function (ex: attention-deficit hyperactivity disorder (ADHD)), psychiatric disorders (ex: schizophrenia) and drug addiction (Kiyatkin, 1995b, Goldman-Rakic, 1998b, Nestler, 2001, Moore, 2003, Girault and Greengard, 2004, Arnsten and Li, 2005, Biederman and Faraone, 2005, Kalivas and Volkow, 2005). Although clinical phenotypes usually become apparent only later in life for many of these disorders, developmental abnormalities in dopaminergic circuit formation and connectivity may contribute as dopamine and its receptors can be detected very early in the developing brain across species (Frederick and Stanwood, 2009). Additionally, dopamine has been shown to directly modulate specific developmental processes in the forebrain that, if altered, can result in abnormal

brain development and potential manifestation of clinical behaviors (Metin et al., 2008, Bhide, 2009, Frederick and Stanwood, 2009).

## Dopaminergic Pathways in the CNS:

In the mammalian system, dopaminergic signaling originates within nine major groups of dopaminergic neurons classified as the A8-A17 cell groups. These groups are divided by their anatomical locations and represent midbrain dopamine neurons (groups A8-A10), diencephalic neurons (A11-A15), olfactory neurons (A16), and dopaminergic cells in the retina (A17) (Bjorklund and Dunnett, 2007). Figure 1 shows the distribution of dopamine neuron cell groups in the rodent brain.

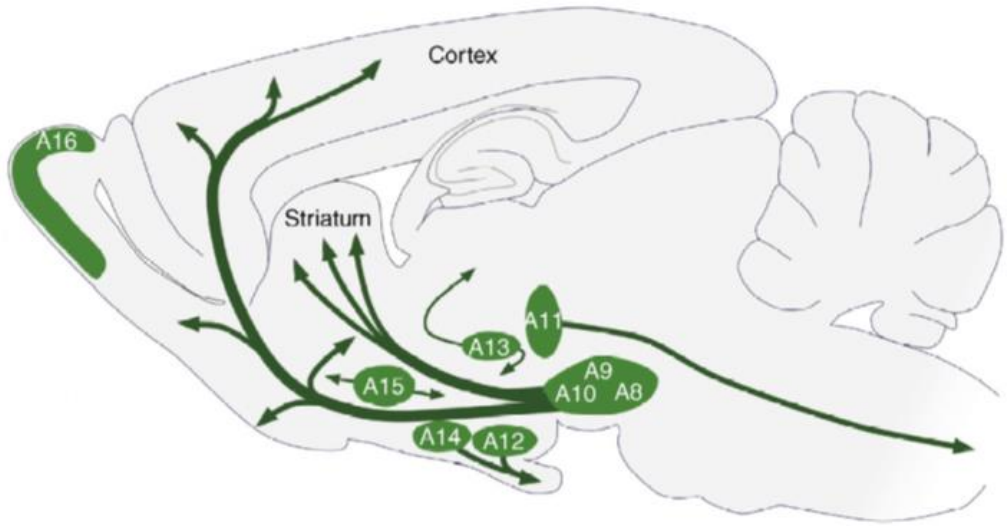


**Figure 1:** Sagittal view through an adult rodent brain depicting the nine dopamine neuron cell groups (A8-17) and their major projections. *(Adapted from Bjorklund and Dunnett, 2007)*

The nigrostriatal tract and the mesocorticolimbic pathways, originating in the A8-A10 cell groups, are two important dopaminergic pathways in the forebrain. The nigrostriatal projections consist of dopamine neurons with cell bodies located primarily in the substantia nigra (SN) pars compacta (A9 cell group) with axonal processes terminating in the dorsal striatum also known as the caudate putamen. The striatum is a component of the subcortical basal ganglia circuitry that plays an essential role in the coordination of voluntary locomotor activity. The mesocorticolimbic pathway arises in the midbrain ventral tegmental area (VTA) (A10 cell group) and can be further divided into the mesocortical and mesolimbic routes. The mesolimbic projections provide input to the nucleus accumbens and surrounding structures and are important for mediating behaviors associated with motivation, reward (endogenous systems and drug abuse) and reinforcement. The cortical projections, on the other hand, terminate in limbic cortical regions including the prefrontal cortex (PFC) and anterior cingulate cortex (ACC) to regulate cognitive and executive functions including attention (Weinberger et al., 1988, Fuxe et al., 2006, Pierce and Kumaresan, 2006, Bjorklund and Dunnett, 2007, Palmiter, 2011).

**Dopamine Receptors: Overview**

The physiological response to dopamine is mediated through five distinct dopamine receptors; the first two of which (the $D_1$ and $D_2$ dopamine receptors) were described in 1979 (Kebabian and Calne, 1979). The other three receptors, the $D_3$, $D_4$, and $D_5$ dopamine receptors, were not cloned from the human genome until over a decade later and were subsequently grouped with either the $D_1$ or $D_2$

receptor based on their pharmacological profiles, sequence homology and signal transduction systems (Sokoloff et al., 1990, Sunahara et al., 1991, Van Tol et al., 1991).

Dopamine receptors are seven-transmembrane guanine nucleotide binding protein (G-protein) coupled receptors (GPCRs). The receptors interact through the third intracellular loop with specific G-proteins to induce intracellular second messenger signaling cascades including regulation of calcium and potassium channels on the postsynaptic cell. Additionally, there is also auto-regulatory influence of dopamine through presynaptic receptor activation of receptors in the dopamine $D_2$-like receptor family (Freissmuth et al., 1989, Strader et al., 1994, Jaber et al., 1996, Missale et al., 1998, Beaulieu and Gainetdinov, 2011). Transmitter action at receptors is terminated by a number of regulatory mechanisms, including desensitization, down-regulation of receptor expression and clearance of the transmitter. Dopamine is cleared by re-uptake into the presynaptic terminal by a high affinity plasma membrane dopamine transporter (DAT) in most regions (or the norepinephrine transporter (NET) in the cortex) and by enzymatic degradation by monoamine oxidase (MAO) or catechol-o-methyl transferase (COMT) (Jaber et al., 1996, Missale et al., 1998, Beaulieu and Gainetdinov, 2011).

**Dopamine Receptors: Classification and Expression**

The $D_1$-like receptor family is comprised of the $D_1$ and $D_5$ receptor subtypes. These receptors share 80% transmembrane homology and have

similar pharmacologic profiles making it difficult for any known compound to fully discriminate between the two receptors (Sunahara et al., 1991, Tiberi et al., 1991, Tiberi and Caron, 1994). The $D_1$ receptor is expressed more globally throughout the brain and at higher levels than any of the other dopamine receptors. The $D_5$ receptor, by comparison, is much more modestly expressed in the brain and is primarily concentrated in the hippocampus, lateral mammillary nucleus and the parafasicular nucleus of the thalamus; areas where the $D_1$ receptor is not significantly expressed (Missale et al., 1998, Beaulieu and Gainetdinov, 2011). In regions such as the prefrontal cortex and striatum where both $D_1$ and $D_5$ receptors are expressed, it has been shown that the receptor subtypes localize to different cellular locations and populations of cells. For example, on cortical pyramidal cells in the primate brain, subtype-specific antibodies were used to demonstrate that $D_1$ receptors are concentrated in dendritic spines while $D_5$ receptors are localized to dendritic shafts. Additionally, $D_5$ receptors have been shown to be expressed on cholinergic interneurons in the cortex and striatum while $D_1$ receptors are located primarily on pyramidal neurons and medium spiny neurons (MSNs), respectively (Bergson et al., 1995, Wang et al., 1997, Khan et al., 2000, Berlanga et al., 2005).

The $D_2$-like receptor family is comprised of the dopamine $D_2$, $D_3$, and $D_4$ receptor subtypes. The $D_2$ and $D_3$ receptors share a high degree of sequence homology with 52% amino acid homology overall and 75% homology within their transmembrane domains (Sokoloff et al., 1990). The dopamine $D_4$ receptor is less conserved; however, sharing only 41% overall and approximately 50%

transmembrane homology with the $D_2$ receptor (Van Tol et al., 1991). Of the three $D_2$-like receptor subtypes, the $D_2$ receptor proper is most widely and abundantly expressed in the mammalian brain, where is it is found predominantly in the striatum, nucleus accumbens, cortex, olfactory tubercle, hippocampus, amygdala and the hypothalamus. The $D_3$ receptor has a more limited and specific pattern of distribution than the $D_2$ receptor; concentrated primarily in limbic brain regions including the shell of the nucleus accumbens, the olfactory tubercle, and the islands of Calleja (Sokoloff et al., 1990, Missale et al., 1998, Stanwood et al., 2000, Beaulieu and Gainetdinov, 2011). The distribution patterns of $D_2$ and $D_3$ receptors are therefore overlapping in some regions, however, receptor binding studies with radioactive ligands have shown that $D_2$ receptor expression is at least twice as abundant as $D_3$ receptor expression in regions where both receptors are expressed (Sokoloff et al., 1990, Levesque et al., 1992, Gurevich and Joyce, 1999). The $D_4$ dopamine receptor subtype has a widespread distribution in the mammalian brain and appears to be concentrated in the frontal cortex, amygdala, hippocampus, hypothalamus and the retina (Van Tol et al., 1991, Defagot et al., 1997, Missale et al., 1998, Beaulieu and Gainetdinov, 2011). Particularly within the cortex, signaling through the $D_4$ receptor subtype has been shown to be important in modulating specific functions (Lauzon and Laviolette, 2010, Rondou et al., 2010, Lauzon et al., 2011) including glutamatergic neurotransmission in pyramidal neurons (Wang et al., 2003, Yuen et al., 2010, Yuen and Yan, 2011), modulation of $GABA_A$ receptor currents (Wang et al., 2002, Graziane et al., 2009) and regulation of calcium

($Ca^{2+}$)/ calmodulin (CaM)-dependent protein kinase II $\alpha$ ($CaMKII\alpha$) (Gu and Yan, 2004, Lauzon et al., 2011). Furthermore, mutations and polymorphic variants in *DRD4*, the gene that encodes the $D_4$ receptor, have been shown to alter receptor function and have been implicated in a variety of disorders including ADHD and schizophrenia (DiMaio et al., 2003, Jonsson et al., 2003, Tarazi et al., 2004, Lauzon and Laviolette, 2010, Rondou et al., 2010, Ptacek et al., 2011).

Additionally, $D_1$ and $D_2$ receptor protein expression has been observed in the SN pars reticulata and the external segment of the globus pallidus; regions of the basal ganglia circuit that receive input from MSNs in the striatum. Dopamine receptor mRNA has not been detected in these regions, however, indicating that dopamine receptors in these regions are present on MSN projections from the striatum (Jaber et al., 1996, Missale et al., 1998).

**Dopamine Receptors: Signaling**

Dopamine receptors couple to G-proteins which serve as signal transducers between the membrane-bound receptors and intracellular effector systems. G-proteins exist in hetero-trimeric complexes composed of a $G_\alpha$ subunit in association with $G_{\beta\gamma}$ which exists as a functional dimer. Activation of the G-protein complex is controlled by a regulatory cycle involving receptor-activated exchange of GDP for GTP on $G_\alpha$, dissociation of the trimer, activation of effector molecules, and inactivation through the GTPase activity of $G_\alpha$ (Freissmuth et al., 1989, Strader et al., 1994).

$D_1$-like receptors couple to stimulatory $G_{\alpha s}$ proteins to increase adenylyl cyclase (AC) activity in most brain regions (Kebabian and Calne, 1979). In the striatum, however, $D_1$ receptor signaling is mediated by $G_{\alpha olf}$, a stimulatory G-protein which exists in greater abundance than $G_{\alpha s}$ in that region (Zhuang et al., 2000, Corvol et al., 2001, Herve et al., 2001). $D_2$-like receptors have antagonistic functions to $D_1$-like receptors; coupling to $G_{\alpha i/o}$ to inhibit cyclase activity (Kebabian and Calne, 1979). Downstream of cyclase activity, the two classes of receptors differentially regulate the activity of cyclic adenosine monophosphate (cAMP) and dependent proteins. One such protein, cAMP-dependent protein kinase A (PKA), is a kinase that phosphorylates a large number of substrates including the dopamine and cAMP-regulated phosphoprotein of 32kDa (DARPP-32), a cytosolic protein that is enriched in dopaminoceptive neurons. PKA phosphorylates DARRP-32 on residue $Thr^{34}$ thus converting DARPP-32 into a potent inhibitor of protein phosphatase 1 (PP1). The balance of PKA/PP1 thereby regulates the phosphorylation of DARPP-32-$Thr^{34}$, which modulates PKA/PP1 in a feedback loop, and the phosphorylation state of many downstream effectors including $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (Greengard, 2001, Neve et al., 2004, Svenningsson et al., 2004). Within the C-terminal domain of the GluR1 subunit, there are two major phosphorylation sites, one of which, residue $Ser^{845}$, is regulated by PKA. Dopamine receptor-mediated regulation of PKA and DARPP-32 therefore has a role in regulating neuronal excitability through influencing the function of AMPA receptor GluR1 phosphorylation state. Phosphorylation at

GluR1-Ser$^{845}$ increases the peak open probability and surface expression of AMPA receptors, thereby enhancing AMPA currents and neurotransmission (Carvalho et al., 2000, Wang et al., 2005, Santos et al., 2009).

The second major GluR1 phosphorylation site is regulated by CaMKII$\alpha$; an enzyme that is highly enriched in the postsynaptic density (PSD) and central to synaptic transmission and plasticity. Following increased calcium signal, $Ca^{2+}$/CaM binds to the regulatory domain of the kinase thereby activating the molecule for phosphorylation of exogenous substrates as well as autophosphorylation at residue Thr$^{286}$. Autophosphorylation at residue Thr$^{286}$ renders activity of CaMKII$\alpha$ independent of $Ca^{2+}$/CaM regulation and persists after the initial calcium signal has subsided. In this way, CaMKII$\alpha$ is able to convert transient calcium signals into longer-lasting changes in synaptic activity (Colbran and Brown, 2004). CaMKII$\alpha$ phosphorylates AMPA GluR1 at Ser$^{831}$ thus increasing AMPA receptor conductance and enhancing neuronal activity (Carvalho et al., 2000, Wang et al., 2005, Santos et al., 2009). Dephosphorylation of CaMKII$\alpha$ is mediated by PP1 which is also localized at the PSD and has a role in regulating AMPAR functions, as previously described (Carvalho et al., 2000, Colbran and Brown, 2004, Wang et al., 2005, Santos et al., 2009).

Dopamine $D_1$ receptor-mediated regulation of intracellular $Ca^{2+}$ mobilization and CaMKII$\alpha$ has been described (Lee et al., 2004, Zhen et al., 2004, So et al., 2005, Rashid et al., 2007, Ng et al., 2010); thus providing a second mechanism by which dopamine can modulate glutamatergic signaling and neurotransmission. In this mechanism, $D_1$ receptors are reported to couple

to $G_{\alpha q}$ to activate phospholipase C (PLC) and generate inositol triphosphate ($IP_3$) and diacylglycerol second messengers from PLC-mediated phosphatidylinositol (PI) metabolism. Liberated $IP_3$ then binds intracellular receptors to release $Ca^{2+}$ from intracellular stores and activate $Ca^{2+}$ sensitive molecules such as CaMKII$\alpha$ (Freissmuth et al., 1989, Undie et al., 1994, Wang et al., 1995, Jin et al., 2001, Jin et al., 2003, Rashid et al., 2007).

Although $D_1$ receptor-mediated PI signaling has been previously described (Undie and Friedman, 1990, Undie et al., 1994, Jin et al., 2003), the mechanism remained elusive as $D_1$ receptor agonist-induced $IP_3$ accumulation and some behaviors thought to be mediated by $D_1$ receptors signaling in this pathway were conserved in $D_1$ receptor knockout mice (Friedman et al., 1997, Clifford et al., 1998, Clifford et al., 1999). In 2007, however, Rashid *et. al.* (Rashid et al., 2007) suggested that $D_1$ receptor-mediated PI signaling was resultant from complexes containing $D_1$ and $D_2$ receptor heteromers coupled to $G_{\alpha q}$. In this mechanism, dopaminergic stimulation of neurons that co-express $D_1$ and $D_2$ receptors would activate the $G_{\alpha q}$ pathway, triggering $Ca^{2+}$ release and activation of CaMKII$\alpha$ (Lee et al., 2004, Zhen et al., 2004, So et al., 2005, Rashid et al., 2007, Ng et al., 2010). Although the biological significance of a $D_1/D_2$ receptor pathway coupled to $G_{\alpha q}$ remains unknown; signaling in this pathway has been implicated in a variety of physiological processes including the production of brain-derived neurotrophic factor (BDNF) (Hasbi et al., 2009), neuronal growth (Hasbi et al., 2009), modulation of fibroblast growth factor (FGF)-2 in astrocytes (Zhang et al., 2009), inhibition of high-voltage $Ca^{2+}$ currents in the striatum (Ma et al., 2009),

facilitation of long-term depression in the hippocampus (Liu et al., 2009b) and spontaneous glutamate release in the cortex (Chu et al., 2010). Additionally, the $D_1/D_2$ receptor heteromer has been implicated in pathological conditions; radioligand binding studies reveal an increased proportion of heteromers in the high-affinity state following repeated amphetamine treatment and in the post-mortem brain tissue of individuals that suffered with schizophrenia (Perreault et al., 2010). Furthermore, increased association between $D_1$ and $D_2$ receptors has also been described in the post-mortem brain tissue of individuals who suffered from depression as assessed using co-immunoprecipitation techniques (Pei et al., 2010).

It is recognized, however, that a signaling mechanism involving heteromer formation between $D_1$ and $D_2$ dopamine receptors is not feasible in all brain regions as $D_1$ and $D_2$ receptors are not co-expressed in most neurons. Use of Bacterial Artificial Chromosome (BAC) transgenic mice that express fluorescent proteins under the control of the $D_1$ or $D_2$ receptor promoter have allowed for the visualization of cells that co-express these receptor subtypes. Within the striatum, for example, it has been convincingly demonstrated that MSNs are largely segregated into two distinct pathways, defined as the direct or indirect pathways based on their terminal projections, which express either $D_1$ or $D_2$ receptors, respectively. Only 5-10% of cells in this region, however, have been shown to co-express both receptors. Co-expression of $D_1$ and $D_2$ receptors has also been demonstrated in the prefrontal cortex of BAC transgenic mice, with 20-25% of cells expressing both receptors (Shuen et al., 2008, Matamales et al.,

2009, Valjent et al., 2009, Zhang et al., 2010). Furthermore, use of double-labeled fluorescence immunohistochemistry and fluorescence resonance energy transfer (FRET) technology has revealed additional regions of $D_1$ and $D_2$ receptor co-localization, including the nucleus accumbens and globus pallidus, while confirming the segregation of receptors into separate populations in the striatum (Perreault et al., 2010). Signal transduction mediated by a $D_1/D_2$ receptor heteromer coupled to $G_{\alpha q}$ would constitute a distinct signaling pathway from either $D_1$ receptor or $D_2$ receptor activation of $G_{\alpha s}/G_{\alpha olf}$ or $G_{\alpha i/o}$, respectively; and one which would be highly regulated in specific brain regions and cells (Sahu et al., 2009, Perreault et al., 2011).

Additionally, $D_5$ receptors have been shown to demonstrate the ability to signal through $G_{\alpha q}$ to accumulate $IP_3$ in hippocampal, striatal and cortical membranes stimulated with dopamine or $D_1$ receptor agonists; an ability that was lost in $D_5$ receptor knockout mice (Sahu et al., 2009). The mechanism of this signaling, however, has been suggested to be distinct from that of the reported $D_1/D_2$ receptor heteromer (So et al., 2009, Hasbi et al., 2010) as the $D_1$ and $D_5$ receptor subtypes are largely expressed on different cell populations in the striatum (Khan et al., 2000, Berlanga et al., 2005).

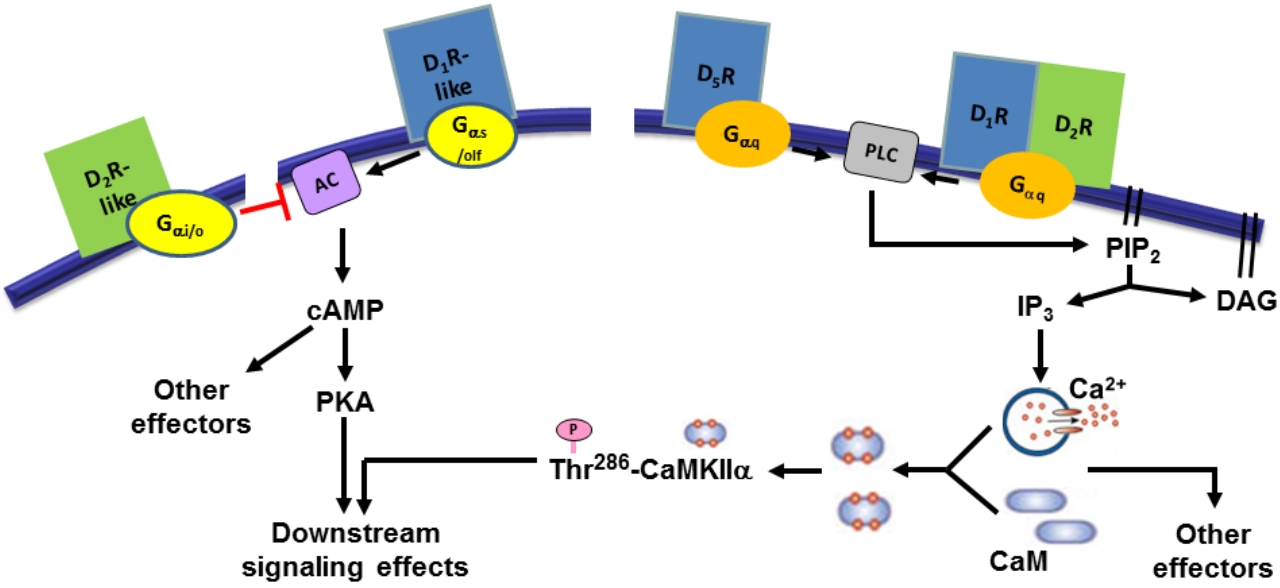


**Figure 2:** Dopamine $D_1$ receptor activation increases adenylyl cyclase activity to increase cAMP and PKA activity. Activation of $D_2$ receptors, on the other hand, has opposing functions and reduces activity of PKA. Additionally, it has been reported that $D_5$ receptors and $D_1/D_2$ receptor heteromers are able to couple and signal through $G_{\alpha q}$ to stimulate PI hydrolysis, $IP_3$ formation and intracellular calcium release.

## Dopamine Receptors: Expression in the Striatum and Direct/Indirect Pathways

The dopamine $D_1$ and $D_2$ receptor subtypes possess distinct cellular distributions within the striatum where they are involved in modulating basal ganglia locomotor circuits. Within the striatum, dopaminergic axons from the SN pars compacta innervate $\gamma$-aminobutyric acid (GABA)ergic MSNs which account for approximately 90% of the cellular population. Also forming synaptic contacts on the dendritic arbors of MSNs are descending corticostriatal glutamatergic inputs and those projections coming from the thalamus. As mentioned, MSNs are largely segregated into two populations based on expression of dopamine receptors and axonal projections. MSNs primarily expressing dopamine $D_1$ receptors, and co-expressing the peptide substance P, project directly to the SN pars reticulata in the striatonigral pathway. Those cells enriched in $D_2$ receptors and additionally expressing enkephalins, project to the SN pars reticulata via an indirect route involving intermediate synapses in the globus pallidus and the subthalamic nucleus; the striatopallidal pathway. The SN pars reticulata then relays signals to the thalamus and motor cortex (stimulatory in the case of the direct pathway or inhibitory from the indirect) for the control of voluntary movement (Gerfen, 1992, Albin et al., 1995, Shuen et al., 2008).

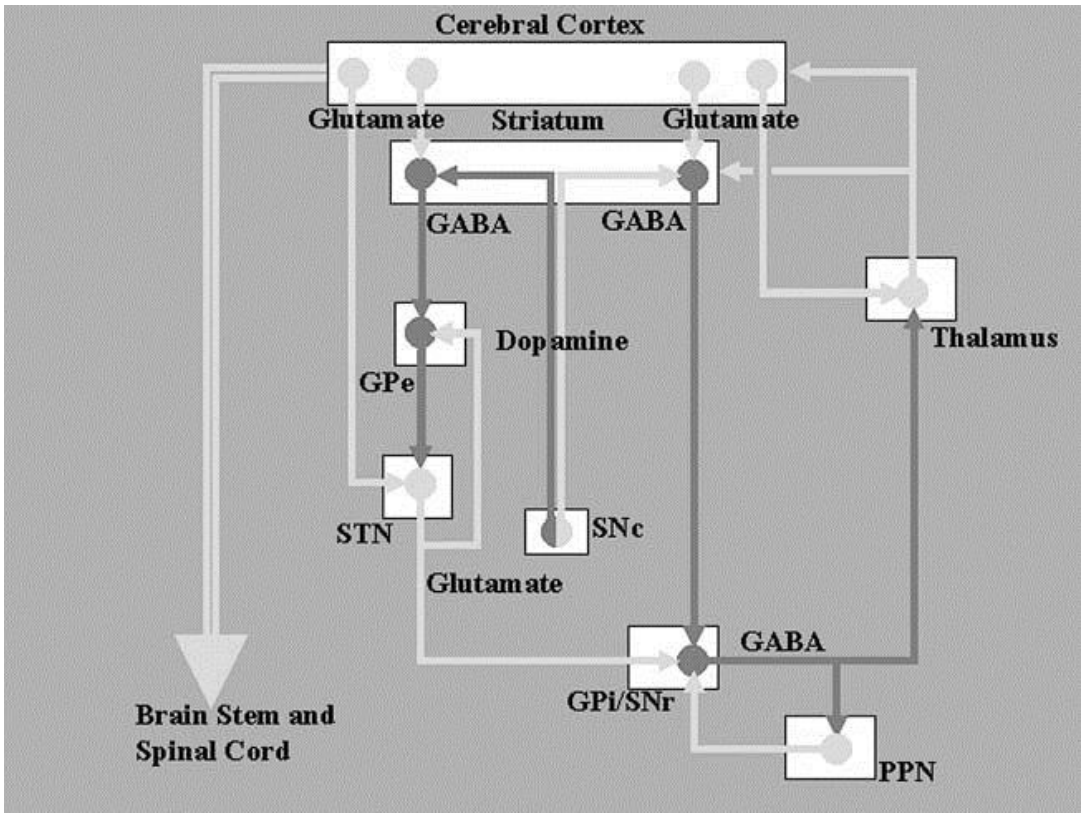


**Figure 3:** Direct and Indirect motor pathways of the basal ganglia are modulated by dopaminergic input. In the direct pathway, $D_1$ receptor stimulation in the striatum acts to increase activity in the circuit, while $D_2$ receptors in the indirect pathway act to reduce input to the cortex. The light grey lines represent excitatory pathways mediated by glutamatergic neurotransmission, and the darker lines show inhibitory projections mediated by GABA; projections from the Substantia Nigra pars compacta (SNc) to the striatum, however, are mediated by dopamine and are depicted as excitatory (light grey) or inhibitory (dark grey) based on the dopamine receptors activated ($D_1$ or $D_2$ receptors respectively) in the striatal MSNs.
*(Adapted from http://functionalneurosurgery.net/parkinsonsurgery.htm)*

**Dopamine and Locomotion:**

Dopamine neurotransmission plays a fundamental role in normal functioning of the basal ganglia and an extensive literature exists on the role of dopaminergic signaling in modulating locomotor behaviors (Beninger, 1983, Fishman et al., 1983, Waddington and O'Boyle, 1987, 1989, Gerfen, 1992, Jackson and Westlind-Danielsson, 1994, Missale et al., 1998, Beaulieu and Gainetdinov, 2011). The identification of selective $D_1$-like and $D_2$-like receptor ligands has been instrumental in dissecting the roles of the individual receptor subtypes as well as the interactions between the two dopamine signaling systems in mediating specific aspects of behavior. In addition, the creation of dopamine receptor knockout mice has provided an invaluable genetic tool to investigate these functions. Furthermore, evaluation of knockout mice has largely revealed phenotypes resembling those observed with pharmacological manipulations using selective ligands.

Multiple lines of evidence, using pharmacological and genetic approaches, indicate that locomotor activity is primarily controlled by post-synaptic $D_1$ receptor stimulation and $D_2$ and $D_3$ dopamine receptors expressed at both pre- and postsynaptic locations (Waddington and O'Boyle, 1989, Jackson and Westlind-Danielsson, 1994, Waddington et al., 1995). It should be noted that there are two splice variants of the $D_2$ dopamine receptor, $D_2$ long ($D_2L$) and $D_2$ short ($D_2S$) receptor variants that are differently distributed within these pre- and post-synaptic neuronal population; $D_2S$ receptors being predominantly presynaptic in

location while the $D_2$L receptor isoforms are found predominantly at postsynaptic sites (Usiello et al., 2000, De Mei et al., 2009).

$D_1$ receptor stimulation by selective agonists increases locomotor activity that can be blocked by the $D_1$ receptor antagonist, SCH23390; indicating the importance of $D_1$ receptors in locomotor activation (Molloy et al., 1986, Waddington and O'Boyle, 1989, Xu et al., 1994b). Furthermore, evaluation of $D_1$ receptor knockout mice revealed a phenotype of normal or increased locomotor activity (depending upon the originating line) (Drago et al., 1994, Xu et al., 1994b, Waddington et al., 2001, Waddington et al., 2005), that was not altered by administration of $D_1$ receptor agonists or SCH23390 (Xu et al., 1994b, Waddington et al., 2001, Waddington et al., 2005). These mice were originally created on a mixed background, however, later studies in mutant mice on a congenic C57Bl/J background also revealed a hyperactive phenotype (McNamara et al., 2003). An additional phenotype of blunted habituation (thereby increasing activity compared to wildtype over time) was also observed in these mice (McNamara et al., 2003). Taken together, these data not only demonstrate a role for the $D_1$ receptor in the locomotor activating properties of dopaminergic ligands, but also validates the *in vivo* selectivity of the $D_1$ receptor ligands that had been previously demonstrated using receptor binding techniques. Additionally, $D_1$ receptor null mice did not increase their locomotor activity in response to cocaine, a psychostimulant drug that elicits a profound locomotor response in wildtype mice (Xu et al., 1994a, Drago et al., 1998).

Modulation of locomotor activity by $D_2$-like receptors is more complex. Presynaptic autoreceptors generally provide an important negative feedback mechanism that adjusts neuronal firing rate, synthesis, and release of dopamine in response to changes in extracellular levels of dopamine. These receptors, therefore, generally decrease dopamine release when stimulated resulting in decreased locomotor activity (Jackson and Westlind-Danielsson, 1994, Beaulieu and Gainetdinov, 2011). Stimulation of postsynaptic receptors, on the other hand, generally increases locomotor activity (Molloy et al., 1986).

Additionally, $D_2$-like autoreceptors are generally activated by lower concentrations of dopaminergic agonists than those necessary to activate postsynaptic receptors; a mechanism by which the same agonist can induce a biphasic locomotor response with decreased activity at low doses and locomotor activation at higher doses (Waddington and O'Boyle, 1989, Jackson and Westlind-Danielsson, 1994, Beaulieu and Gainetdinov, 2011). Consistent with the ability of $D_2$ receptors to increase locomotor responses, behavioral characterization of $D_2$ receptor knockout mice revealed an akinetic phenotype with significantly reduced spontaneous movement; one that resembled the extrapyramidal symptoms associated with Parkinson's disease (although the severity varied dependent upon the line assessed) (Baik et al., 1995, Kelly et al., 1998, Clifford et al., 2000, Wang et al., 2000, Waddington et al., 2005). This phenotype was also recapitulated by administration of $D_2$-like receptor antagonists (Wadenberg et al., 2000, Waddington et al., 2005) further indicating that $D_2$ receptor stimulation is critical for locomotor activation.

Furthermore, it is clear that there is a synergic interaction between simultaneous activation of $D_1$-like and $D_2$-like dopamine receptors that is necessary for maximal stimulation of locomotor activity (Carlson et al., 1987, White et al., 1988, Jackson and Westlind-Danielsson, 1994). Mutant mice with simultaneous knockout of the $D_1$ and $D_3$ receptor subtypes or the $D_2$ and $D_3$ receptors have been evaluated to address these interactions between receptor subtypes (Hu and White, 1994, Jung et al., 1999, Karasinska et al., 2000, Wong et al., 2003). Additionally, $D_1$/$D_2$ receptor knockouts have been created, however concurrent deletion of the $D_1$ and $D_2$ receptors is not compatible with postnatal life (Kobayashi et al., 2004).

Pharmacological manipulation of $D_3$ receptor signaling with $D_3$ receptor-preferring ligands (over $D_2$ receptors) and $D_3$ receptor knockout mice suggests that activation of $D_3$ dopamine receptors exerts an inhibitory effect on locomotion; either by acting as autoreceptors or through the involvement of postsynaptic receptor populations. $D_3$ receptor-preferring agonists have been shown to inhibit locomotor activity (Daly and Waddington, 1993, Sokoloff and Schwartz, 1995) whereas $D_3$-preferring antagonists evoke motor activation (Waters et al., 1993, Sokoloff and Schwartz, 1995). Additionally, $D_3$ receptor knockout mice have been shown to exhibit heighted basal locomotor activity in some studies (Accili et al., 1996, Steiner et al., 1997, Xu et al., 1997, Joseph et al., 2002), although this phenotype has not been observed in all strains tested (Waddington et al., 2005).

Lastly, the role of the $D_5$ and $D_4$ receptor subtypes in modulating locomotor activity has yielded inconclusive results. Initial phenotypic

characterization of $D_5$ receptor knockout mice revealed a hyperactive phenotype, while other characterizations revealed no differences or reduced activity (Sibley, 1999, Holmes et al., 2001, Elliot et al., 2003, Waddington et al., 2005). Additionally, the lack of $D_5$ receptor-selective ligands makes it impossible to pharmacologically assess the selective contribution of this receptor to locomotor responses. Similarly, in $D_4$ receptor knockout mice, initial evaluation indicated a hypoactive phenotype in these mice (Rubinstein et al., 1997), however that was later shown to be likely due to novelty and not a pure motor phenotype (Dulawa et al., 1999, Falzone et al., 2002). Furthermore, the $D_4$ receptor is not highly expressed in the basal ganglia, therefore the contribution of $D_4$ receptors to locomotor behaviors likely originates from modulation of glutamatergic input from the cortex (where $D_4$ receptors are highly expressed) into the striatum (Rondou et al., 2010).

**Functional Selectivity of GPCRs:**

Functional selectivity (also referred to as biased agonism, protean agonism, agonist-directed trafficking of receptor stimulus, collateral efficacy, differential engagement, or stimulus trafficking) describes the ability of agonists to stabilize different active conformations of the same GPCR; thereby resulting in differential activation of signaling pathways and cellular responses mediated by a single receptor (Mailman, 2007, Urban et al., 2007, Stallaert et al., 2011). This idea has been slow to gain popularity in the field, as classical pharmacology theory would suggest that agonist-induced activation of GPCRs is generally a “linear” process;

that is, all agonists that activate a particular GPCR do so by stabilizing the same conformation of the receptor, which then couples to the same G-protein(s) or accessory effector proteins and subsequently activates (or inhibits) the same set of intracellular responses (Figure 4A). The idea of functional selectivity, therefore, is seemingly irreconcilable with this premise of classical receptor signaling. More recently, however, an evolving body of literature has demonstrated that agonists that activate the same GPCR are capable of producing different physiological response profiles through activation of different types of G-proteins *in vitro* and *in vivo* (Figure 4B) (Mailman, 2007, Urban et al., 2007, Stallaert et al., 2011). Additionally, it has been shown that some agonists trigger different regulatory processes (including differential recruitment of arrestins and G protein-coupled receptor kinases) even when they activate the same G-proteins; thereby producing different cellular responses by undergoing different mechanisms of desensitization and GPRC-independent signaling (Kelly et al., 2008, Porrello et al., 2011, Stallaert et al., 2011). To date, there are a number of examples in the literature of functionally selective ligands at various GPCR targets that have been shown to contribute to physiological or pathophysiological processes. Additionally, functionally selective interactions across dimers has also been described (Mailman, 2007, Urban et al., 2007, Mailman and Murthy, 2010, Patel et al., 2010, Gesty-Palmer and Luttrell, 2011, Porrello et al., 2011, Urizar et al., 2011).

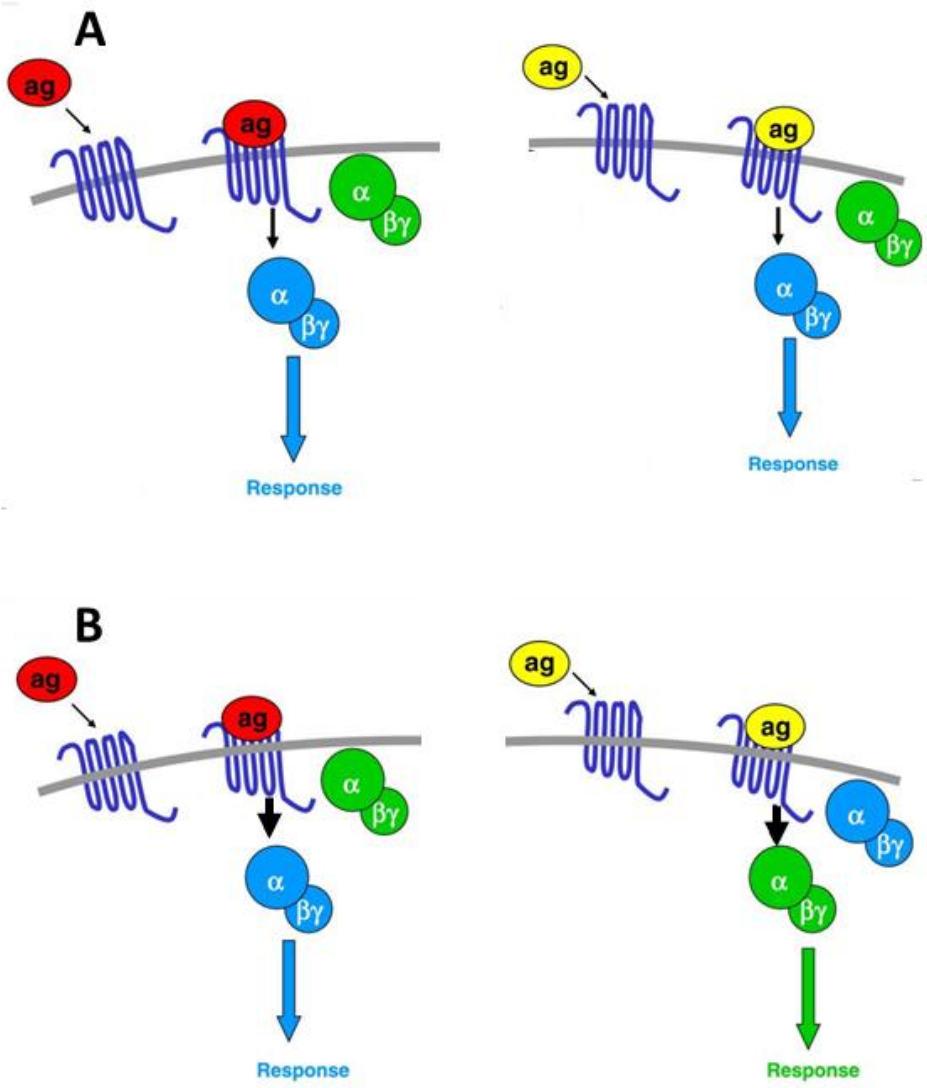


**Figure 4:** Functional selectivity of agonists (ag) at GPCRs. (**A**) is a schematic of the traditional "linear" model of agonist action at a GPCR where both the red and yellow agonists produce the same cellular responses through the same set of G-proteins (blue). Functional selectivity is depicted in (**B**) where the red and yellow agonists each stabilize a different conformation of the same GPCR, thereby coupling to different G-proteins (blue or green, respectively) and producing different cellular responses. (*Adapted from Kelly et. al., 2008*)

**Functional Selectivity in the Dopamine System:**

Functionally selective ligands, because of their ability to modulate differential signaling, can "fine-tune" pharmacological responses; a significant advantage for engineering drugs that not only target specific GPCRs, but specific signaling pathways for greater therapeutic efficacy with reduced side-effects from off-target signaling. Within the dopamine system, functional selectivity of dopamine receptors has been described at the level of G-protein coupling, mechanisms of desensitization and GPCR-independent signaling; thereby increasing the diversity of mechanisms by which dopamine can modulate cellular processes (Jin et al., 2001, Beaulieu et al., 2005, Ryman-Rasmussen et al., 2005, Rashid et al., 2007, Ryman-Rasmussen et al., 2007, Mailman and Murthy, 2010, Beaulieu and Gainetdinov, 2011). Particularly of interest to us are the $D_1$-like receptor ligands which have been reported to preferentially activate $D_1$ receptors coupled to alternative signaling through $G_{\alpha q}$ (Arnt et al., 1992, Andringa et al., 1999, Cools et al., 2002, Jin et al., 2003, Rashid et al., 2007).

The high-affinity benzazepine compound SKF83959 is one such ligand that has been reported to show functional selectivity at dopamine $D_1$ receptors. SKF83959, a derivative of the prototypic $D_1$ receptor agonist SKF38393 (Setler et al., 1978), was classified as a $D_1$ receptor partial agonist, however with a very different pharmacologic profile than previous ligands in this class. In 1992, Arnt and colleagues reported that SKF83959 had no significant agonist activity in stimulating adenylyl cyclase in their *in vitro* system and actually inhibited the cyclase-stimulating activity of 100 μM dopamine, similar to the reference

antagonist SCH23390. Seemingly contradictory, however, SKF83959 retained agonist activity behaviorally; inducing circling behavior and oral stereotypies in unilateral 6-hydroxydopamine (6-OHDA) lesioned rats, a rodent model of Parkinson's disease, with similar efficacy as SKF38393 (Arnt et al., 1992). These initial biochemical characterizations of SKF83959 were subsequently echoed by other laboratories demonstrating antagonistic properties of SKF83959 both *in vitro* and *in vivo* (Andringa et al., 1999, Cools et al., 2002, Jin et al., 2003). It was difficult to reconcile biochemical antagonism with the behavioral properties of this ligand as studies from a variety of laboratories interested in the clinical efficacy of SKF83959 demonstrated that it produced behavioral responses similar to other cyclase-stimulating $D_1$ receptor agonists. Behaviorally, SKF83959 was shown to elicit intense grooming behavior and orofacial movements in rodent models (Downes and Waddington, 1993, Fujita et al., 2010), have efficacy in reversing some parkinsonian motor symptoms in both rodent and non-human primate (MPTP treated) models of Parkinson's disease (Arnt et al., 1992, Gnanalingham et al., 1995a, Gnanalingham et al., 1995c, b, Zhang et al., 2007), and reduce the abuse-related effects of cocaine including its self-administration (Bergman et al., 2000, Khroyan et al., 2000, Platt et al., 2001).

As it appeared that the efficacy of SKF83959 in stimulating adenylyl cyclase did not correlate with its behavioral efficacy, the possibility that SKF83959 might activate other signaling systems was explored. There was already evidence in the literature suggesting that dopamine, and some $D_1$ receptor agonists (SKF38393 for example) could modulate PI metabolism in the

amygdala, hippocampus, striatum and frontal cortex (Mahan et al., 1990, Undie and Friedman, 1990, 1992, Undie et al., 1994). These actions were blocked by the $D_1$ receptor antagonist SCH23390 (Undie and Friedman, 1990, 1992), co-stimulation of cAMP with forskolin (Undie and Friedman, 1994) or co-activation of PKA with Sp-cAMPS (Undie and Friedman, 1994), but not by the $D_2$ receptor antagonist sulpiride (Undie and Friedman, 1990, 1992). Dopamine-mediated modulation of intracellular calcium currents was also demonstrated (Mahan et al., 1990, Tang and Bezprozvanny, 2004, Ming et al., 2006, Rashid et al., 2007, Liu et al., 2009a).

As increasing numbers of $D_1$ receptor agonists were assessed for biochemical profiling, there was mounting evidence suggesting that $D_1$ receptor ligands possessed differential efficacies for activating adenylyl cyclase and/or PI metabolism with no correlation between the potencies in the two assays; evidence potentially indicating that $D_1$-like receptors mediating PI signaling were pharmacologically distinct from classic $D_1$ receptors coupled to adenylyl cyclase activity (Felder et al., 1989, Undie and Friedman, 1994, Undie et al., 1994). In 1995, Wang *et. al.* demonstrated the ability of $D_1$ receptors to couple to $G_{\alpha q}$ in rat striatum thereby providing a putative mechanism for dopamine-stimulated PI signaling through alternate G-protein coupling (Wang et al., 1995). $D_1$ receptor-$G_{\alpha q}$ coupling (and $G_{\alpha i}$ to some degree) was later demonstrated in the amygdala, hippocampus, and frontal cortex and was additionally shown to be blocked by SCH23390 but not by sulpiride (Jin et al., 2001, Jin et al., 2003, Mannoury la Cour et al., 2007). Furthermore, SKF83959 was shown to enhance PI hydrolysis

in rat and monkey brain tissue with greater potency than SKF38393, thereby attributing a potential signaling mechanism to the behavioral actions of the ligand (Panchalingam and Undie, 2001, Jin et al., 2003). Interestingly, however, SKF83959-induced PI hydrolysis was not induced in PC12 cells expressing dopamine $D_1$ receptors (Jin et al., 2003). Additionally, dopamine and SKF38393-induced $IP_3$ accumulation and co-immunoprecipitation of [$^3$H]SCH23390 binding sites with $G_{\alpha q}$ were shown to be retained in the striatum of $D_1$ receptor knockout mice. Accumulation of cAMP, on the other hand, was lost and $G_{\alpha s}$ binding sites were greatly reduced (Friedman et al., 1997); further evidence suggesting that the classical dopamine $D_1$ receptor may not be the receptor responsible for mediating PI hydrolysis.

In 2007, the veil of mystery surrounding the molecular identity of the $D_1$-like dopamine receptor linked to $G_{\alpha q}$ signaling appeared to be lifted. Rashid *et. al.* reported evidence suggesting that dopaminergic modulation of $G_{\alpha q}$ was resultant from activation of heteromeric complexes containing $D_1$ and $D_2$ dopamine receptors in the striatum. When activated by the $D_1$ receptor agonist SKF81297, an intracellular calcium signal was produced that could be blocked by either SCH23390 or the $D_2$ receptor antagonist, raclopride, indicating involvement of the $D_2$ receptor (Rashid et al., 2007). There was some evidence in the literature suggesting that SKF83959 had moderate affinity for other receptors, including dopamine $D_2$ receptors and alpha adrenergic receptors (Andringa et al., 1999, Neumeyer et al., 2003), however previous studies failed to establish a role for these receptors in the signaling of SKF83959 (Undie and

Friedman, 1990, 1992, Jin et al., 2001). It was further demonstrated in these studies that the $D_2$ receptor agonist quinpirole was unable to stimulate calcium release; however, it potentiated the response when co-applied with SKF81297 or SKF83959 thereby indicating that $D_2$ receptors were only involved in the signaling if co-activated with $D_1$ receptors (Rashid et al., 2007). Furthermore, Rashid *et. al.* reported the ability of specific $D_1$ receptor ligands to differentially stimulate coupling to $G_{\alpha s}$ or $G_{\alpha q}$; thereby identifying functionally selective ligands for activating AC-coupled or PI hydrolysis-coupled $D_1$ receptors (Rashid et al., 2007). Consistent with previous reports, SKF83959 was reported to preferentially activate $D_1$ receptors coupled to $G_{\alpha q}$ and PI hydrolysis while a second benzazepine analog SKF83822 was claimed to selectively activate $D_1$ receptors coupled to $G_{\alpha s}$ and cyclase activity (Figure 5) (Rashid et al., 2007). To date, SKF83959-induced signaling has been implicated in a variety of physiological processes (Kuroiwa et al., 2008, Chen et al., 2009, Liu et al., 2009a, Fujita et al., 2010, Stolzenberg et al., 2010, Chu et al., 2011, Nimitvilai et al., 2012) and some chemical optimization has been done around the ligand for development of new compounds with increased potential for drug development (Neumeyer et al., 2003, Desai et al., 2007, Zhang et al., 2008).

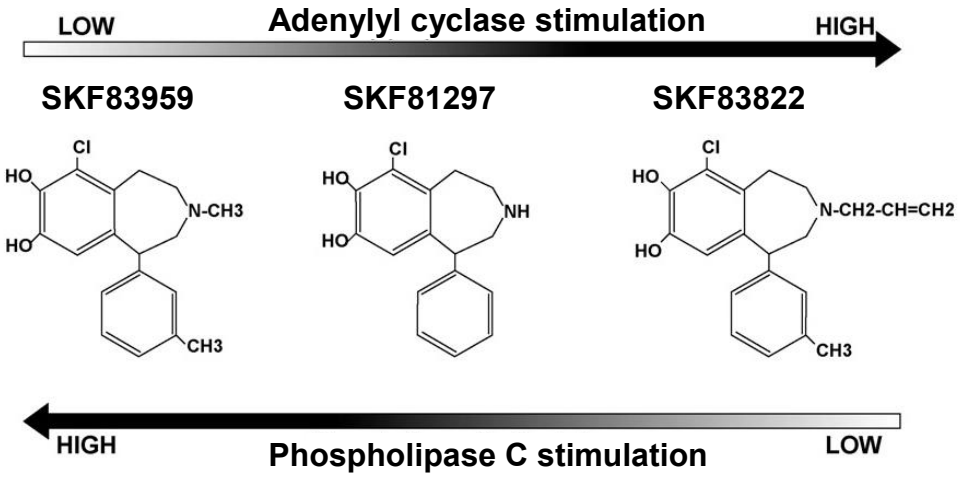


**Figure 5:** Specific $D_1$-like receptor agonists have been reported to preferentially activate $G_{\alpha q}$ and/or $G_{\alpha s}$ signaling pathways. *(Schematic adapted from Rashid et. al., 2007)*

**Summary:**

SKF83959 is a high affinity dopamine $D_1$ receptor agonist that has been reported to preferentially activate $D_1$ receptors coupled to $G_{\alpha q}$. This pathway results in Pl hydrolysis, intracellular calcium mobilization, and potential activation of CaMKII$\alpha$, an important regulator of synaptic transmission. Although the exact mechanism remains unclear, one recent model suggests that SKF83959 activates $D_1/D_2$ receptor heteromeric complexes coupled to $G_{\alpha q}$. In order to test the hypothesis that SKF83959 exerts its actions by activating $D_1/D_2$ receptor heteromers coupled to $G_{\alpha q}$ and CaMKII$\alpha$- $Thr^{286}$ phosphorylation (as a proxy for downstream activation), we used pharmacologic approaches (selective antagonist compounds) and genetic models (dopamine receptor knockout mice,

$G_{\alpha q}$ knockout mice and CaMKII$\alpha$- $Thr^{286}$Ala knockin mice) to define the signaling specificity of SKF83959 using behavioral endpoints (locomotor response and orofacial grooming assessment). Additionally, since $G_{\alpha q}$ knockout mice exhibit a motor phenotype, we have further characterized the neurobehavioral phenotype and drug-induced locomotor responses of this mutant in order to place our SKF83959-induced findings in the $G_{\alpha q}$ null mice into better context. Lastly, we have extended the behavioral characterization of SKF83959 on motor output and additionally defined SKF83959-induced effects on anxiety and depressive-like behaviors.

# CHAPTER II

## NEUROBEHAVIORAL PHENOTYPING OF Gαq KNOCKOUT MICE REVEALS IMPAIRMENTS IN MOTOR FUNCTIONS AND SPATIAL WORKING MEMORY WITHOUT CHANGES IN ANXIETY OR BEHAVIORAL DESPAIR

### Introduction:

A large number of neurotransmitter receptors containing seven transmembrane domains, including the group I metabotropic glutamate receptors mGluR1 and mGluR5, $\alpha_1$ adrenergic receptors and $5HT_2$ serotonergic receptors, mediate their physiological responses by activating heterotrimeric GTP-binding (G-) proteins with alpha subunits in the $G_q$ family ($G_{\alpha q}$) (Pin and Duvoisin, 1995, Millan et al., 2008, Cotecchia, 2010, Ribeiro et al., 2010). The $G_{\alpha q}$ family consists of four members: $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 15/16}$; of which $G_{\alpha q}$ and $G_{\alpha 11}$ represent the major isoforms in the adult brain (Strathmann and Simon, 1990, Wilkie et al., 1991). These proteins are co-expressed almost ubiquitously in the central nervous system, share 88% amino acid sequence homology and couple receptor stimulation to the activation of PLC-β isoforms, PI hydrolysis, and downstream second messenger signaling systems (Strathmann and Simon, 1990, Smrcka et al., 1991, Taylor et al., 1991, Mailleux et al., 1992, Offermanns et al., 1994, Exton, 1996). Although $G_{\alpha q}$ and $G_{\alpha 11}$ are expressed together in almost every cell type, the relative levels of expression vary across brain regions with $G_{\alpha q}$ expression being 2-5 times higher than $G_{\alpha 11}$ in most areas (Milligan, 1993). Given that the functions of these two proteins are largely redundant,

genetic inactivation of both *Gnaq* and *Gna11*, the genes that encode for $G_{\alpha q}$ and $G_{\alpha 11}$ respectively, results in embryonic lethality at embryonic day 10.5 due to developmental defects of the cardiovascular system (Offermanns et al., 1998). Genetic inactivation of either $G_{\alpha q}$ or $G_{\alpha 11}$ results in mice that are viable with more pronounced phenotypes observed in the $G_{\alpha q}$ knockouts which harbor impairments in cerebellar maturation, motor coordination and primary hemostasis (Offermanns et al., 1997a, Offermanns et al., 1997b, Offermanns et al., 1998).

Gross anatomical deficits in the morphology and development of the nervous system have not been reported in $G_{\alpha q}$ knockout mice other than postnatal alterations in the innervation of the cerebellum (Offermanns et al., 1997a). As such, loss of $G_{\alpha q}$-mediated synaptic pruning in the cerebellum has been suggested to underlie the altered behavioral output in motor function and ataxia observed in these animals. These findings raise the question of whether deficits in $G_{\alpha q}$ signaling in forebrain locomotor circuitry may also be involved. In these circuits, dopaminergic axons from the SN pars compacta innervate GABAergic MSNs in the dorsal striatum (caudate putamen). Also forming synaptic contacts on the dendritic arbors of the MSNs are descending corticostriatal glutamatergic inputs and those projections coming from the thalamus. MSNs in the dorsal striatum are largely segregated into two populations based on expression of dopamine receptors and axonal projections. MSNs expressing dopamine $D_1$ receptors project directly to the SN pars reticulata while those expressing $D_2$ receptors project to the SN pars reticulata via an indirect route involving intermediate synapses in the globus pallidus and

the subthalamic nucleus. The SN pars reticulata then relays signals to the thalamus and motor cortex (stimulatory in the case of the direct pathway or inhibitory from the indirect) for the control of voluntary movement (Gerfen, 1992, Albin et al., 1995, Shuen et al., 2008). The thalamus and motor cortex are points of convergence between the cerebellar and basal ganglia circuits (Nakano, 2000), suggesting that deficits in either or both of these circuits could result in motor deficits from loss of $G_{\alpha q}$ signaling. In fact, $G_{\alpha q}$ is highly expressed in both the caudate putamen and the cerebellum (Mailleux et al., 1992).

Recent evidence also suggests that dopamine $D_1$-like receptors, which are typically thought of as coupling with $G_{\alpha s/olf}$, may also be capable of coupling to $G_{\alpha q}$ (Wang et al., 1995, Jin et al., 2001). Additionally, given the broad diversity in receptors that couple to $G_{\alpha q}$ and the range in biological processes in which these receptors are involved, there may be other circuits that are behaviorally relevant that might be impacted by constitutive loss of $G_{\alpha q}$ signaling. For example, the prefrontal cortex, a brain region that has been shown to directly regulate working memory and other cognitive functions (Goldman-Rakic, 1995, Chudasama, 2011, Kesner and Churchwell, 2011) as well as influencing a variety of other behaviors including mood regulation and emotional processing (Drevets et al., 2008, White et al., 2009, Etkin, 2010) also expresses high levels of $G_{\alpha q}$ protein (Milligan, 1993). The functions of the prefrontal cortex have been previously shown to be sensitive to local changes in neurochemical content and receptor signaling (Rinaldi et al., 2007, Vijayraghavan et al., 2007, Arnsten, 2011). Here, we

address these questions and more precisely define the phenotype and drug responsivity of $G_{\alpha q}$ knockout mice using a systems-level approach.

## Methods:

### *Animals*

The generation of $G_{\alpha q}$ knockout mice has previously been described (Offermanns et al., 1997a). For the present experiments, heterozygous $G_{\alpha q}$ males were mated to heterozygous females to generate litters containing wildtype, heterozygous and knockout mice. The genotypes of all mice were determined by polymerase chain reaction (PCR) analysis of genomic DNA obtained from tail biopsies using methods previously described with minor adaptations (Offermanns et al., 1997a, Stanwood et al., 2005). For each experiment, an average of 6-24 wildtype, 6-20 heterozygous and 6-15 knockout mice were analyzed with the exception of the forced swim test in which four knockout mice were analyzed. Tail biopsies were obtained at the time of weaning, postnatal day (P)21, for initial assignment of genotypes and once again at the time of sacrifice for confirmation.

Male mice were housed in cages of 2-5 with their littermates under standard housing conditions on a 12 h light/dark cycle (lights on 0600-1800 h) with a*d libitum* food and water. Their cages contained environmental enrichment huts (Otto Environmental, Milwaukee, WI) and their diet was high-energy irradiated LabDiet 5LJ5 (Tusculum, Nashville, TN). All behavioral testing was conducted during the light phase on mice that were at least (P)60 at the time of

initial testing. Mice were extensively handled for at least one week prior to the beginning of testing and were habituated to the testing rooms for ~30 min prior to beginning of every experiment. Mice were also weighed prior to the beginning of each experiment and there were no significant changes in weight as a result of the behavioral testing. All procedures were approved by the Vanderbilt University Animal Care and Use Committee.

### *Drugs*

The drugs used in this study were the dopamine $D_1$-like receptor agonists SKF83959 (3-methyl-6-chloro-7,8-dihydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine; Tocris Biosciences, Minneapolis, MN) used at 1 mg/kg and SKF83822 ([*R/S*]-6-chloro-7, 8-dihydroxy-3-allyl-1-[3-methyl-phenyl]-2,3,4,5-tetrahydro-1*H*-3-benzazepine, NIMH Chemical Synthesis Program, Research Triangle Park, NC) used at 0.4 mg/kg. Additionally, the NMDA receptor antagonist MK-801 ([5R,10S]-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, Sigma, St. Louis, MO) was used at 0.2 mg/kg and cocaine HCl (NIH/NIDA, Bethesda, MD) was used at 30 mg/kg. All drugs were dissolved in 0.9% saline and injected intraperiotoneally (i.p.).

### *Rotarod*

Motor coordination and balance were measured using a commercially available accelerating rotarod apparatus (Ugo Basile model 7650, Collegeville,

PA). Mice were placed on the rotating cylinder (3 cm in diameter) and confined to a section approximately 6.0 cm long by gray plastic dividers. The rotational speed of the cylinder was increased from 5 to 40 rpm over a 5 min period. The latency at which mice fell off the rotating cylinder was measured. Each mouse was tested on three independent trials per day (with a 15 min inter-trial period) over a three day testing period.

***Inverted Screen***

For the inverted screen test, 2-4 littermates were placed on a metal grid screen (10 cm x 14 cm) with separate compartments. After placement, the mice were allowed time to grip the grid before it was inverted 60 cm over a plastic cage containing fresh bedding. Latency to fall was recorded up to 60 s, at which point mice were removed from the apparatus and returned to the home cage. Three independent trials were conducted approximately 15 min apart on one day of testing, and data from all three trials were averaged together.

***Elevated Zero Maze***

The elevated zero maze is a modification of the elevated plus maze used for assessing anxiety-related behaviors. Use of the circular maze removes any ambiguity in data interpretation as there is no center zone (Lister, 1990, Shepherd et al., 1994, Rodgers et al., 1997). The elevated circular platform (40 cm off the ground, 50 cm in diameter) had two enclosed arenas opposite each other (5 cm wide with 15 cm high walls) and two open arenas (5 cm wide). At the

start of the test, each mouse was lowered by its tail into the open arena of the maze and allowed to explore the maze for 300 sec. Activity of the mouse was monitored via an overhead camera connected to a computer in a separate room using video acquisition and ANY-maze analysis software (Stoelting, Wood Dale, IL). Data analyzed included percentage of time spent in the open versus closed arenas and the total distance traveled in the maze.

***Y-maze***

The Y-maze containing 3 clear arms (34.5 cm × 5.2 cm) joined in the center was placed on an opaque table about 91 cm above the ground in a room containing several large immovable objects to use as spatial cues. At the start of the test, each mouse was lowered by its tail into the center junction of the maze and allowed to explore the maze for 360 s. Activity of the mouse was monitored via an overhead camera connected to a computer in a separate room using video acquisition and ANY-maze analysis software (Stoelting, Wood Dale, IL). The sequence of individual arm entries was scored by the observer in real time and used to calculate the percentage of spontaneous alternations for each animal (consecutive entry into each of the three arms) as previously described (Thompson et al., 2005). The Y-maze assesses spatial working memory as animals tend to alternate between arms based on their memory of the previously visited arms. Chance performance is 22.2% in this paradigm.

### *Forced Swim Test*

Behavioral despair was assessed in the forced swim test using plastic cylinders (50 cm in diameter, 21 cm in height) filled approximately ¾ full with room temperature water. Mice were individually placed into the cylinder for a 6 min test and were recorded on video for the duration of the test. After testing, the mice were placed into a heating cage to dry before returning to the home cage. The water was changed between tests and the temperature of the water was recorded. Videos were later analyzed for time spent immobile for each mouse by an observer blinded to genotype.

### *Open Field*

Locomotor activity in a novel open field and locomotor responses to SKF83959, SKF83822, cocaine and MK-801 were measured using commercial open field activity chambers (Med Associates, 29 x 29 x 20.5 cm) that were contained within light- and air-controlled environmental chambers (Med Associates, St. Albans, VT; 64 × 45 × 42 cm) (Stanwood and Levitt, 2007). Location and movement were detected by the interruption of infrared beams by the body of the mouse (16 photocells in each horizontal direction, as well as 16 photocells elevated 4 cm to measure rearing) and were measured by the Med Associates Activity Monitoring program. On Days 1 and 2 of testing, mice were placed into activity chambers for 30 min for baseline measurements, removed from the chambers, injected with 0.9% saline, and returned to the chambers for 60 min. On Day 3 of testing, the mice were injected with SKF83959 (1 mg/kg)

instead of 0.9% saline. This extended protocol was designed to extensively habituate the mice to the chambers before drug administration. For testing of additional compounds, a 2-day protocol was implemented where the mice received 0.9% saline on Day 1 and the test compound (0.4 mg/kg SKF83822, 0.2 mg/kg MK-801 or 30 mg/kg cocaine; i.p.) on Day 2. Experiments were conducted at least one week apart and animals were handled during the period of time during which they were not tested. For simplicity of analysis and display, the baseline and post-injection periods were averaged and are represented as bar graphs.

***Data Analysis and Statistics***

Except when otherwise noted, data were subjected to one- or-two way analysis of variance (ANOVA) using genotype as a between-group factor using GraphPad Prism (GraphPad Software, San Diego, CA). Post-hoc Tukey's multiple comparison tests were used to compare groups to each other except for the rotarod analysis, where Bonferroni comparisons were employed. Normality was not observed within the inverted screen dataset, due to many null mice immediately falling from the screen. For these data, therefore, a nonparametric Kruskal-Wallace test and posthoc Dunn's comparisons were employed. Graphs are marked with an asterisk (*) to denote statistical significance ($p < 0.05$). For data with $p < 0.01$ or $p < 0.001$, the graphs are marked with two (**) or three (***) asterisks, respectively. For data with a $p > 0.05$ but less than $p = 0.20$, the data was noted as exhibiting a trend. In the inverted screen test, genotype differences

were assessed by unpaired Student's *t*-test with significance defined as two-tailed $p < 0.05$.

## Results:

### *$G_{\alpha q}$ knockout mice exhibit alterations in body weight*

Visual inspection revealed that $G_{\alpha q}$ knockout mice are significantly smaller than their wildtype littermates (Figure 6A). Figure 6B shows the average weights of adult $G_{\alpha q}$ null, heterozygous and wildtype mice at the start of behavioral testing. Consistent with their smaller sizes, $G_{\alpha q}$ knockout mice weigh almost half as much as wildtype mice ($F(2,28) = 12.33$, $p < 0.001$) and this phenotype is maintained across their lifespan (data not shown).

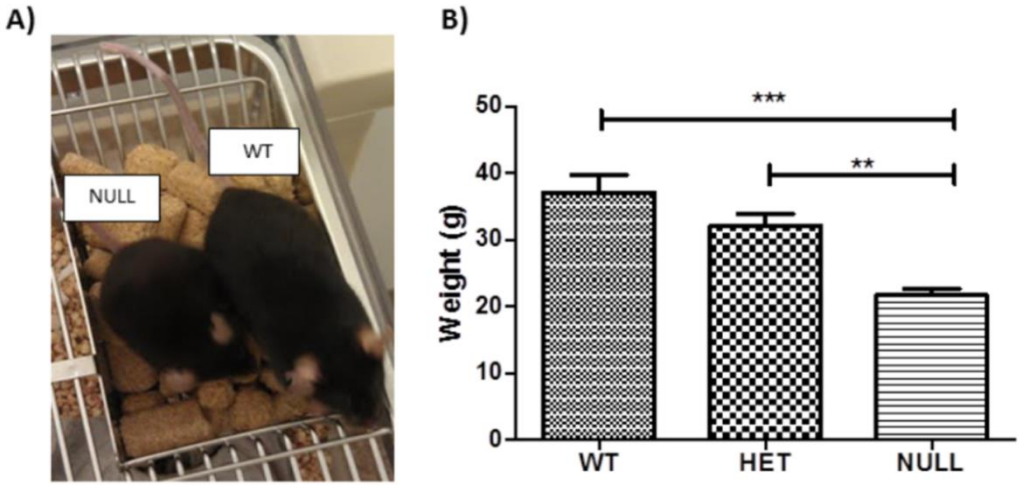


**Figure 6: Weight analysis.**

$G_{\alpha q}$ knockout mice are smaller than their wildtype littermates as shown in the photomicrograph in **(A). (B)** shows the average weight of each genotype at the time of initial testing (~2–3 months of age). $G_{\alpha q}$ knockout mice weigh significantly less than wildtype [$F_{(2, 28)} = 12.33$, $p < 0.001$] and heterozygous [$F_{(2, 28)} = 12.33$, $p < 0.01$] mice and this phenotype is maintained throughout the life of the animals (data not shown). Each column represents the average of 8–12 animals.

### *Abnormal motor function in $G_{\alpha q}$ knockout mice*

Mice homozygous for a deletion in $G_{\alpha q}$ have previously been described as exhibiting deficits in motor function including loss of balance during walking and rearing, spastic and uncontrolled movements and ataxia upon visual inspection ((Offermanns et al., 1997a) and data not shown). Quantifiable deficits in motor function and coordination are revealed on an accelerating rotarod where $G_{\alpha q}$ knockout mice fell from the device in significantly less time than controls on each of three consecutive testing days (Figure 7A; factorial ANOVA, post-hoc Bonferroni comparisons $p < 0.05$ on Day 1, $p < 0.01$ on Days 2 and 3),confirming previous findings (Offermanns et al., 1997a). There were also significant differences in performance observed between the heterozygous and null mice on each day of testing (Figure 7A; $p < 0.05$ on Day 1, $p < 0.001$ on Days 2 and 3) with no significant differences between the heterozygous and wildtype mice. Similarly, $G_{\alpha q}$ knockout mice performed significantly worse than wildtype and heterozygous animals on an inverted screen test, confirming motor and/or coordination impairments in the null animals ($p < 0.05$; Figure 7B).

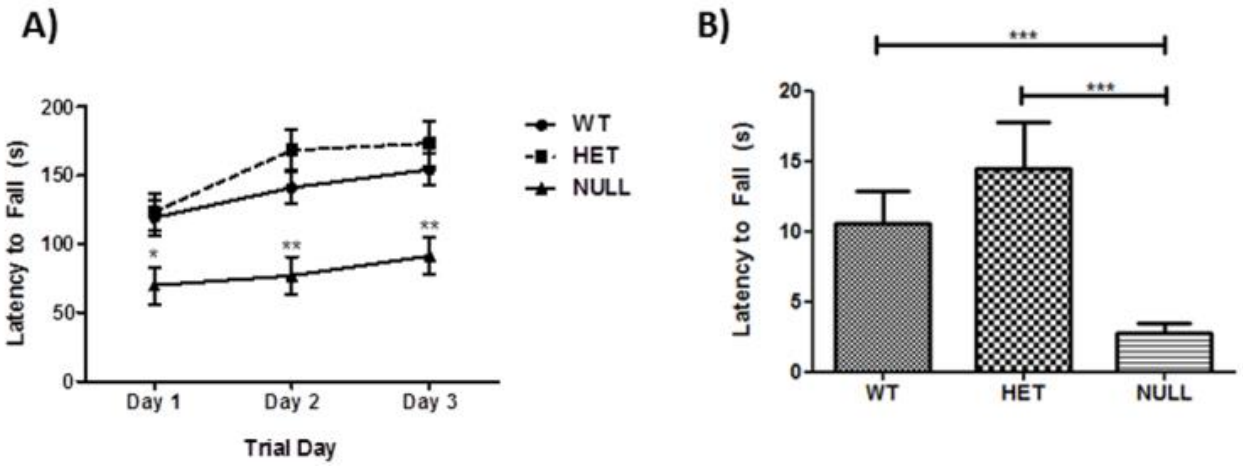


**Figure 7: Rotarod and inverted screen tests.**

$G_{\alpha q}$ knockout mice spend significantly less time on an accelerating rotarod than their wildtype and heterozygous littermates (**A**; $p < 0.05$ between the knockouts and the other two genotypes on day 1 of testing; $p < 0.01$ on days 2 and 3 by Two-Way ANOVA with Bonferroni *post-hoc* comparison test). $n = 6$ for each genotype and each individual animal was tested in three trials on three consecutive days of testing. The data shown here represents the three trial averages across genotypes on each day of testing. **(B)** shows the latency of each genotype to fall from an inverted screen. Wildtype and heterozygous mice are able to grip the screen almost three times longer than $G_{\alpha q}$ null mice indicating reduced muscle and/or grip strength in the knockouts (overall $p < 0.001$ by Kruskal–Wallis test; null mice are significantly different from both wildtype and heterozygote mice by Dunn's test, $p < 0.05$). Each column represents the average of 13–22 animals.

### *$G_{\alpha q}$ knockout mice appear normal in tests of anxiety and behavioral despair*

$G_{\alpha q}$ knockout mice exhibited a significant hypolocomotive phenotype in the elevated zero maze as evidenced by the reduction in total ambulatory distance traveled in the maze compared to wildtype and heterozygote animals (Figure 8A). Both $G_{\alpha q}$ knockout and wildtype mice spent significantly more time in the closed arenas than the open arenas with no significant difference between the genotypes with respect to the percentage of time spent in the open (33.9 +/- 3.8% for wildtype, 25.3 +/- 6.1% for $G_{\alpha q}$ knockout) or closed arenas (66.1 +/- 3.8% for wildtype, 74.7 +/- 6.1% for $G_{\alpha q}$ knockout) of the maze (Figure 8B). This is consistent with previous reports suggesting that wildtype mice spend approximately 20-30% of their time in the open arenas of the zero maze (Shepherd et al., 1994). Although not statistically significant ($F(2,32) = 2.3$, $p = 0.11$), heterozygous animals spent somewhat more time in the open arenas (42.1 +/- 6.8%) than the other two genotypes. While in the open arenas, wildtype and heterozygous animals traveled at significantly faster speeds than the knockout mice (Figure 8C; $F(5,30) = 8.5$, $p < 0.001$) which moved slower in the maze overall (Figure 8D; $F(2,33) = 11.96$, $p < 0.001$) compared to the other two genotypes.

Figure 9 displays the results of the forced swim test, a commonly used assay of behavioral despair used to predict the antidepressant potential of compounds or drug targets in animal models (Porsolt et al., 1977, Shepherd et al., 1994). The forced swim test revealed no significant differences between the

genotypes, indicating that loss of $G_{\alpha q}$ protein expression does not confer antidepressant effects.

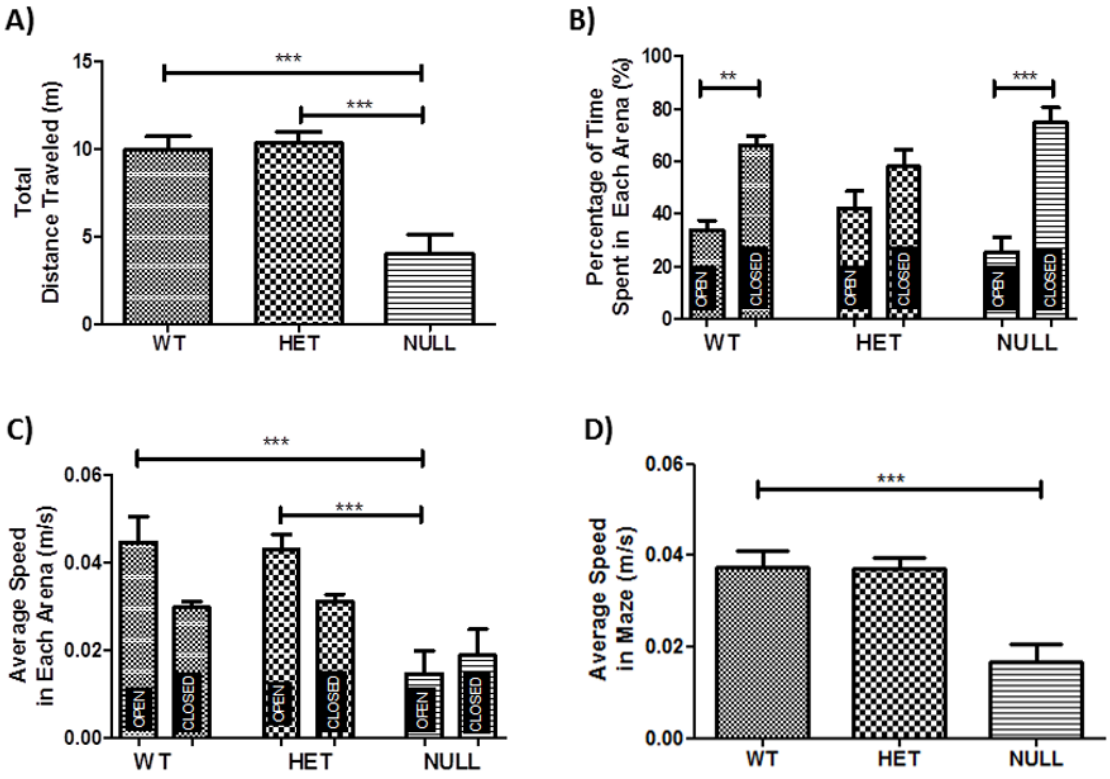


**Figure 8: Elevated zero maze.**

$G_{\alpha q}$ knockout mice exhibit a hypoactive phenotype on the elevated zero maze and travel significantly less distance in the maze than their wildtype and heterozygous littermates (**A**; $F_{(2, 15)} = 16.55$, $p < 0.001$). There is not a significant difference between the genotypes in the percentage of time spent in the open areas **(B)** indicating a normal anxiety phenotype in the null mice. $n = 12$ for wildtype and heterozygous mice, $n = 11$ for knockout mice in these experiments. While in the open arenas, wildtype and heterozygous mice move at significantly faster speeds than knockout mice (**C**; $F_{(5, 30)} = 8.5$, $p < 0.001$), which move slower in the maze overall (**D**; $F_{(2, 33)} = 11.96$, $p < 0.001$).

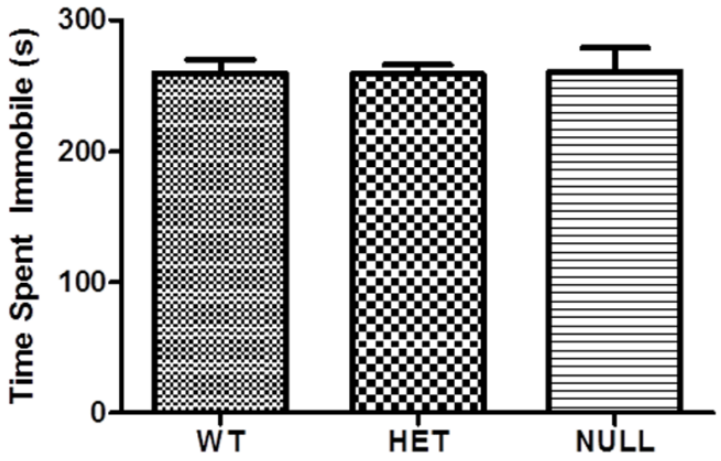


**Figure 9: Forced swim test.**

Forced swim test analysis reveals no significant differences ($p > 0.05$) between the genotypes in this assay of behavioral despair. Each column represents the average of 4–6 animals.

### *$G_{\alpha q}$ knockout mice exhibit deficits in spatial working memory*

$G_{\alpha q}$ knockout mice again exhibited significant hypoactivity in the Y-maze as evidenced by the significant reduction in total arm entries committed by this genotype compared heterozygous and wildtype littermates (Figure 10A). In addition, acquisition of this spatial task was severely impaired in $G_{\alpha q}$ knockouts compared to wildtype ($F(2,4) = 13.3$, $p < 0.001$). $G_{\alpha q}$ knockout mice exhibited a significant reduction in the number of spontaneous alternations (entry of the maze's three arms in sequence), and the percentage of alternations, a measure which takes into account the hypoactive phenotype of the $G_{\alpha q}$ null mice in this task (Figure 10B and C). Wildtype animals spontaneously alternated at 62.5 +/- 2.9 % compared to 44.1 +/- 7.5 % observed in $G_{\alpha q}$ knockout mice.

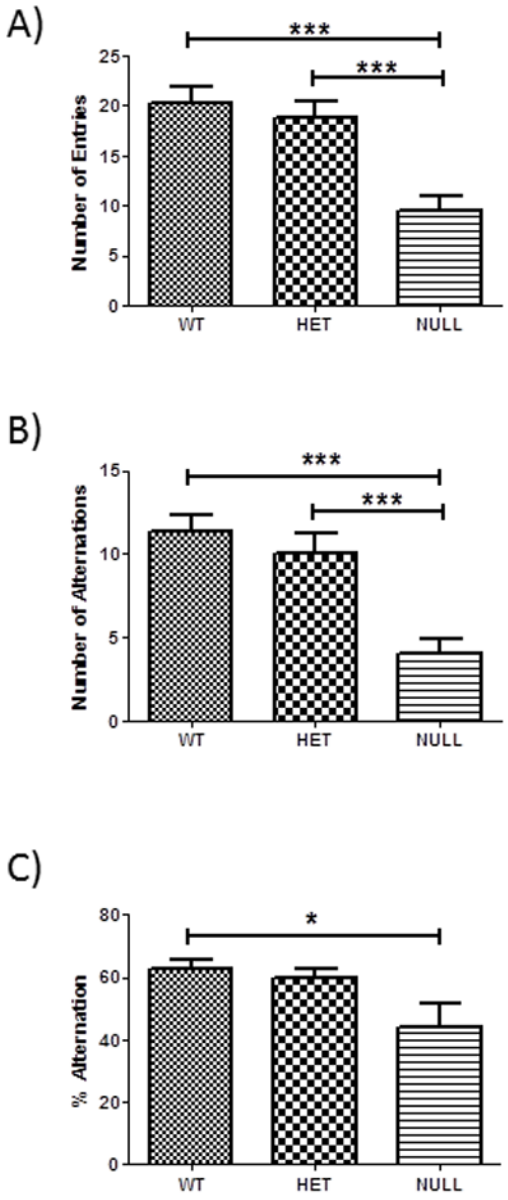


**Figure 10: Y-maze test.**

In this test of spatial working memory, the reduced number of arms entered by $G_{\alpha q}$ knockout mice compared to wildtype and heterozygous littermates again reveals a hypoactive phenotype **(A)**. In addition, acquisition of this spatial task was severely impaired in $G_{\alpha q}$ knockout mice which exhibited a significant reduction in the number of spontaneous alternations (**B**; $F_{(2, 44)} = 14.42$, $p < 0.001$) and percentage of alternations (**C**; $F_{(2, 44)} = 4.44$, $p < 0.05$ between wildtype and null) in this task. Percentage of alternations was determined by dividing the number of alternations by the number of possible alternations [(# total arm entries − 2) × 100], thereby taking into account the locomotor phenotype of $G_{\alpha q}$ knockout mice. Each column represents the average of 12–18 animals.

### *Drug-induced locomotor responses appear intact in $G_{\alpha q}$ knockout mice*

In order to gauge the integrity of basal ganglia locomotor circuitry, we investigated the spontaneous locomotor activity of $G_{\alpha q}$ knockout, heterozygous and wildtype mice in open field chambers and their acute locomotor response to pharmacological compounds known to modulate locomotor output (primarily by modulating dopaminergic signaling in basal ganglia circuits). Our data show that in a novel open field, $G_{\alpha q}$ knockout mice are initially hypoactive compared to their wildtype and heterozygous littermates (as assessed by distance traveled) and travel significantly less distance than wildtype animals during the 90 min session (Figure 11A; ($F(2,30) = 4.2$, $p < 0.05$). These data further support our earlier observations regarding the activity level of the null animals in both the elevated zero and Y-maze tasks. On the second day of habituation (Figure 11B), as the wildtype and heterozygous animals acclimate further to the chambers and reduce their level of activity, there is no longer a significant difference in the total distance traveled by each genotype, although there is still a trend ($F(2,31) = 4.2$, $p = 0.16$) toward hypoactivity in the null mice.

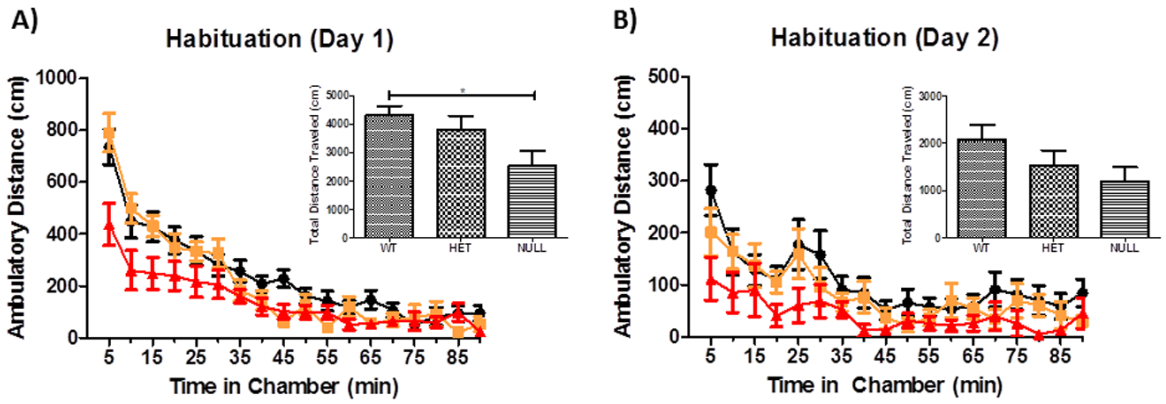


**Figure 11: Open field test.**

Locomotor activity in a novel open field was assessed. **(A,B)** show the ambulatory distance traveled as a function of time (5 min blocks over a 90 min testing period) by each genotype on 2 days of habituation. Mice were removed from the chamber after 30 min and administered 0.9% saline (i.p.) before returning to the chamber for the last 60 min of testing. The inset in each panel shows the total distance traveled by each genotype, which is significantly reduced for $G_{\alpha q}$ knockout mice on day 1 (**A**; $F_{(2, 30)} = 4.2$, $p < 0.05$). $n$ = 12–18 for each genotype.

Drug-induced locomotor responses were first assessed using cocaine, a prototypical psychomotor stimulant that increases locomotor activity by blocking high affinity monoamine transporters. Figure 12A shows the ambulatory distance traveled by each genotype as a function of time in the open field chamber. The first 30 min represent the baseline period during which the animals were allowed time to habituate to the chamber and the last 60 min (minutes 35-90 on the graph) represent the post-injection period. The data for the baseline and post-injection periods are then averaged and displayed as Figure 12B. These data illustrate that injection of 30 mg/kg cocaine (i.p.) elicited a significant locomotor response in all three genotypes relative to the pre-injection baseline period ($F(5,21) = 186.0$, $p < 0\ .001$). The raw distance traveled for the knockout mice in the post-injection period, however, was significantly reduced compared to responses observed in both the wildtype and heterozygous animals ($F(5,21) = 186.0$, $p < 0.001$). There was also a small but significant blunting of the post-injection response of the heterozygous animals ($F(5,21) = 186.0$, $p < 0.001$). However, when normalized for percentage change from baseline, there were no significant differences between the genotypes. If anything, there was a trend ($F(2,25) = 2.23$, $p = 0.13$) toward a greater percentage change from baseline in the null animals because their baseline activity was very low (Figure 12C). Taken together, these results indicate that the cocaine-induced locomotor response is largely intact in $G_{\alpha q}$ knockout mice, despite their profound basal hypoactivity.

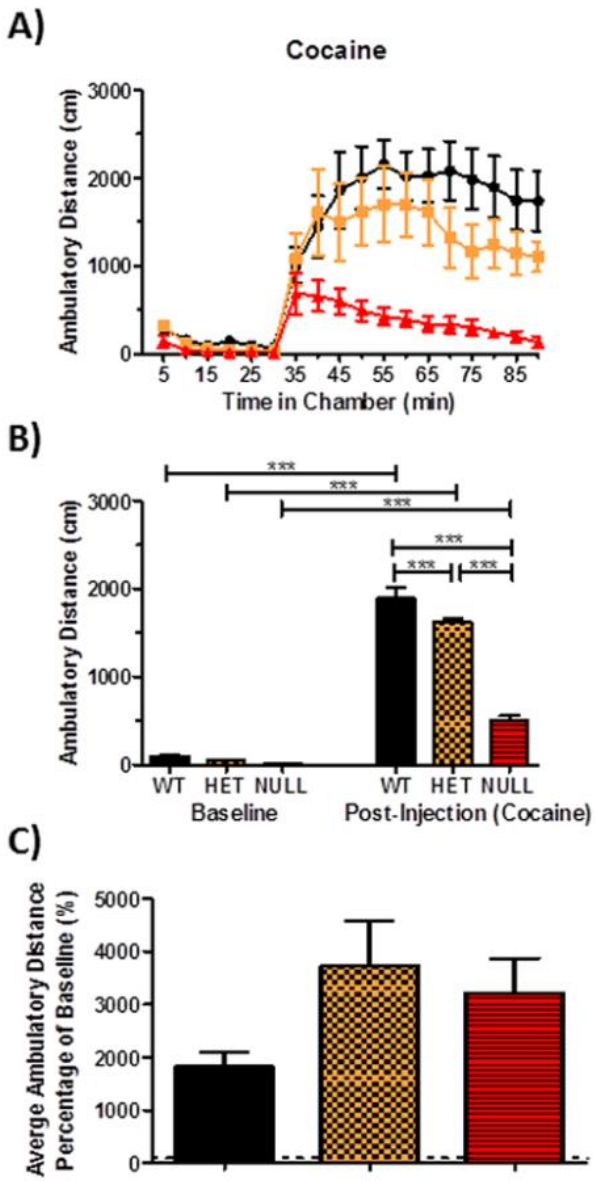


**Figure 12: Locomotor response to acute cocaine.**

We investigated the integrity of locomotor responses to variety of pharmacological compounds with the ability to modulate dopaminergic signaling (directly or indirectly) and motor output. **(A)** shows the ambulatory distance traveled as a function of time (5 min blocks over a 90 min testing period) by each genotype during a 30 min baseline period and 60 min after the animals were injected with cocaine (30 mg/kg; i.p.). The data is represented as bar graphs in **(B)** with the data from min 10–30 collapsed for each genotype as the baseline measurement and min 40–60 representing the post-injection period. There is a significant increase in locomotor activity in each genotype following exposure to cocaine, however there is a significant difference between the post-injection

locomotor response of the knockout mice compared to the other two genotypes [$F_{(5, 21)} = 186.0$, $p < 0.001$]. The percentage change from baseline for each genotype is displayed in **(C)** with no significant changes. $n = 6–12$ for each genotype in these experiments.

Next, we assessed the locomotor responses to direct stimulation of dopamine $D_1$ receptors by the high affinity benzazepine-derived agonist SKF83822. SKF83822 has been reported to activate dopamine $D_1$ receptors coupled to $G_{\alpha s/olf}$ and downstream cyclase activity (Undie et al., 1994, Rashid et al., 2007). Behaviorally, SKF83822 has been shown to produce a locomotor response in both rodent and non-human primate models without affecting stereotypy, intense grooming or dyskinesia (Peacock and Gerlach, 2001, O'Sullivan et al., 2004). In our analyses, an acute injection of SKF83822 (0.4 mg/kg; i.p.) induced a greater than three-fold increase in locomotor activity relative to the baseline period for each genotype (Figure 13A and B; $F(5,18) = 139.2$, $p < 0.001$). Again, as observed with acute cocaine, there were significant differences between the post-injection response of the knockout animals compared to their wildtype and heterozygous littermates (Figure 13B; $F(5,18) = 139.2$, $p < 0.001$) without significant changes in the percentage change from baseline between the genotypes (Figure 13C).

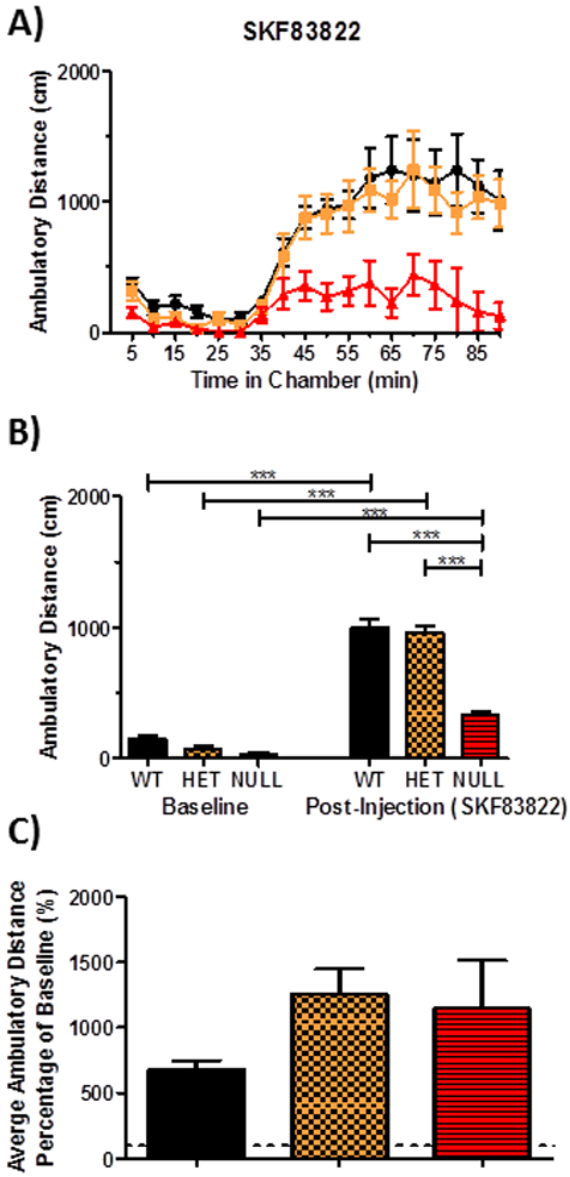


**Figure 13: Locomotor response to acute SKF83822.**

**(A,B)** represent the ambulatory distance traveled by each genotype in response to an acute injection of the dopamine $D_1$ receptor agonist SKF83822 (0.4 mg/kg; i.p.). There is a significant increase in locomotor activity in each genotype following exposure to SKF83822 [$F_{(5, 18)} = 139.2$, $p < 0.001$]. There is, however, a significant difference between the locomotor response of the knockouts compared to the other two genotypes [$F_{(5, 18)} = 139.2$, $p < 0.001$] without significant changes in the percentage change from baseline between the genotypes **(C)**. $n$ = 6–12 for each genotype in these experiments.

We then assessed a second benzazepine-derived $D_1$ receptor agonist, SKF83959, for activity in the open field. Unlike SKF83822, SKF83959 has been reported to antagonize dopamine-mediated stimulation of adenylyl cyclase (Arnt et al., 1992, Andringa et al., 1999, Cools et al., 2002, Jin et al., 2003) and may preferentially activate $D_1$ receptors linked to stimulation of PI hydrolysis (Arnt et al., 1992, Panchalingam and Undie, 2001, Jin et al., 2003). Initial studies assessing the locomotor response to varying doses of SKF83959 suggested that 1 mg/kg (i.p.) elicited a maximal response in wildtype mice (data not shown). This response was still fairly modest, however, and increased locomotor activity approximately 2-fold over the baseline level (data not shown). In response to an acute injection of SKF83959 (1 mg/kg; i.p.), we again observed significant increases in post-injection locomotor responses in wildtype, heterozygous and knockout mice (Figure 14A and B; $F(5,18) = 123.9$, $p < 0.001$). We did observe significant differences in the post-injection response of the knockout animals compared to the wildtype and heterozygous animals ($F(5,18) = 123.9$, $p < 0.001$), however the magnitude of the locomotor response was more robust in the knockout ($F(2,33) = 7.0$, $p < 0.05$) and heterozygous ($F(2,33) = 7.0$, $p < 0.001$) animals compared to wildtype when considering the percentage change from baseline (Figure 14C).

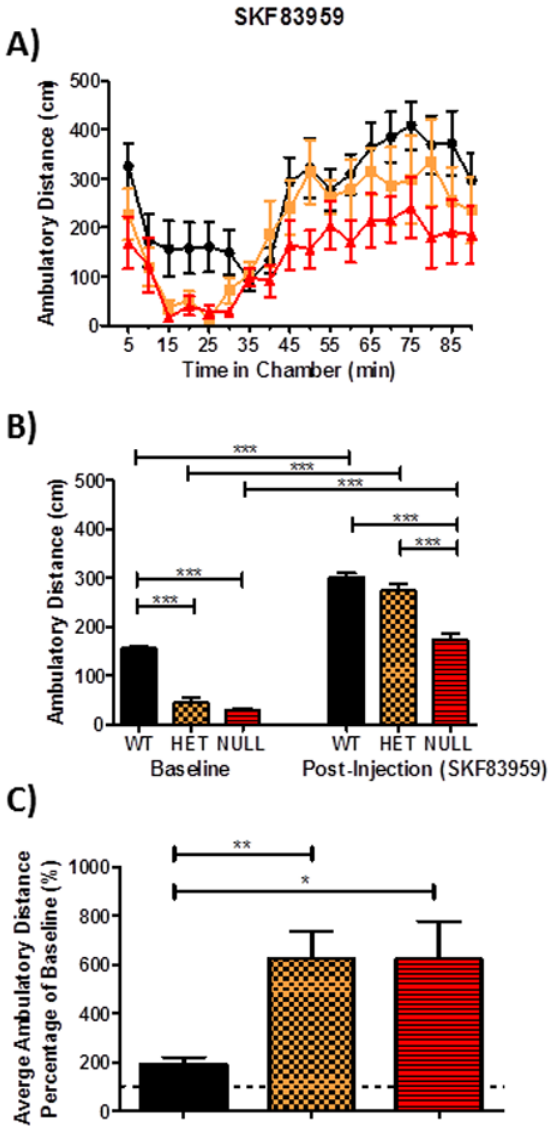


**Figure 14: Locomotor response to acute SKF83959.**

SKF83959 (1 mg/kg; i.p.), a dopamine $D_1$ receptor agonist that has been reported to activate $D_1$ receptors coupled to $G_{\alpha q}$, significantly increases locomotor activity in $G_{\alpha q}$ knockout, heterozygous, and wildtype mice (**A,B**; $F_{(5, 18)} = 123.9$, $p < 0.001$). There is also a significant difference in the post-injection locomotor response of the null mice compared to their wildtype and heterozygous littermates (**B**; $F_{(5, 18)} = 123.9$, $p < 0.001$) and a significant difference in the percentage change from baseline of the knockouts [**C**; $F_{(2, 33)} = 7.0$, $p < 0.05$] and heterozygotes [$F_{(2, 33)} = 7.0$, $p < 0.001$] compared to wildtype. $n = 12–18$ for each genotype.

Lastly, we set out to evaluate locomotor activity by modulating glutamatergic input with the non-competitive N-Methyl-D-Aspartate (NMDA) receptor antagonist MK-801. In response to acute MK-801 (0.2 mg/kg; i.p.), we observed significant increases in post-injection locomotor responses in wildtype and $G_{\alpha q}$ heterozygotes ($F(5,36) = 9.8$, $p < 0.05$) but not in $G_{\alpha q}$ knockout mice (Figure 15A and B). There were however, as observed previously with all compounds tested, significant differences between the post-injection response of the knockout animals compared to their wildtype ($F(5,36) = 9.8$, $p < 0.001$) and heterozygous ($F(5,36) = 9.8$, $p < 0.01$) littermates (Figure 15B) following acute MK-801. Similarly, as observed with all compounds tested except SKF83959, there were no significant changes in the percentage change from baseline between the genotypes (Figure 15C).

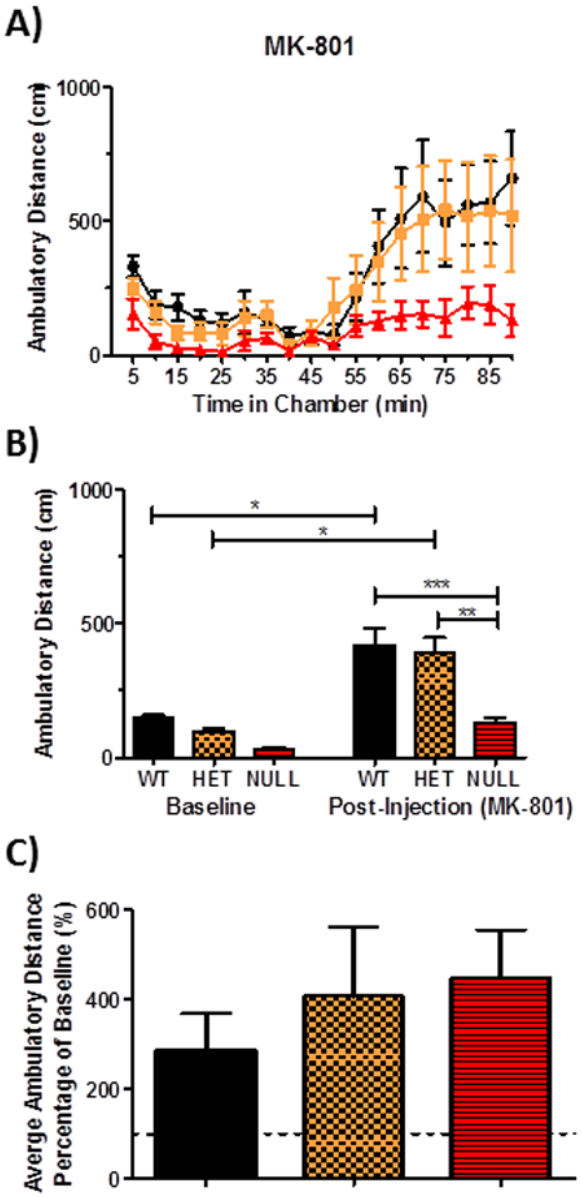


**Figure 15: Locomotor response to acute MK-801.**

The NMDA receptor antagonist MK-801 (0.2 mg/kg; i.p.) induced a significant locomotor response in wildtype and heterozygous mice [$F_{(5, 36)} = 9.8$, $p < 0.05$] but not in $G_{\alpha q}$ knockouts **(A,B)**. Additionally, there were no significant changes observed in the percentage change from baseline between the genotypes **(C)**. $n$ = 6–12 for each genotype in these experiments.

**Discussion:**

As predicted, $G_{\alpha q}$ knockout mice performed significantly worse than wildtype mice on tests of motor coordination and strength revealing a phenotypic motor dysfunction in these animals. These results confirm the visual phenotype of the null mice which hints at ataxia, inability to coordinate movements and uncontrolled locomotion. $G_{\alpha q}$ knockout mice are also much smaller and weigh less than heterozygous or wildtype mice; a phenotype which is consistent with the previously reported role of $G_{\alpha q}$ signaling in the normal functioning of the hypothalamus to regulate the production of growth hormone and feeding behavior (Wettschureck et al., 2005).

Motor impairments in $G_{\alpha q}$ knockout mice could result from deficits in motor circuits controlled by cerebellar output as previously hypothesized (Offermanns et al., 1997a) and/or the involvement of other motor pathways including those involving the basal ganglia locomotor circuitry, which we assessed indirectly using locomotor stimulant drugs. In attempting to holistically evaluate $G_{\alpha q}$ knockout mice for circuit-level deficits in brain function, we used simple, well-defined behavioral paradigms to probe the contribution of $G_{\alpha q}$ signaling capacity in complex behaviors that may be relevant to mental health disorders.

Anxiety and depression, for example, are two common emotional disorders accounting for a substantial proportion of the burden of mental health disorders in the United States (Weissman et al., 1996, Kessler et al., 2005). Although the neural circuits underlying these disorders are not completely understood, dysfunctions in the amygdala, hippocampus, basal ganglia and

prefrontal cortex are commonly implicated (Clark et al., 2009, Aupperle and Paulus, 2010, Clark and Beck, 2010, Harro et al., 2011, McEwen et al., 2012). In the elevated zero maze, a useful task for assessing anxiety-related behavior in rodent models, we saw no significant differences between the genotypes with respect to the percentage of time spent in the open arenas. These results indicate a normal anxiety-like phenotype in the knockout mice although we do not know if there are compensations within the circuit from global loss of $G_{\alpha q}$ that result in lack of an observed phenotype. Additionally, we observed no significant differences in the forced swim test assessing depressive-like phenotypes. These results were unexpected as there is evidence in the literature suggesting that inhibiting the PLC-protein kinase C signaling transduction pathway or intracellular calcium release (which can be activated by $G_{\alpha q}$-coupled receptors) produces antidepressant effects in the forced swim test (Galeotti et al., 2006, Galeotti and Ghelardini, 2011). There is also evidence suggesting that chronic stress in rodent models alters transcript levels of *Gnaq* (Alfonso et al., 2006); another implication of alterations in $G_{\alpha q}$ signaling in rodent models of depression. It is possible, however, to activate these signaling pathways via other mechanisms including signaling initiated by $G_{\alpha 11}$ coupling which remains intact in $G_{\alpha q}$ knockout animals.

The Y-maze task is often employed as a test of spatial working memory whereby mice will alternate between the three arms of the maze based on their interest in exploring the novel environment and their memory of the last arm entered. The influence of the prefrontal cortex in spontaneous alternation

behaviors has previously been demonstrated in rodent models (Kolb, 1984) in addition to the roles of other brain regions including the hippocampus, basal forebrain, dorsal striatum and cerebellum in mediating this behavior (Lalonde, 2002). We observed significant differences in the ability of $G_{\alpha q}$ knockout mice to perform this task indicating that functional $G_{\alpha q}$ signaling in the prefrontal cortex may be necessary for acquisition of this task. These results are interesting with respect to the extensive literature detailing the importance of catecholamine signaling, in particular the role of dopamine, in mediating prefrontal cortex function and working memory (Vijayraghavan et al., 2007, Arnsten, 2011). Additionally, there is evidence in the literature suggesting that dopamine $D_1$ receptors in the prefrontal cortex are able to couple to $G_{\alpha q}$ (Jin et al., 2001) and thus the performance of $G_{\alpha q}$ null mice could be explained by lack of dopamine signaling in this pathway. Alternatively, lack of signaling through other $G_{\alpha q}$-coupled receptors in the cortex could be contributing to the observed phenotype.

In evaluating the intactness of the basal ganglia locomotor circuitry, we assessed the drug-induced locomotor responses of wildtype, heterozygous and $G_{\alpha q}$ null animals to a variety of pharmacologic compounds. The psychostimulant cocaine acts indirectly to increase dopaminergic signaling by blocking the dopamine transporter, thus inhibiting dopamine re-uptake into pre-synaptic nerve terminals. As a result, dopamine accumulates at MSN synapses in the dorsal striatum, thus increasing and prolonging receptor activation primarily through $D_1$ receptors signaling in the direct motor pathway to increase locomotor output (Kolb, 1984, Karasinska et al., 2005, Bateup et al., 2010). We were successful in

generating locomotor responses in all three genotypes in response to acute cocaine suggesting that the functioning of the basal ganglia motor pathways remains largely intact in the absence of $G_{\alpha q}$. Although $G_{\alpha q}$ protein expression does not appear to be necessary for the acute locomotor response to cocaine, it does appear to be involved in the expression of cocaine withdrawal in rodent models. It has previously been shown that rats undergoing withdrawal for 2 days after receiving twice-daily cocaine injections (15 mg/kg; i.p.) exhibited increased levels of membrane-associated $G_{\alpha 11}$ and $G_{\alpha q}$ proteins in the amygdala after 1 or 3 days (for peak expression, respectively) of cocaine exposure (Carrasco et al., 2004). Additionally, following 5 days of cocaine treatment, membrane-bound $G_{\alpha 11}$ and $G_{\alpha q}$ were also increased in the paraventricular nucleus of the hypothalamus (Carrasco et al., 2004), but no changes were observed in brain regions such as the frontal cortex even after 14 days of cocaine exposure (Carrasco et al., 2003, Carrasco et al., 2004). These changes in $G_{\alpha 11}$ and $G_{\alpha q}$ protein expression are transient, however, and are reversed back to baseline levels when assessed after 7 days of withdrawal (Carrasco et al., 2003).

We were also successful in generating a locomotor response in $G_{\alpha q}$ knockout mice by directly stimulating dopamine $D_1$ receptors in the direct motor output pathway with the $D_1$ receptor partial agonists SKF83822 and SKF83959. These results were expected with SKF83822 as this compound has been previously shown to produce a locomotor response typical of classical dopamine agonists stimulating adenylyl cyclase activity (Peacock and Gerlach, 2001, O'Sullivan et al., 2004). Interestingly, however, we also observed a locomotor

response in $G_{\alpha q}$ knockout mice following administration of SKF83959, a dopamine $D_1$ receptor agonist that has been reported to activate $D_1$ receptors coupled to PI hydrolysis and intracellular calcium mobilization. Previous reports assessing the behavioral effects of SKF83959 have shown the drug to elicit intense grooming behavior and orofacial movements in rodent models (Downes and Waddington, 1993, Fujita et al., 2010) and prove effective in reversing parkinsonian symptoms in rodent (unilateral 6-OHDA lesioned) and non-human primate (MPTP treated) models of Parkinson's disease (Arnt et al., 1992, Gnanalingham et al., 1995a, Gnanalingham et al., 1995c, b, Zhang et al., 2007).

One recent model in the literature detailing a mechanism by which SKF83959 stimulates PI activity involves activating a $D_1/D_2$ receptor heteromer complex coupled to $G_{\alpha q}$ protein (Rashid et al., 2007) and subsequent signaling systems. We therefore expected to observe minimal SKF83959-induced locomotor responses in $G_{\alpha q}$ knockout mice, if in fact, SKF83959 does activate $D_1$ receptors signaling through $G_{\alpha q}$. Contrary to our hypotheses, however, SKF83959-induced locomotor responses were intact in $G_{\alpha q}$ null animals. In fact, when their different baselines are taken into account, $G_{\alpha q}$ heterozygous and knockout mice may actually be more sensitive to the effects of SKF83959 in that both genotypes exhibited a significantly greater percentage change from baseline in their post-injection locomotor response compared to wildtype animals. Taken together, these results suggest that SKF83959 may not be exclusively activating receptors coupled to the $G_{\alpha q}$ signaling pathway. The most likely explanation is that SKF83959 is not a selective as thought, and may activate $D_1$ receptors

coupled to $G_{\alpha s/olf}$ signaling pathways. It is conceivable that even if SKF83959 does activate $D_1$-$G_{\alpha s/olf}$ coupled receptors, there could be alternative cyclase-independent pathways feeding into $IP_3$ dependent-calcium mobilization that would support the initial biochemical characterization of this drug (Arnt et al., 1992, Andringa et al., 1999, Jin et al., 2003).

Our attempts to induce a significant locomotor response in $G_{\alpha q}$ knockout mice by modulating glutamatergic tone with the non-competitive NMDA receptor antagonist MK-801 proved unsuccessful. Experiments using MK-801 were designed to indirectly modulate locomotor output as descending glutamatergic inputs from the cortex project to the striatum where they synapse on the dendritic spines of MSNs in close proximity to the synaptic contacts of the ascending midbrain dopamine neurons from the SN pars compacta. The glutamatergic and dopaminergic nerve terminals form synaptic triads where they contact MSNs: points of contact whereby the two signaling systems likely converge to modulate MSN output (Schmidt and Kretschmer, 1997, Klug et al., 2011). Additionally, there is evidence suggesting that MK-801 produces indirect activation of dopaminergic neurons to dose-dependently induce locomotor activity up to 0.5 mg/kg with a peak response around 0.2 mg/kg. At higher doses of MK-801 (> 0.5 mg/kg) a characteristic motor syndrome is produced involving ataxia and stereotypic behaviors including head weaving and body rolling (Liljequist et al., 1991). Although the locomotor response induced by MK-801 was not statistically significant in the null animals in our assessments, there was a trend toward increased activity in these mice. In addition, the response observed in the

wildtype animals was fairly modest after a 0.2 mg/kg exposure and it may be necessary to move to slightly higher doses for a more robust response. At higher doses, however, other behaviors are also elicited which could confound the clarity of the locomotor response. Furthermore, there is some evidence suggesting that proper postnatal signaling of the $G_{\alpha q}$-coupled mGluR1 receptor is required for proper maturation of glutamatergic synapses in the ventral tegmental area, a midbrain nucleus containing dopaminergic cell bodies (Bellone et al., 2011). A mechanism such as this could potentially be necessary in other brain regions and may partially explain the results observed in the $G_{\alpha q}$ knockout mice.

**Conclusions:**

We have replicated and extended findings showing clear motor deficits in $G_{\alpha q}$ knockout mice as assessed on an accelerating rotarod and the inverted screen test. Also, we have shown that $G_{\alpha q}$ knockout mice exhibit a significant hypoactive phenotype in the Y-maze, elevated zero maze and the open field, further supporting deficits in motor output. Drug-induced locomotor activity in $G_{\alpha q}$ knockout mice, however, remains intact with stimulation by dopaminergic agonists but not with the glutamatergic antagonist, MK-801. These findings indicate that basal ganglia locomotor circuitry is largely functional in the absence of $G_{\alpha q}$ signaling capacity. Motor impairments in these animals, therefore, likely originate in the cerebellum or other brain regions important in initiating motor output or discrete regions in areas such as the thalamus that are involved in signal integration and relay of motor signals to the cortex.

Additionally, we observed normal phenotypes in both the elevated zero maze and the forced swim test indicating that anxiety and depression-related circuitry appears intact after loss of $G_{\alpha q}$ expression. Lastly, use of the Y-maze revealed spatial memory deficits in $G_{\alpha q}$ knockout mice, indicating that functional $G_{\alpha q}$-coupled receptor signaling is necessary for proficiency in this task, most likely in the prefrontal cortex. However, our use a global mutant line and systemic injections clearly requires a very cautious interpretation, particularly with regard to specific brain regions.

# CHAPTER III

## LOCOMOTOR AND GROOMING RESPONSES TO SKF83959 REQUIRE DOPAMINE D1 BUT NOT D2 RECEPTORS: EVIDENCE AGAINST SKF83959 ACTIVATING A D1-D2 RECEPTOR OLIGOMER

### Introduction:

The dopamine $D_1$ receptor is the most highly expressed subtype among the dopamine receptor family ($D_1$-$D_5$ receptors) and plays critical roles in a variety of functions including motor control, motivated behaviors and cognition (Kiyatkin, 1995a, Goldman-Rakic, 1998a, Girault and Greengard, 2004, Arnsten and Li, 2005). While $D_1$-like receptors, consisting of the $D_1$ and $D_5$ receptor subtypes (Grandy et al., 1991, Sunahara et al., 1991), are positively coupled to adenylyl cyclase activity through the stimulatory G-protein $G_{\alpha s}$ (Stoof and Kebabian, 1981), it is also recognized that $D_1$-like receptors are also capable of coupling to $G_{\alpha q}$ (Wang et al., 1995, Jin et al., 2001). Additionally, it has been shown that specific $D_1$-like receptor ligands can activate alternative signal transduction systems resulting in PI hydrolysis and accumulation of inositol triphosphate *in vitro* (Undie and Friedman, 1990, Undie et al., 1994, Undie et al., 2000). Furthermore, there is some evidence suggesting that specific dopaminergic ligands can increase intracellular calcium mobilization resulting in the activation of CaMKII$\alpha$, an important regulator of synaptic transmission (Zhen et al., 2004, Rashid et al., 2007, Ng et al., 2010).

SKF83959, a dopamine $D_1$-like receptor partial agonist and phenylbenzazepine derivative of SKF38393, is one ligand that has been reported to preferentially activate $D_1$-like receptors linked to stimulation of PI hydrolysis in native tissue preparations (Arnt et al., 1992, Panchalingam and Undie, 2001, Jin et al., 2003). Additionally, this reported biased ligand has also been documented to have minimal cyclase-stimulating activity and antagonize dopamine-mediated stimulation of adenylyl cyclase *in vitro* (Arnt et al., 1992, Andringa et al., 1999, Cools et al., 2002, Jin et al., 2003). One recent model in the literature suggested that dopaminergic modulation of PI activity and downstream signaling systems occurs through activation of a $D_1/D_2$ receptor heteromeric complex coupled to $G_{\alpha q}$ protein (Rashid et al., 2007). Such functional selectivity in dopamine receptor-G protein signaling was suggested to be highly regulated; occurring only in specific brain regions and cells (Perreault et al., 2010, Zhang et al., 2010, Perreault et al., 2012).

Although the biological significance of signaling mediated by the proposed $D_1/D_2$ receptor heteromer remains largely unknown, the complex has been implicated in drug addiction (Perreault et al., 2010) and psychiatric disorders (Grymek et al., 2009, Pei et al., 2010, Perreault et al., 2010). Furthermore, SKF83959 has been previously shown to reverse some motor symptoms in animal models of Parkinson's disease (Arnt et al., 1992, Gnanalingham et al., 1995a, Gnanalingham et al., 1995c, Zhang et al., 2007). Here, we assessed the necessity of functional $D_1$ and $D_2$ receptors in the responses induced by SKF83959. Additionally, we evaluated the contribution of $D_5$ receptors, $G_{\alpha q}$ and

the role of CaMKII$\alpha$-Thr$^{286}$ autophosphorylation in mediating signaling initiated by SKF83959.

## Materials and Methods:

### *Animals*

C57Bl/6J mice (Jackson) were utilized for dose-response experiments and those studies examining the effects of dopaminergic antagonists on SKF83959-mediated behaviors. All other mice ($D_1$, $D_2$ and $D_5$ receptor knockouts, $G_{\alpha q}$ knockouts and CaMKII$\alpha$-Thr$^{286}$Ala knockin mice) were bred at Vanderbilt University using strategies previously described for generation of mixed litters and assignment of genotypes (Frederick et al., 2012). All lines were fully backcrossed (>10 generations) to a C57Bl/6 background.

Male mice were housed under standard housing conditions on a 12 h light/dark cycle with conditions previously described (Frederick et al., 2012). All behavioral testing was conducted on mice that were at least (P)60 at the time of initial testing. Mice were extensively handled prior to testing and were habituated to the testing rooms for ~30 min prior to beginning of every experiment. All procedures were approved by the Vanderbilt University Animal Care and Use Committee.

### *Drugs*

The dopamine $D_1$-like receptor agonist SKF83959 (3-methyl-6-chloro-7,8-dihydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine; Tocris

Biosciences, Minneapolis, MN) was dissolved in 0.9% saline solution at 0.2 mg/cc (1 mg/kg) and injected intraperiotoneally (i.p.) for the majority of experiments. For the dose response experiments, additional doses of SKF83959 (0.05 and 0.25 mg/kg) were also used. The $D_2$-like receptor antagonist raclopride (3,5-Dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide (+)-tartrate salt, Sigma, St. Louis, MO) was used at 0.5 mg/kg and the $D_1$-like receptor antagonist SCH23390 (R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride, Sigma, St. Louis, MO) was used at 0.01 mg/kg or 0.25 mg/kg. Additional doses of SCH23390 (0.05 and 0.1 mg/kg) were also tested for their effects on locomotor responses (See Appendix Figure 33). The selective $D_3$ receptor antagonist PG 01037 (*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-*trans*-but-2-enyl)-4-(pyridine-2-yl)benzamide hydrochloride; Tocris Biosciences, Minneapolis, MN) was used at 0.5, 5 and 10 mg/kg. Additionally, the mGluR5 antagonist MTEP ([(2-methyl-1, 3-thiazol-4-yl) ethynyl]pyridine; Ascent Scientific, Princeton, NJ/Abcam Biochemicals, Cambridge, MA) was used at 10 mg/kg while the $\alpha_2$ adrenergic receptor antagonist atipamezole (5-(2-Ethyl-2,3-dihydro-1*H*-indene-2-yl)-1*H*-imidazole hydrochloride; Tocris Biosciences, Minneapolis, MN) was used at 0.01, 0.05, 0.1, 0.25 and 0.5 mg/kg for initial dose finding (See Appendix Figure 34), and 0.25 mg/kg for experiments with SKF83959.

### *Open Field*

Locomotor responses to SKF83959 were measured using commercial

open field activity chambers (Med Associates, 29 x 29 x 20.5 cm) that were contained within light- and air-controlled environmental chambers (Med Associates, St. Albans, VT; 64 × 45 × 42 cm). Location and movement were detected by the interruption of infrared beams by the body of the mouse (16 photocells in each horizontal direction, as well as 16 photocells elevated 4 cm to measure rearing) and were measured by the Med Associates Activity Monitoring program. A three-day protocol was employed where the mice received injections of 0.9% saline for two days and SKF83959 on the third day as previously described (Frederick et al., 2012). For the studies with dopaminergic antagonists, the antagonist solution was injected (i.p.) 10 min prior to injection with SKF83959 (1 mg/kg; i.p.) while the mice were in the home cage. Following the SKF83959 injection, the mice were returned to the activity chambers for a 60 min assessment of locomotor activity.

### *Grooming Analysis*

During the open field testing, mice were additionally monitored by an overhead camera for later analyses of grooming behavior. Grooming was analyzed from the video recordings by assessing the number of grooming events every 30 s for a 5 min period during the baseline session and 5 min from the post-injection period. The observer was blind to genotype at the time of analysis.

### *Data Analysis and Statistics*

Data were subjected to one- or-two way analysis of variance (ANOVA)

using genotype as a between-group factor using GraphPad Prism (GraphPad Software, San Diego, CA). Post-hoc Tukey's multiple comparison tests or Bonferroni comparisons were used to compare groups to each other. Graphs are marked with an asterisk (*) to denote statistical significance ($p < 0.05$). For data with $p < 0.01$ or $p < 0.001$, the graphs are marked with two (**) or three (***) asterisks, respectively.

**Results:**

In wildtype mice, a peripheral injection of SKF83959 (0.05 - 1 mg/kg) produced a dose-dependent increase in horizontal locomotor activity that was maximal at 1 mg/kg (Figure 16). This response was still fairly modest, however, and increased locomotion approximately 2-fold over the baseline level of activity (Figure 16C; $F(4,39) = 39.9$, $p < 0.05$). In addition to the locomotor response, SKF83959 (1 mg/kg; i.p.) also elicited a specific motor stereotypy involving orofacial grooming as previously described (Downes and Waddington, 1993, Deveney and Waddington, 1995) and Figure 17. The grooming response was maximal at 0.05 mg/kg (Figure 17B; $F(3,23) = 10.3$, $p < 0.001$) compared to vehicle) although there was no significant difference between the responses elicited from the 0.05 mg/kg and the 1 mg/kg dose.

The SKF83959-induced locomotor response was blocked by the $D_2$-like receptor antagonist raclopride (Figure 18A and B) and the $D_1$-like receptor antagonist SCH23390; a result which showed dose-dependency (Figure 18C - E). SKF83959-induced grooming behavior was also blocked by $D_1$-like and $D_2$-

like receptor antagonists (Figure 18G and H) as previously described (Deveney and Waddington, 1995, Perreault et al., 2010).

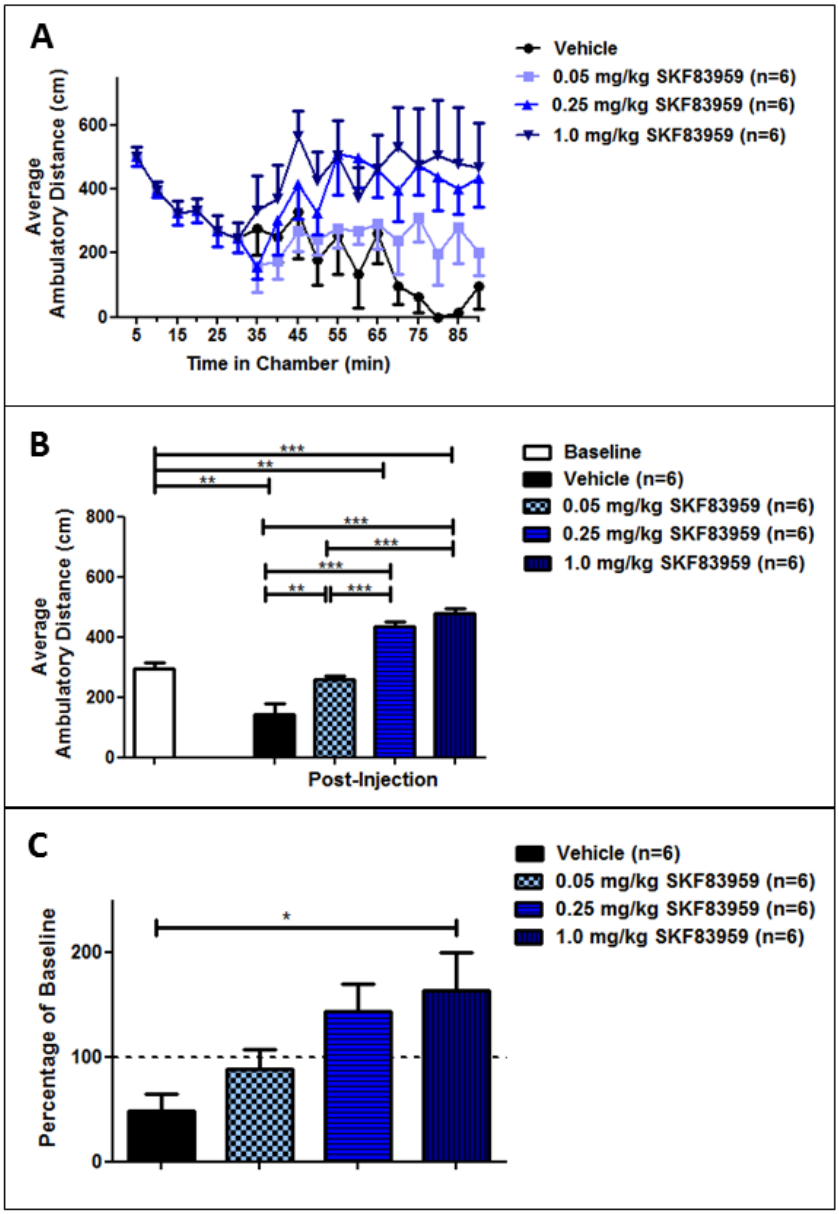


**Figure 16**

SKF83959 (0 - 1.0 mg/kg; i.p.) dose-dependently increases locomotor activity in C57/Bl6J mice (**A, B and C**). (**A**) shows the raw time-course data while (**B**) depicts the data as a bar graph. The percentage change from baseline for each dose tested is shown in (**C**) with 1.0 mg/kg producing a significant increase in activity compared to vehicle.

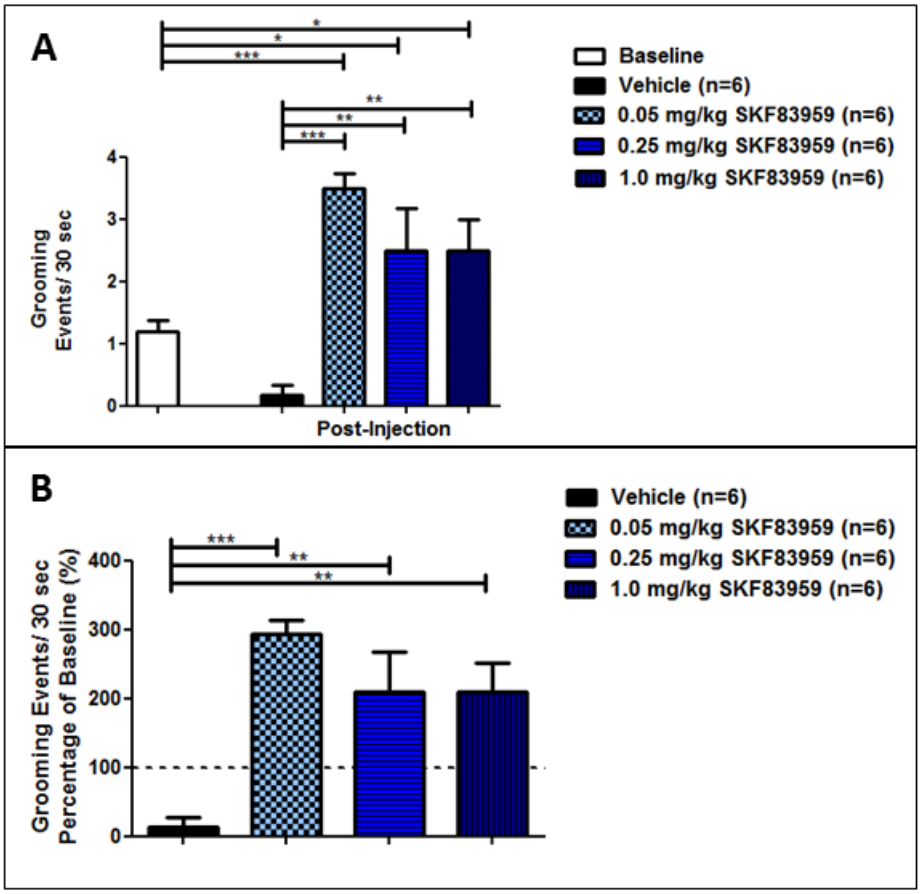


**Figure 17**

SKF83959 (0 - 1.0 mg/kg; i.p.) increases orofacial grooming behavior in C57/Bl6J mice with a peak in grooming at the 0.05 mg/kg dose (**A and B**). (**A**) shows the number of grooming events per 30sec during the 5 min periods evaluated for the baseline and post-injection periods. The percentage change from baseline for each dose tested is shown in (**B**) with all three doses producing a significant increase in grooming compared to vehicle.

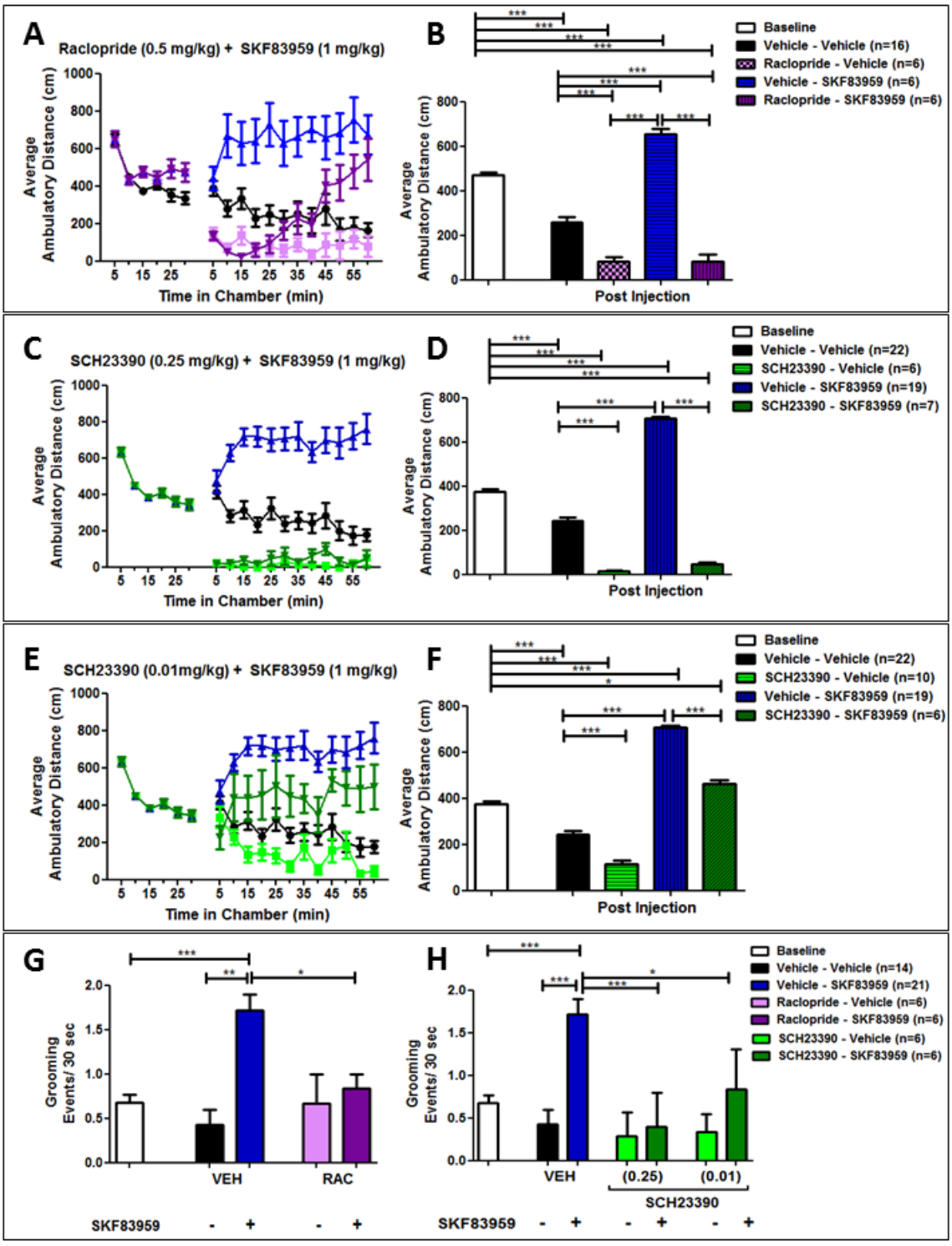


**Figure 18**

SKF83959-induced locomotor activity and orofacial grooming is blocked by $D_1$-like and $D_2$-like receptor antagonists in C57/Bl6J mice. (**A, C and E**) show the

ambulatory distance traveled in open field chambers as a function of time (5 min blocks over a 90 min testing period) by each test group during a 30 min baseline period and 60 min after the animals were injected with SKF83959 (1 mg/kg; i.p.) or 0.9% saline. Ten minutes prior to receiving SKF83959 or saline control, animals were injected with the $D_2$-like receptor antagonist raclopride (**A, B and G**; 0.5 mg/kg; i.p.), the $D_1$-like receptor antagonist SCH23390 (**C, D and H**; 0.25 mg/kg; and **E, F and H**; 0.01 mg/kg; i.p.) or 0.9% saline. The data is represented as bar graphs in (**B, D and F**), with the data from min 10-30 collapsed for the baseline measurement and min 40-60 representing the post-injection period for statistical comparisons. Grooming analyses are displayed in (**G** and **H)**.

In order to assess the complement of dopamine receptors necessary for mediating SKF83959-induced signaling and behavior, we investigated the SKF83959-induced locomotor activity of dopamine receptor knockout mice. Our data show that in open field chambers, $D_1$ receptor knockout mice were initially hyperactive compared to their wildtype and heterozygous littermates during the baseline session (Figure 19A and B; $p < 0.001$ by Two-way ANOVA, Bonferroni post-test). During the post-injection period, wildtype and heterozygous $D_1$ receptor mutant mice both significantly increased their locomotor activity in response to SKF83959 (Figure 19A and B; $p < 0.001$). The null mice, on the other hand, were seemingly unaffected and continued to habituate to the chambers to reduce their level of activity (Figure 19A and B; $p < 0.001$).

In the $D_5$ receptor mutant line, SKF83959 elicited a significant locomotor response in all three genotypes tested (Figure 19C and D; $p < 0$ .001). The post-injection response for the knockout and heterozygous mice, however, was significantly reduced compared to wildtype (Figure 19C and D, $p < 0.05$). Similarly, SKF83959 also significantly increased locomotor activity in all three genotypes of the $D_2$ receptor mutant line (Figure 19E and F; $p < 0$ .001). Here, the $D_2$ receptor knockout mice initially exhibited significant hypoactivity compared to the other two genotypes in the baseline session (Figure 19E and F; $p < 0$ .001) resulting in a more robust response in the knockout mice when considering the percentage change from baseline (data not shown).

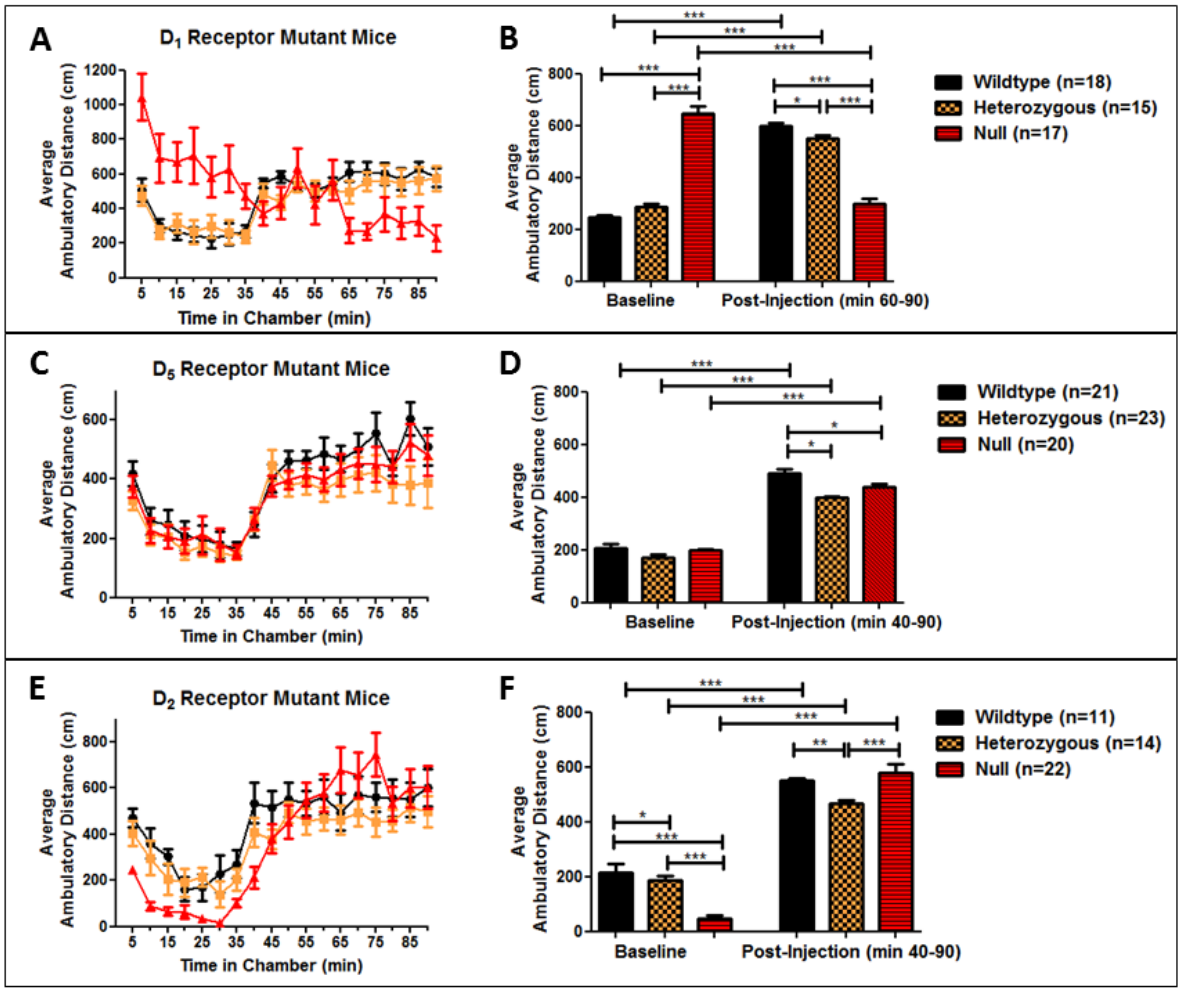


**Figure 19**

SKF83959 does not induce locomotor activity in $D_1$ receptor knockout mice but significantly increases activity in $D_5$ and $D_2$ receptor knockouts. (**A, C and E**) show the ambulatory distance traveled as a function of time (5 min blocks over a 90 min testing period) by each genotype for the three dopamine receptor mutant lines assessed. Mice were removed from the chamber after 30 min and administered SKF83959 (1 mg/kg; i.p.) before returning to the chamber for the last 60 min of testing. The data are represented as bar graphs in (**B, D and F**).

SKF83959-induced grooming responses largely paralleled the observations made with locomotor activation in the mutant lines. SKF83959 elicited a significant response in wildtype mice (Figure 20A; $p < 0$ .001) bot not in $D_1$ receptor knockout mice which were significantly different from wildtype when assessing the number of post-injection grooming events (Figure 20A; $p < 0$ .001) and the percentage change from baseline (Figure 20B; $p < 0$ .05). SKF83959-induced grooming was significantly increased in $D_5$ receptor knockout mice (Figure 20C; $p < 0.01$); however, the response in the null mice was significantly blunted compared to wildtype (Figure 20C and D; $p < 0.05$). Lastly, SKF83959 also significantly increased grooming activity in mice lacking functional $D_2$ receptors (Figure 20E; $p < 0.05$) with no difference in percentage change from baseline between the knockouts and wildtype mice (Figure 20F).

In addition to evaluating the contribution of the dopamine receptors to SKF83959-mediated actions, we also assessed the contribution of the G-protein $G_{\alpha q}$. Locomotor and grooming responses to SKF83959 were assessed in $G_{\alpha q}$ knockout mice with similar results as those observed in the $D_2$ receptor mutant line. Here, we show that SKF83959 significantly increased grooming in $G_{\alpha q}$ knockout mice (Figure 20G, $p < 0.05$) with a trend toward a greater percentage change from baseline in the knockouts compared to wildtype (Figure 20H, $p = 0.19$). These data parallel our previously published data showing that SKF83959-induced locomotor activity is also intact in these mice (Frederick et al., 2012).

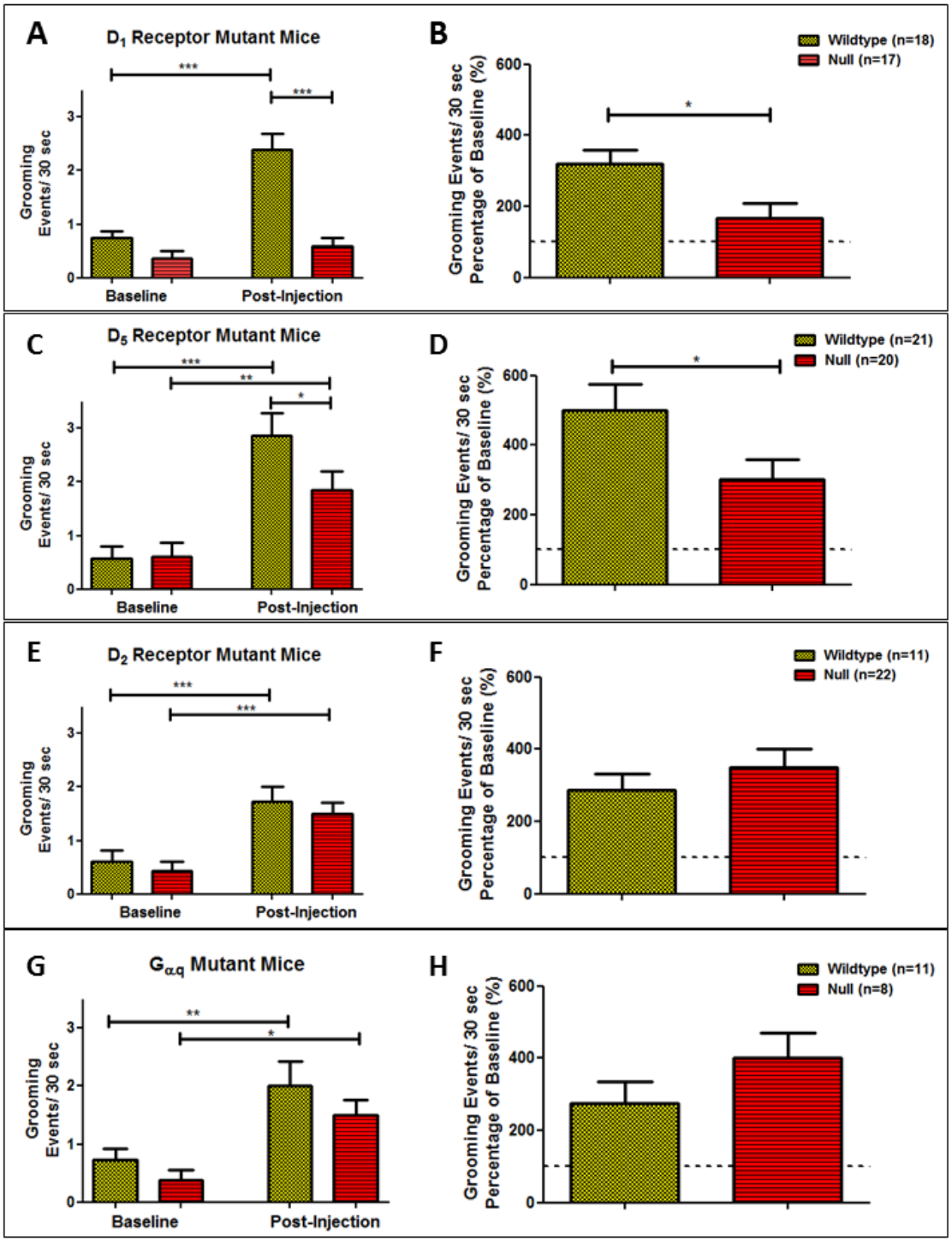


**Figure 20**

SKF83959-induced grooming is absent in mice lacking the $D_1$ receptor but present in $D_5$ and $D_2$ receptor knockouts and $G_{\alpha q}$ null mice. Mice were monitored by an overhead camera while in the open field and grooming was later assessed

every 30 s for a 5 min period during the baseline and post-injection periods. (**A, C, E and G**) show the baseline and post-SKF83959 grooming events committed by each genotype for each of the mutant lines assessed while (**B, D, F and H**) shows the data represented as a percentage of the baseline.

In order to assess the hypothesis that dopamine $D_3$ receptors may be involved in mediating SKF83959-induced actions, we tested whether we could block SKF83959-induced locomotion by inhibiting $D_3$ receptor signaling with the selective $D_3$ receptor antagonist PG 01037. In wildtype mice, we found that PG 01037 did not attenuate SKF83959-induced locomotor activity at the three doses tested: 0.5, 5 and 10 mg/kg (Figure 21A - F). At the 0.5 and 5 mg/kg doses, PG 01037 had no effect on locomotor responses when administered alone, however we did observed a significant increase in locomotor activity compared to vehicle at the 10 mg/kg dose (Figure 21E and F, $p < 0.001$ by Two-way ANOVA, Bonferroni post-test). This locomotor response, however, was not significantly different from the baseline level of activity (Figure 21E and F, comparison between baseline and PG 01037-vehicle).

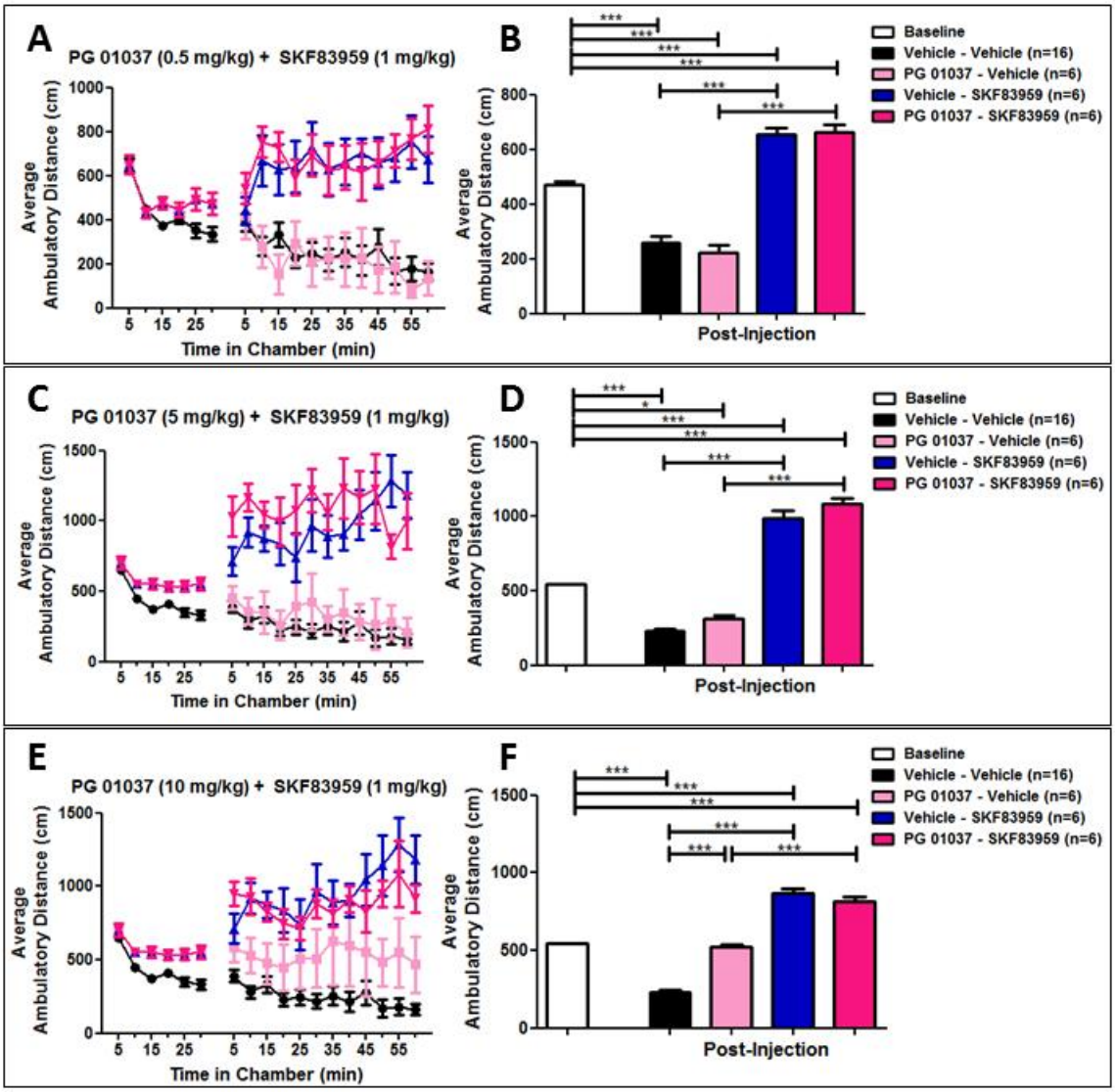


**Figure 21**

SKF83959-induced locomotor activity is not blocked by the selective $D_3$ receptor antagonist PG 01037 in C57/Bl6J mice. (**A, C and E**) show the ambulatory distance traveled in open field chambers as a function of time by each test group during a 30 min baseline period and 60 min after the animals were injected with SKF83959 (1 mg/kg; i.p.) or 0.9% saline. Ten minutes prior to receiving SKF83959 or saline, animals were injected with saline or PG 01037 (**A and B**; 0.5 mg/kg; i.p.), (**C and D**; 5 mg/kg) or (**E and F**; 10 mg/kg; i.p.). The data is represented as bar graphs in (**B, D and F**), with the data from min 10-30 collapsed for the baseline measurement and min 40-90 representing the post-injection period for statistical comparisons.

Since we did not observe any effects when $D_3$ receptors were selectively blocked in wildtype mice (and $D_2$ receptors were still functional), we then decided to test the hypothesis that signaling through both the $D_2$ and $D_3$ receptor subtypes needed to be blocked in order to attenuate SKF83959-induced actions. In the $D_2$ receptor mutant line, we observed that PG 01037 had no effect on locomotor activity when administered alone (Figure 22A and B) as we have previously observed in our earlier experiments with wildtype mice (see Figure 21). Additionally, the $D_2$ receptor knockout mice were significantly hypoactive when compared to wildtype as we have observed previously in this line (see Figure 19) and all three genotypes continued to habituate in the chambers after they received PG 01037 (10 mg/kg; i.p.) (Figure 22A and B). To our surprise, however, when $D_2$ receptor knockouts received PG 01037 (10 mg/kg, i.p.) ten minutes prior to receiving an acute injection of SKF83959 (1 mg/kg, i.p.), the response was greatly potentiated in these mice compared to wildtype (Figure 22C and D). Furthermore, we replicated these results using a second compound, raclopride, to block $D_3$ receptors in the $D_2$ receptor knockouts and obtained similar results (Figure 23C and D). In these experiments, however, we observed that raclopride significantly reduced locomotor activity when administered at 0.5 mg/kg (i.p.) to wildtype and heterozygous $D_2$ receptor mutant mice (Figure 23A and B) and blocked the SKF83959-induced locomotor response in these mice (Figure 23C and D). The $D_2$ receptor knockout mice, on the other hand, produced the potentiated response (Figure 23C and D), similarly as observed previously with the PG 01037 compound.

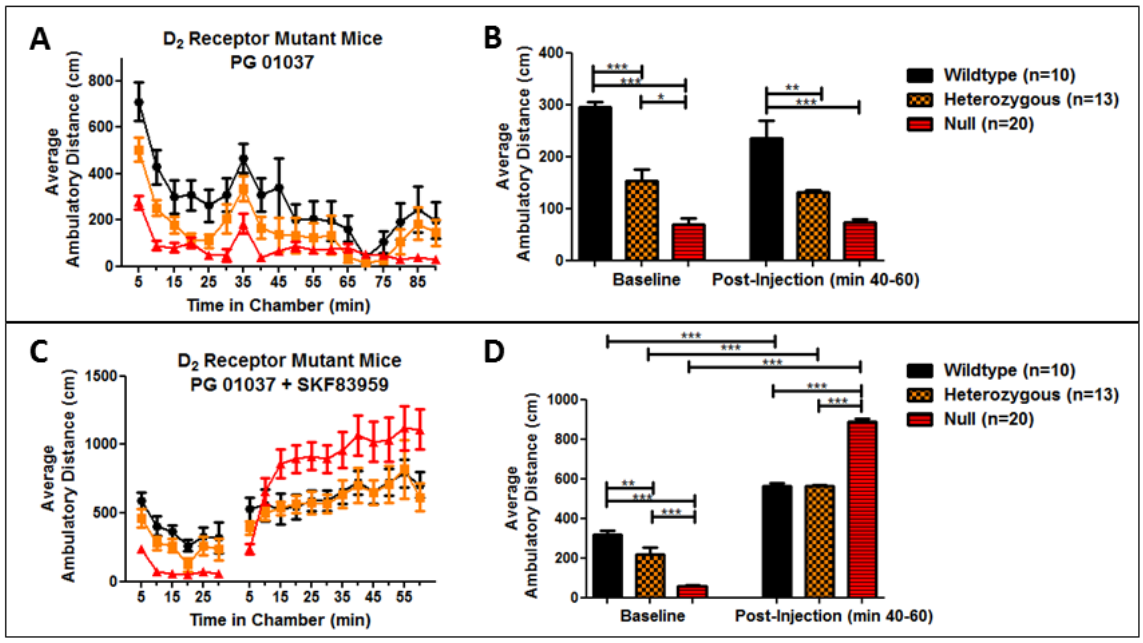


**Figure 22**

(**A and B**) show that the selective $D_3$ receptor antagonist PG 01037 does not affect locomotor responses when administered (10 mg/kg; i.p.) to $D_2$ receptor wildtype, heterozygous or knockout mice. The compound potentiates the locomotor response in $D_2$ receptor knockout mice, however, when administered prior to an acute injection of SKF83959 as shown in (**C and D**).

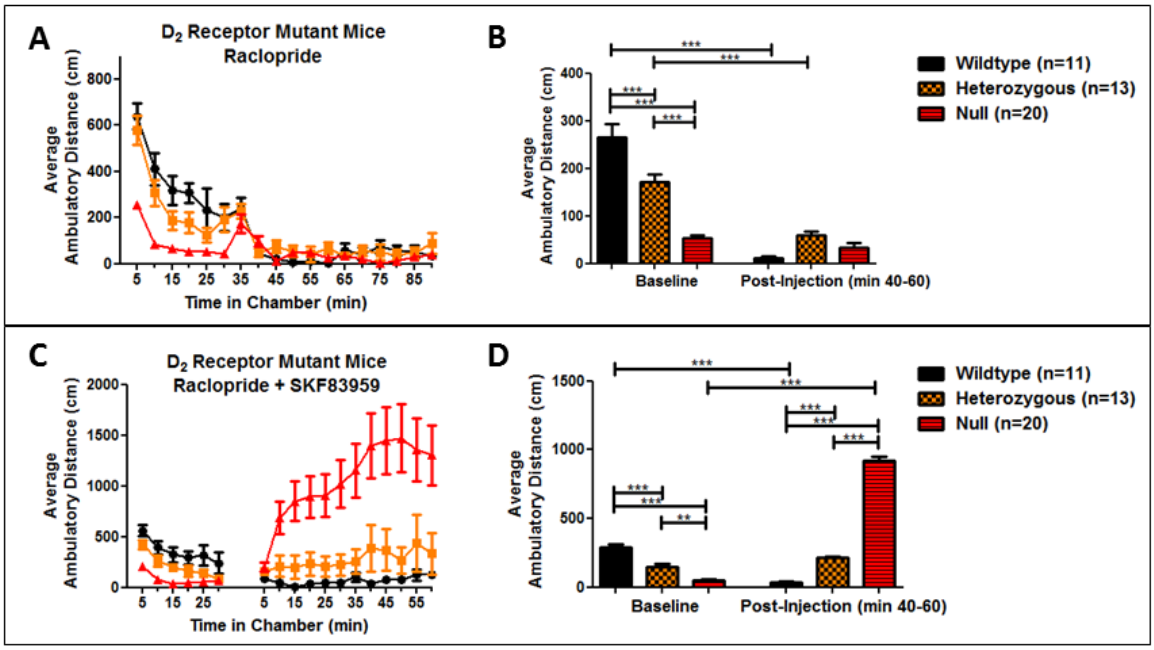


**Figure 23**

(**A and B**) show that the $D_2$-like receptor antagonist raclopride (0.5 mg/kg; i.p.) inhibits locomotor activity in $D_2$ receptor wildtype and heterozygous mice, thereby bringing them to the same level of activity as the hypoactive null mice. (**C and D**) show that raclopride blocks SKF83959-induced locomotor activity in the wildtype and heterozygous mice, however, locomotor responses are potentiated in the null mice.

Additionally, we also assessed the contribution of non-dopaminergic receptors in the signaling of SKF83959. First, we assessed the contribution of signaling through the metabotropic glutamate receptor subtype 5 (mGluR5) to SKF83959-induced locomotor behavior by blocking mGluR5 signaling with the non-competitive antagonist MTEP. Next, we evaluated the contribution of

$\alpha_2$ adrenergic receptors by blocking receptor signaling with the highly potent and selective antagonist atipamezole. We observed an attenuation of SKF83959-induced locomotor activity by both MTEP (Figure 24A and B; 10 mg/kg) and by atipamezole (Figure 24C and D; 0.25 mg/kg) when administered ten minutes prior to administration of an acute dose of SKF83959 (1 mg/kg; i.p.).

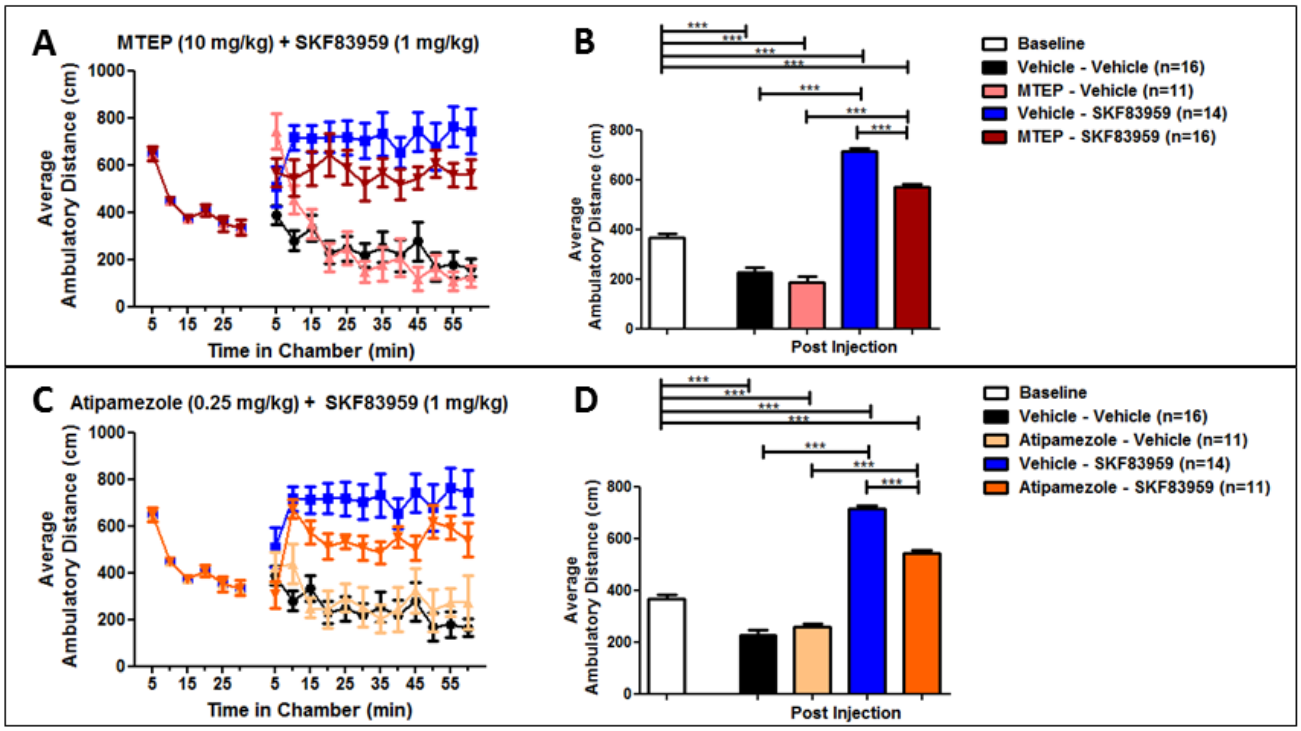


**Figure 24**

SKF83959-induced locomotor responses are blunted but not completely blocked by the mGluR5 receptor antagonist MTEP (**A and B**) and by the $\alpha_2$ adrenergic receptor antagonist atipamezole (**C and D**).

Lastly, we assessed the role of CaMKIIα in mediating the behavioral responses to SKF83959 to address previous reports indicating that phosphorylation of CaMKIIα is a critical downstream component in the signaling of SKF83959 (Zhen et al., 2004, Rashid et al., 2007, Ng et al., 2010). We evaluated CaMKIIα-Thr$^{286}$Ala knockin mice which are not able to be phosphorylated at the Thr$^{286}$ residue (Giese et al., 1998, Gustin et al., 2011) to address this question. Our data show that both SKF83959-induced locomotor and grooming responses are intact in CaMKIIα-Thr$^{286}$Ala knockin mice (Figure 25A and D) as these mice respond similarly to wildtype in both assessments.

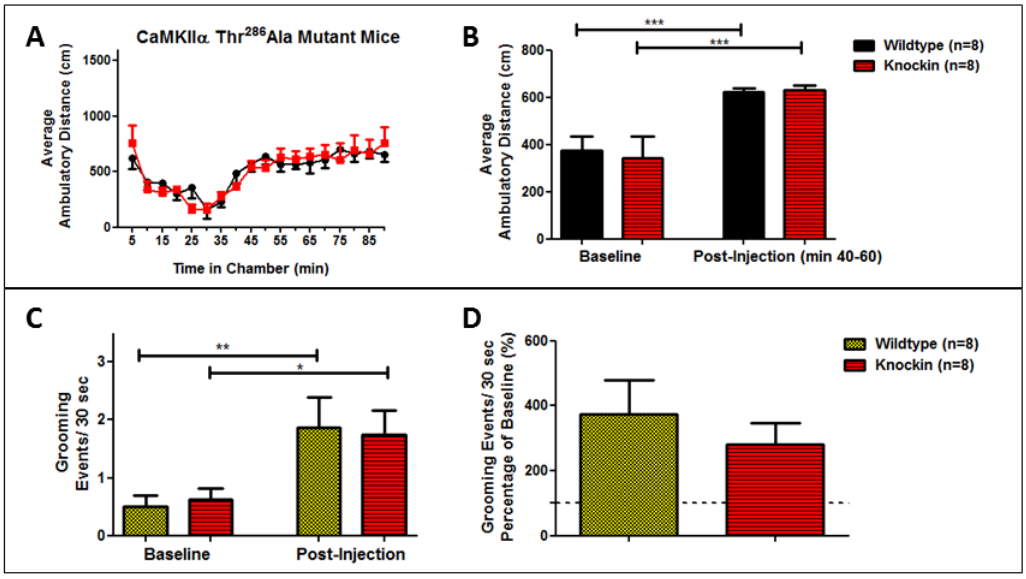


**Figure 25**

SKF83959-induced behaviors are intact in autophosphorylation - deficient CaMKIIα-Thr$^{286}$Ala knock-in mice. (**A and B)** show the locomotor response to SKF83959 as a time-course and bar graph, respectively. The SKF83959-induced grooming response is displayed in (**C and D).**

**Discussion:**

We have shown that SKF83959-induced locomotion was dose-dependently increased over a range of doses (0 - 1 mg/kg; i.p.) and a maximal locomotor response was elicited from 1 mg/kg SKF83959 when compared to vehicle. The orofacial grooming response, however, was maximal at a lower dose of SKF83959 (0.05 mg/kg; i.p.) although significant grooming was still elicited at the higher doses of SKF83959 (0.25 and 1 mg/kg; i.p.). It is unclear at this time why there is a large separation (at least a 20 fold difference) between the doses of SKF83959 needed to elicit maximal responses in the locomotor and grooming responses. Additionally, although we have observed that SKF83959 elicits parallel responses for these two behaviors, we do not know whether the same circuitry subserves both the locomotor and grooming responses to SKF83959. Studies utilizing localized injections of SKF83959 into specific brain regions would be necessary to further address the question of whether these behaviors are mediated by activation of the same (or different) neural circuits. To this end, there is evidence in the literature demonstrating that local injections of locomotor-stimulating agents such as cocaine or amphetamine into the nucleus accumbens or ventral striatum, but not the caudate putamen (dorsal striatum), increases locomotor activity in rodent models. Furthermore, lesions to the ventral striatum have been shown to reduce locomotion elicited by systemic cocaine or amphetamine (Kelly and Iversen, 1976, Staton and Solomon, 1984, Delfs et al., 1990, Ikemoto, 2002). Moreover, it has also been demonstrated in the literature that microinjections of amphetamine into the dorsal striatum

increase stereotypic responses (of which grooming is included) over changes in locomotion (Staton and Solomon, 1984, Kelley et al., 1988). It is therefore reasonable to hypothesize that the SKF83959-induced locomotor and grooming responses are primarily mediated by the ventral and dorsal striatum, respectively.

SKF83959-induced behavioral responses were attenuated by the $D_1$-like receptor antagonist SCH23390 and absent in $D_1$ receptor knockout mice, thereby confirming the role of the dopamine $D_1$ receptor in mediating the effects of SKF83959. Additionally, since SKF83959 also has affinity for $D_5$ receptors, we also assessed the SKF83959-induced behaviors in $D_5$ receptor null mice. SKF83959-induced locomotor activity and grooming were largely intact in $D_5$ receptor knockouts, further confirming the role of the $D_1$ receptor as the primary $D_1$-like receptor target for the behavioral effects of SKF83959. There was, however, a significant difference between the response of wildtype and $D_5$ receptor knockouts, suggesting a possible role for the $D_5$ receptor in mediating the signaling to some degree.

The $D_1$ and $D_5$ dopamine receptors, sharing 80% homology in their transmembrane domains and similar pharmacologic profiles, are not differentiated from one another by any known pharmacologic compound. It is recognized, however, that the $D_5$ receptor displays higher baseline activity, lower agonist-induced stimulation of cAMP and a higher affinity for dopamine than the $D_1$ receptor (Sunahara et al., 1991, Tiberi et al., 1991, Tiberi and Caron, 1994, Missale et al., 1998, Beaulieu and Gainetdinov, 2011). Additionally, the anatomical distribution of the $D_1$ and $D_5$ receptors are largely distinct from each

other in expression density and localization as the $D_5$ receptor is poorly expressed in most regions when compared to the $D_1$ receptor. The $D_5$ receptor is expressed at low to moderate levels in multiple brain regions including the cerebral cortex, substantia nigra, hypothalamus, striatum, nucleus accumbens, cerebellum and olfactory tubercle and more distinctly in the hippocampus, the lateral mammillary nucleus, and the parafasicular nucleus of the thalamus, where the $D_1$ receptor is not significantly expressed (Bergson et al., 1995, Khan et al., 2000, Berlanga et al., 2005). Particularly, within the striatum, $D_5$ receptors have been shown to be poorly expressed on GABAergic MSNs, where $D_1$ receptors are predominately expressed, but instead are more abundantly expressed on large aspiny cholinergic interneurons which account for approximately 2% of the entire striatal neuronal population (Bergson et al., 1995, Khan et al., 2000, Berlanga et al., 2005). The localization of $D_5$ receptors on cholinergic cells allows for direct dopaminergic modulation of acetylcholine levels in the striatum. In fact, $D_5$ receptor activation of striatal cholinergic interneurons has been shown to enhance $Zn^{2+}$ sensitive $GABA_A$ currents thereby modulating GABA neurotransmission in the striatum (Yan and Surmeier, 1997). Loss of $D_5$ receptor signaling, therefore, could blunt SKF83959-induced behaviors through a reduction in $GABA_A$ currents facilitated through $D_5$ receptors on cholinergic cells thereby altering maximal striatal output. Additionally, cholinergic interneurons have been shown to modulate striatal signaling and synaptic plasticity via activation of muscarinic receptors on MSNs and modulation of long-term potentiation at striatal synapses (Howe and Surmeier, 1995, Calabresi et al.,

1999, Alcantara et al., 2001); mechanisms which could also be altered in the knockout mice.

Interestingly, and much to our surprise, we observed behavioral responses to SKF83959 in both the $D_2$ receptor knockout mice and $G_{\alpha q}$ knockout mice, suggesting that these proteins are not necessary for SKF83959 signaling and drug-induced behavioral responses. These results contradict the current model in the literature regarding the signaling mechanism of SKF83959. In this mechanism, SKF83959 acts through a $D_1/D_2$ receptor heteromer coupled to $G_{\alpha q}$ and downstream signaling systems involving PI hydrolysis and intracellular calcium release. If, in fact, SKF83959 does signal through such a mechanism, then we would have expected to observe minimal SKF83959-induced locomotor and grooming responses in the $D_2$ receptor and $G_{\alpha q}$ null mice as we did with the $D_1$ receptor knockout mice. Contrary to our hypotheses however, $D_2$ receptor and $G_{\alpha q}$ knockout mice appear to be more sensitive to SKF83959; both exhibiting greater percentage change from baseline in the locomotor assay compared to wildtype mice. We did, however, replicate previous reports indicating that SKF83959-induced behaviors can be blocked by the $D_2$-like receptor antagonist raclopride. These results, however, are confounded by the fact that raclopride alone at 0.5 mg/kg, the dose that has previously been reported to block SKF83959-induced grooming behaviors, induces significant catalepsy in wildtype mice (see Figure18A and B and (Wadenberg et al., 2000, Perreault et al., 2010)).

SKF83959-induced behaviors were conserved in $D_2$ receptor knockout mice suggesting that $D_2$ receptors are not necessary for SKF83959-induced

actions. We therefore next assessed the possible role that dopamine $D_3$ receptors could be playing in mediating these effects. Although the $D_2$ and $D_3$ receptor subtypes share only 46% amino acid sequence homology, they do share structural and signaling similarities with 75% homology in their transmembrane domains; the regions primarily involved in constructing the ligand-binding site. Additionally, they share a similar long third intracellular loop common to GPCRs that interact with $G_{\alpha i}$ isoforms that signal to inhibit activity of adenylyl cyclase (Missale et al., 1998, Beaulieu and Gainetdinov, 2011).

Because of the high degree of homology between the $D_2$ and $D_3$ receptor ligand binding sites, it has been difficult to obtain specific compounds that can selectively bind to either the $D_2$ or the $D_3$ receptor subtype. The receptors have some distinguishing pharmacological features, however, including differences in the efficacy of response for specific ligands. Dopamine, in fact, has 20 times greater affinity for $D_3$ receptors than for $D_2$ receptors, likely related to sequence differences between the two receptors in the third intracellular loop altering the efficiency of coupling to G-proteins (Missale et al., 1998, Beaulieu and Gainetdinov, 2011). More recently, with focused drug-discovery efforts, selective compounds have been developed that can distinguish between the two receptor subtypes. (Pilla et al., 1999, Grundt et al., 2005, Boeckler and Gmeiner, 2006, Micheli and Heidbreder, 2008). These ligands allow for the subtype-specific evaluation of receptor functions and subtype-specific contributions to various modalities of behavioral output.

The $D_2$ and $D_3$ receptor subtypes also differ in their neuroanatomical localization and expression levels. The $D_3$ receptor has a more limited and specific pattern of distribution than the $D_2$ receptor; concentrated primarily in limbic brain regions including the shell of the nucleus accumbens, the olfactory tubercle, and the islands of Calleja. Additionally, the $D_3$ receptor is expressed at very low, but detectable levels in the ventral pallidum, substantia nigra pars compacta, ventral tegmental area and hippocampus (Gurevich and Joyce, 1999, Stanwood et al., 2000, Beaulieu et al., 2005). Based on its anatomical localization, $D_3$ receptors could play important roles in mediating limbic-related functions and motor/sensory processing functions. Particularly of interest here due to its expression in the striatum, the $D_3$ receptor may contribute to extrapyramidal motor functions and more specifically, SKF83959-induced locomotor and grooming responses. Previous reports indicate that SKF83959 has similar affinity for dopamine $D_3$ receptors as it does for $D_2$ receptors (Neumeyer et al., 2003), so it feasible that $D_3$ receptors could compensate for the more abundantly expressed $D_2$ receptor in their absence in the $D_2$ receptor knockout mice. The ability of SKF83959 to signal through a $D_1/D_3$ receptor heteromer has not been previously tested and experiments with raclopride completed by our laboratory and others could implicate the involvement of $D_2$ and/or $D_3$ receptors since raclopride has high affinity at both receptors (Seeman and Van Tol, 1993).

The novel high-affinity and selective $D_3$ receptor antagonist PG 01037 [*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)-*trans*-but-2-enyl)-4-(pyridine-2-

yl)benzamide hydrochloride] was used for our studies due to its established >100-fold binding selectivity at the $D_3$ dopamine receptor compared to the $D_2$ receptor subtype (Grundt et al., 2005). This compound has also been previously shown to be a selective $D_3$ receptor antagonist *in vivo* and readily enters the brain to localize in brain regions rich in $D_3$ receptor expression (Grundt et al., 2005). Furthermore, the metabolism and pharmacokinetic profile of this compound have already been established in the literature (Mason et al., 2010). Behaviorally, PG 01037 has been shown to be effective in antagonizing $D_3$ receptors in animal models of psychostimulant abuse to alter drug-seeking behaviors (Grundt et al., 2007, Higley et al., 2011) and to attenuate abnormal involuntary movements associated with L-Dopa therapy in animal models of Parkinson's disease (Kumar et al., 2009).

In wildtype mice, we were unable to block SKF83959-induced locomotor responses with the selective $D_3$ receptor antagonist PG 01037 at the three doses tested: 0.5, 5 and 10 mg/kg. We believe that we were in an adequate dose range to observe a response, if any, as the compound has been shown to enter the brain at concentrations of 2-10 mg/kg (Grundt et al., 2007, Mason et al., 2010). Additionally, the 10 mg/kg dose has previously been shown to be efficacious in behavioral paradigms in animal models (Collins et al., 2005, Kumar et al., 2009, Higley et al., 2011) and has also been shown to be the preferred dose for administration by the i.p. route (Mason et al., 2010).

We then tested the hypothesis that signaling through both the $D_2$ and $D_3$ receptor subtypes needed to be blocked in order to attenuate SKF83959-induced

actions. We decided to utilize a combined pharmacologic and genetic approach by antagonizing the $D_3$ receptor population with the selective compound PG 01037 in the $D_2$ receptor knockout mice which lack functional $D_2$ receptors. Such an approach, in theory, would be similar to blocking both receptors with the mixed antagonist raclopride, which we have previously shown to block SKF83959-induced behaviors. Additionally, $D_2/D_3$ receptor double knockout mice have been characterized for their locomotor phenotype and have been shown to exhibit a basal hypoactive phenotype (Jung et al., 1999).

In the $D_2$ receptor mutant line, we observed, to our surprise, a greatly potentiated response in the $D_2$ receptor knockouts compared to wildtype when they received PG 01037 (10 mg/kg; i.p.) ten minutes prior to administration of SKF83959 (1 mg/kg; i.p.). The wildtype and heterozygous mice responded to co-administration of PG 01037 and SKF83959 with a 2-3 fold increase in locomotor activity as expected, however the $D_2$ receptor null mice increased their activity approximately 15 fold. These results were contrary to our hypothesis that SKF83959-induced locomotor activity would be diminished if we blocked signaling through both the $D_2$ and $D_3$ receptor subtypes. Furthermore, we replicated these results using a second compound, raclopride, to block $D_3$ receptors in the $D_2$ receptor knockouts and obtained similar results. Taken together, use of the selective $D_3$ receptor antagonist PG 01037 did not clear up whether both $D_2$ and $D_3$ receptors could potentially both be involved in the signaling of SKF83939 as the potentiated response in the knockout mice confounds the results and hints at altered $D_3$ receptor expression in the $D_2$

receptor null mice that warrants further investigation. In order to further explore the potential role of the $D_3$ receptor subtype in SKF83959 signaling using a behavioral approach, we would need to investigate the SKF83959-induced behaviors in $D_3$ receptor knockouts and $D_2/D_3$ receptor double knockout mice. We do not know, however, if there would be alterations in expression of other receptors in these mice, such as the $D_4$ receptor subtype, which shares 53% transmembrane homology with the $D_2$ receptor, which could confound the results. The $D_4$ receptor subtype, however, is not highly expressed in the striatum (Missale et al., 1998, Beaulieu and Gainetdinov, 2011).

Additionally, we assessed the contribution of non-dopaminergic receptors in the signaling of SKF83959. First, since proper functioning of the striatum depends on the ability of MSNs to integrate inputs from dopaminergic neurons and descending glutamatergic cortical neurons, we assessed the contribution of signaling through mGluR5. The mGluR5 subtype is abundantly expressed in the striatum (Shigemoto et al., 1993, Romano et al., 1995, Muly et al., 2003) where it has been shown to be involved in modulating dopamine release (Ohno and Watanabe, 1995, Verma and Moghaddam, 1998, Bruton et al., 1999). Additionally, mGluR5s have been shown to function cooperatively with dopamine $D_1$ receptors in striatal neurons to enhance the accumulation of cAMP (Paolillo et al., 1998) and regulate the phosphorylation state of key signaling molecules: cAMP-response element binding protein (CREB) and the extracellular signal-regulated kinase 2 (ERK2) (Voulalas et al., 2005).

Furthermore, due to the abundant expression of mGluR5 in limbic brain regions (Shigemoto et al., 1993, Romano et al., 1995), and its convergence upon dopaminergic reward pathways, the mGluR5 subtype is believed to play an important role in associative learning, motivational processes and synaptic plasticity. In particular, mGluR5 may be a key neuroanatomical target and modulator of the reinforcing properties of cocaine and other drug of abuse as well mediating other stages of the addiction cycle including withdrawal and reinstatement (Chiamulera et al., 2001, Gubellini et al., 2004, Kauer and Malenka, 2007, Carroll, 2008, Schotanus and Chergui, 2008, Olsen et al., 2010, Huang et al., 2011, Wang et al., 2012). In fact, when mGluR5 function is selectively knocked-down in dopamine $D_1$ receptor-expressing neurons with an interfering RNA peptide in a mouse model, a critical interaction between mGluR5s and dopamine $D_1$ receptors in the formation of reward associations and reinforcement of motivated behaviors is revealed (Novak et al., 2010).

We also assessed the contribution of signaling through $\alpha_2$ adrenergic receptors as interactions between these receptors and dopamine receptor signaling have been previously demonstrated in multiple species (Cornil and Ball, 2008). The morphological and functional features of the $\alpha_2$ adrenergic receptor, in fact, appear to be similar in phylogeny to that of the dopamine $D_2$ receptor (Donnelly et al., 1994, Vernier et al., 1995); the receptor that was proposed to interact with the $D_1$ receptor in the signaling of SKF83959. Additionally, $\alpha_2$ receptors have been shown to operate as hetero-receptors to modulate dopamine release in the striatum (Starke et al., 1989, Trendelenburg et al., 1994,

Bucheler et al., 2002) and dopaminergic activation of these receptors has been shown to inhibit activity of adenylyl cyclase (Zhang et al., 1999). Furthermore, the effects of $\alpha_2$ receptor ligands on dopaminergic modulation of motor function have been investigated in animal models of Parkinson's disease and shown to modulate locomotor responses to amphetamine, methylphenidate and apomorphine (Mavridis et al., 1991, Chopin et al., 1999, Haapalinna et al., 2003).

The $\alpha_2$ adrenergic receptor family is comprised of three subtypes; the $\alpha_{2a}$, $\alpha_{2b}$ and $\alpha_{2c}$ receptors of which the $\alpha_{2a}$ and $\alpha_{2c}$ subtypes are the major forms expressed in the brain (Nicholas et al., 1993, Happe et al., 2004). The $\alpha_{2c}$ receptor has a more limited expression pattern and has been shown to be concentrated in the basal ganglia among other regions; however, it is expressed at much lower densities than the widely distributed $\alpha_{2a}$ receptor (Nicholas et al., 1996, Happe et al., 2004). Selective compounds and receptor knockout mice have been developed to investigate the functions of individual $\alpha_2$ receptor subtypes selectively (Link et al., 1996, Altman et al., 1999, Knaus et al., 2007, Quaglia et al., 2011); future studies could dissect the role of $\alpha_2$ receptor signaling at this level of detail.

In order to assess the contribution of mGluR5 to SKF83959-induced locomotor behavior, we blocked mGluR5 signaling with the non-competitive antagonist MTEP. MTEP has been shown to be a highly selective ligand with no known off-target activity at other receptors tested (Cosford et al., 2003, Busse et al., 2004). Additionally, MTEP has been previously shown to reach full receptor occupancy at a dose of 10 mg/kg; therefore, this dose was selected for our

behavioral assessments. For studies evaluating $\alpha_2$ adrenergic receptor contribution, the highly potent and selective $\alpha_2$ receptor antagonist atipamezole was used. In receptor binding studies, atipamezole was shown to have 100 times higher affinity on $\alpha_2$ adrenergic receptors than reference compounds and was additionally shown to be greater than 200 times more selective than yohimbine, an $\alpha_2$ receptor antagonist that was previously the standard compound reported in the literature (Virtanen et al., 1989). Although atipamezole has been assessed in behavioral models, the doses used (> 0.5 mg/kg) have been reported to produced effects on locomotor response (Kauppila et al., 1991). We therefore conducted dose response experiments to determine a suitable dose for use in our locomotor experiments (see Appendix Figure 34) and selected a dose of 0.25 mg/kg.

We observed an attenuation of SKF83959-induced locomotor activity by both the mGluR5 receptor antagonist MTEP (10 mg/kg; i.p.) and by the $\alpha_2$ adrenergic receptor antagonist atipamezole (0.25 mg/kg; i.p) suggesting that signaling through non-dopaminergic receptors may contribute to producing a maximal SKF83959-mediated response. These receptors, however, are not required for SKF83959-induced locomotor activity.

Lastly, we assessed the role of CaMKII$\alpha$ in mediating the behavioral responses to SKF83959 to address previous reports indicating that phosphorylation of CaMKII$\alpha$ is a critical downstream component in the signaling of SKF83959 (Zhen et al., 2004, Rashid et al., 2007, Ng et al., 2010). We observed intact SKF83959-induced locomotor and grooming responses in

CaMKII$\alpha$-Thr$^{286}$Ala knockin mice, thereby negating the necessity of CaMKII$\alpha$ phosphorylation at the autophosphorylation site (Thr$^{286}$) in the signaling mechanism of SKF83959.

**Conclusions**:

When taken together, these data suggest that SKF83959 does not exclusively activate $D_1$ and $D_2$ receptors coupled to a $G_{\alpha q}$ signaling pathway as has been previously claimed in the literature. We therefore need to be cautious when interpreting data collected from studies utilizing this ligand and re-evaluate the literature with respect to these new findings. Additionally, these results indicate that alternative mechanisms for the signaling of SKF83959 should be explored as its effects clearly do not require the presence of $D_1/D_2$ receptor heteromers.

# CHAPTER IV

## SKF83959 ALTERS RESPONSES IN ANIMAL MODELS OF ANXIETY AND BEHAVIORAL DESPAIR

### Introduction:

Depression and anxiety are common emotional disorders accounting for a substantial proportion of the burden of mental health disorders in the United States (Weissman et al., 1996, Kessler et al., 2005). Although several subtypes of these disorders are described in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), they all share general symptoms and treatment regimens and are highly comorbid. The estimated lifetime prevalence for depression and anxiety is approximately 17% and 25-30%, respectively, with both these disorders occurring more often in women. Additionally, both disorders often have onset during adolescence or early adulthood and become either recurrent or chronic conditions; thereby reducing quality of life and occupational productivity in the affected clinical population (Weissman et al., 1996, Kessler et al., 2005, Aina and Susman, 2006).

Although the neural circuits underlying these disorders are not completely understood and there is no unitary model for their molecular and cellular origins, dysfunctions in the amygdala, hippocampus, basal ganglia and prefrontal cortex are commonly implicated (Clark et al., 2009, Aupperle and Paulus, 2010, Clark and Beck, 2010, Harro et al., 2011, McEwen et al., 2012). In depression, for example, some cognitive theories propose that the interplay between reduced

activation in subcortical regions produce the altered emotional state while increased activation in cortical circuits dysregulates higher-order control of emotion and behavioral output (Clark and Beck, 2010, Harro et al., 2011). Regardless of the neural circuits ultimately responsible for the manifestation of symptoms, current drug therapies take a systems-level approach to increase levels of certain neurotransmitters throughout the brain; primarily serotonin and norepinephrine (Bauer et al., 2007, Bandelow et al., 2008, Dell'Osso et al., 2010, Racagni and Popoli, 2010). Additionally, GABAergic neurotransmission has been targeted by drugs such as benzodiazepines used primarily for the treatment of anxiety disorders (Kalueff and Nutt, 2007, Ravindran and Stein, 2010) and more recent drug discovery efforts have been aimed at developing compounds that target the glutamate system for treatment of depression (Cryan and O'Leary, 2010, Autry et al., 2011, Duman et al., 2012).

Although pharmacologic intervention is the first line of treatment for patients suffering with mood and stress-related disorders, these therapies prove largely unsatisfactory in a significant portion of the affected population. According to the large, multi-center Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study that was completed a few years ago, only 33% of depressed patients ($n > 2800$) went into remission following 14 weeks of treatment with the selective serotonin reuptake inhibitor (SSRI) citalopram; a widely prescribed antidepressant drug. Furthermore, for those that did achieve successful remission initially, there were high rates of relapse which increased dramatically for patients that required several different drug treatment strategies

to reach remission. Unfortunately, approximately 1/3 of patients received no benefit from any medication prescribed; revealing a substantial population of treatment-resistant individuals with unmet medical needs (Rush et al., 2006, Trivedi et al., 2006, Pigott et al., 2010, Sinyor et al., 2010). Similar numbers have been reported for the number of patients suffering with anxiety disorders that do not respond sufficiently to first-line treatment; approximately 25% of the patient population (Bandelow et al., 2008).

Alternative treatment options are limited for those that fail to respond to current pharmacologic regimens and are often more time-consuming and can be (semi-) invasive; however, psychotherapy, electroconvulsive therapy, transcranial magnetic stimulation and more recently, deep brain stimulation are options for medication-resistant cases (Bauer et al., 2007, Bandelow et al., 2008, Mathew, 2008, Hollon, 2011, Anderson et al., 2012, Lee et al., 2012).

Undoubtedly, there is much room in the field for the discovery and verification of new therapeutic interventions for depression and anxiety; either as stand-alone options or to augment those treatments currently available. Recently, modulation of the dopamine neurotransmitter system has been highlighted as a potential target in the treatment of depression. In particular, a potential interaction between dopamine $D_1$ and $D_2$ receptors has been a point of focus as it has been demonstrated that increased co-immunoprecipitation of these receptors can be achieved in post-mortem tissue of individuals who suffered from depression (Pei et al., 2010). Furthermore, dopamine has been previously implicated in depression as it is known to be potent modulator of mood

and several studies (both from animal models of depression and human studies) have implicated dopamine deficiency in the pathogenesis of the disorder (Swerdlow and Koob, 1987, Kapur and Mann, 1992, Dunlop and Nemeroff, 2007). For example, in a learned-helplessness model of depression in rats, reduced dopamine content was observed in the striatum and nucleus accumbens concurrent with the expression of the "depressed" phenotype. These effects were prevented by prior administration of a $D_1$ receptor agonist but exacerbated by pre-treatment with $D_1$ receptor antagonist (Anisman et al., 1979, Tombaugh et al., 1980, Sherman et al., 1982). Additionally, the use of dopaminergic agonists, dopamine re-uptake inhibitors, dopamine precursors and antidepressant drugs has shown some efficacy in other animal models of depression (Muscat et al., 1990, Kapur and Mann, 1992).

In humans, a decreased level of the dopamine metabolite homovanillic acid in the cerebrospinal fluid of depressed patients has been a consistent finding, indicating reduced dopamine turnover in this population. Additionally, antidepressant treatments and ECT have shown to enhance dopamine function in some patients (Linnoila M, 1983, Kapur and Mann, 1992, Dunlop and Nemeroff, 2007). Furthermore, some symptoms observed in depression, such as lack of motivation and anhedonia may originate in brain regions directly modulated by dopaminergic input. In fact, both depression and anxiety are often comorbid with Parkinson's disease; a disorder characterized by degeneration of dopaminergic projections into the striatum (Kapur and Mann, 1992, Aarsland et al., 2012, Anderson et al., 2012).

Calcium, and molecules sensitive to calcium signaling such as CaMKII$\alpha$, are central to neuronal excitability and synaptic plasticity; physiological processes that are likely altered in human depression (Colbran and Brown, 2004, Castren and Rantamaki, 2010, Drago et al., 2011). SKF83959, therefore, with its reported specify to activate dopamine receptors linked to calcium regulation, could have unique therapeutic potential for modulating calcium selectively in dopaminergic pathways. In fact, SKF83959 has been previously shown to have some therapeutic efficacy in improving motor symptoms in animal models of Parkinson's disease (Arnt et al., 1992, Downes and Waddington, 1993, Gnanalingham et al., 1995a, Gnanalingham et al., 1995c, Zhang et al., 2007, Fujita et al., 2010). Additionally, as putative $D_1/D_2$ receptor heteromers have also been implicated in depression (Pei et al., 2010), SKF83959 may be a ligand that could target signaling specifically in that context. Here, we characterized the behavioral responsiveness to SKF83959 in animal models of anxiety and behavioral despair to assess the potential efficacy of this compound for treatment of anxiety and depression.

## Materials and Methods:

### *Animals*

C57Bl/6J mice (Jackson) were utilized for the majority of experiments. $D_2$ receptor knockout mice (on a C57Bl/6J background) from colonies maintained at Vanderbilt University were additionally assessed in the tail suspension test.

Breeding strategies and standard procedures for genotype assignment have previously been described (Frederick et al., 2012).

Male mice were housed under standard housing conditions on a 12 h light/dark cycle with conditions previously described (Frederick et al., 2012). All behavioral testing was conducted on mice that were at least (P)60 at the time of initial testing. Mice were extensively handled prior to testing and were habituated to the testing rooms for ~30 min prior to beginning of every experiment. All procedures were approved by the Vanderbilt University Animal Care and Use Committee.

***Drugs***

The dopamine $D_1$-like receptor agonist SKF83959 (3-methyl-6-chloro-7,8-dihydroxy-1-[3-methylphenyl]-2,3,4,5-tetrahydro-1H-3-benzazepine; Tocris Biosciences, Minneapolis, MN) was dissolved in 0.9% saline solution at 0.2 mg/cc (1 mg/kg) and injected intraperiotoneally (i.p.) 30 minutes prior to testing on the elevated zero maze, forced swim test or tail suspension test. Desipramine (3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-*N*-methylpropan-1-amine; Sigma, St. Louis, MO) and fluoxetine ((±)-*N*-Methyl-γ-[4-(trifluoromethyl)phenoxy] benzenepropanamine hydrochloride; Sigma, St. Louis, MO) were used at 20 mg/kg for acute dosing. For chronic testing in the novelty-induced food suppression test, mice were injected daily with 0.9% saline, 0.5 mg/kg SKF83959, or 10 mg/kg fluoxetine for 21 days. SKF83959 was prepared fresh every other day, based on studies we conducted testing the stability of the

compound in solution (see Appendix Figure 35), while fluoxetine was prepared fresh every 3 days for chronic administration.

### *Elevated Zero Maze*

Anxiety-related behavior was assessed in the elevated zero maze. The elevated circular platform (40 cm off the ground, 50 cm in diameter) had two enclosed arenas opposite each other (5 cm wide with 15 cm high walls) and two open arenas (5 cm wide). At the start of the test, each mouse was lowered by its tail into the open arena of the maze and allowed to explore the maze for 300 sec. Activity of the mouse was monitored as previously described and the percentage of time spent in each arena was calculated.

### *Forced Swim Test*

Behavioral despair was assessed in the forced swim test using plastic cylinders (50 cm in diameter, 21 cm in height) filled approximately ¾ full with room temperature water. Mice were assessed for time spent immobile as previously described (Frederick et al., 2012) and the observer was blinded to treatment group at the time of assessment.

### *Tail Suspension Test*

The tail suspension test was used as a second measure of behavioral despair. In these experiments, each mouse was suspended by its tail from an aluminum bar (11.5 cm x 2.3 cm) inside the testing apparatus (Med Associates,

33 x 33 x 32cm). The level and the duration of force placed on the bar by each mouse was measured using an automated computer monitoring program (Med Associates) during a 7 min test. Applied force that measured below the lower threshold was considered as immobility and used to calculate the time spent immobile for each mouse. At the conclusion of the testing period, mice were removed from the bars and placed into their home cages. The data shown represents the amount of time spent immobile during the last 5 min of the test.

***Novelty-Induced Food Suppression Test***

The novelty-induced food suppression test is a predictive measure of anxiety that has also been shown to be sensitive to chronic antidepressant administration (Dulawa et al., 2004, Dulawa and Hen, 2005). In this paradigm, the latency to consume a palatable substance (Vanilla Ensure, Abbot Laboratories, Abbot Park, IL) and the amount consumed are assessed in a novel environment after a defined treatment period with the potential anti-depressant/anxiety drugs of interest. For these experiments, mice were handled daily for one week prior to beginning a 21 day regimen of daily injections. Cages of mice were assigned to receive either 0.9% saline (negative control), 0.5 mg/kg SKF83959 (test drug) or 10 mg/kg fluoxetine (positive control). On day 17, mice were singly housed and cage bedding was not changed until the testing was concluded. On days 18-20, mice were trained to drink Ensure from a sipper (replacing the water bottle) for 30 min per day under red lighting. The latency to consume and amount consumed by each mouse were noted during these

training sessions. Animals that failed to train by the end of the third day were subsequently omitted from the study. On days 21-22, the testing days, the mice were assessed in the home cage under red light (a familiar condition) or in a novel cage under bright light (a potentially anxiety-provoking condition). In order to avoid ordering effects, on the first day of testing, half of the mice from each experimental drug condition were tested in the home cages first and the other half were first tested in the novel cages. On the next day, the mice were switched and tested under the second condition. Data analyzed included the latency to the first sip of Ensure (in seconds) as well the amount consumed (in grams).

***Data Analysis and Statistics***

Data were subjected to one- or-two way analysis of variance (ANOVA) using genotype as a between-group factor using GraphPad Prism (GraphPad Software, San Diego, CA). Post-hoc Tukey's multiple comparison tests or Bonferroni comparisons were used to compare groups to each other. Graphs are marked with an asterisk (*) to denote statistical significance ($p < 0.05$). For data with $p < 0.01$ or $p < 0.001$, the graphs are marked with two (**) or three (***) asterisks, respectively.

## Results:

### *Acute SKF83959 does not affect anxiety*

In the elevated zero maze, we observed no significant differences in performance between saline-treated mice and those that received an acute injection of SKF83959 (1 mg/kg; i.p.) thirty minutes prior to testing in the elevated zero maze (Figure 26A and B). Both saline and drug-treated mice spent significantly more time in the closed arenas than the open arenas (Figure 26B; $F(3,23) = 16.5$, $p < 0.001$) with no significant difference between the treatment groups in the percentage of time spend in the open arenas.

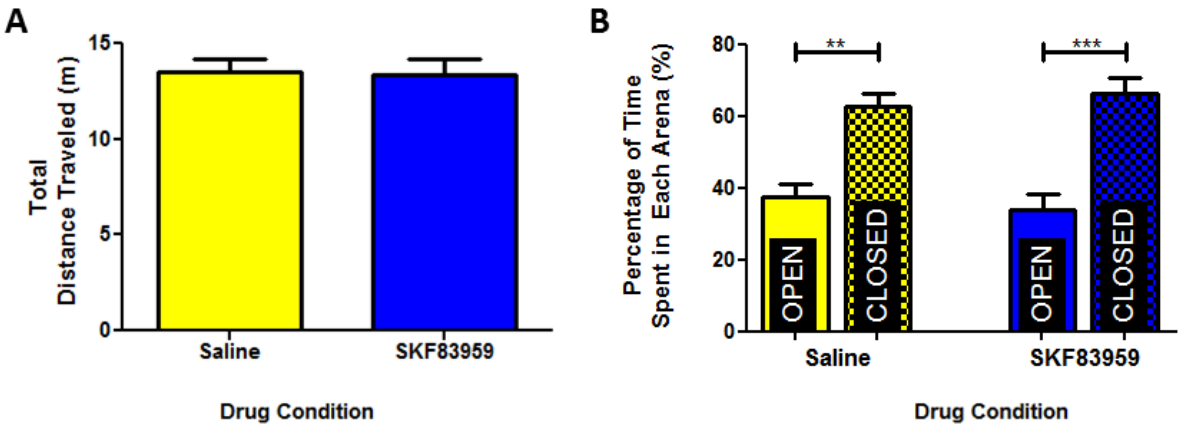


**Figure 26: Elevated zero maze.**

Wildtype mice treated with saline or SKF83959 (1 mg/kg; i.p.) both traveled the same distance in the elevated zero maze (**A**) and spent the same percentage of time in the open portions of the maze (**B**). *n* = 6 for each treatment group in these experiments.

***Acute SKF83959 reduces behavioral despair***

In the forced swim test of behavioral despair, wildtype mice that received the 1 mg/kg dose of SKF83959 tended to spend less time immobile than mice that were treated with saline, desipramine (20 mg/kg; i.p.) or SKF83959 (3 mg/kg; i.p.) (Figure 27). These results, however, were not significant by statistical comparisons ($F(3, 23) = 2.0$, $p = 0.14$).

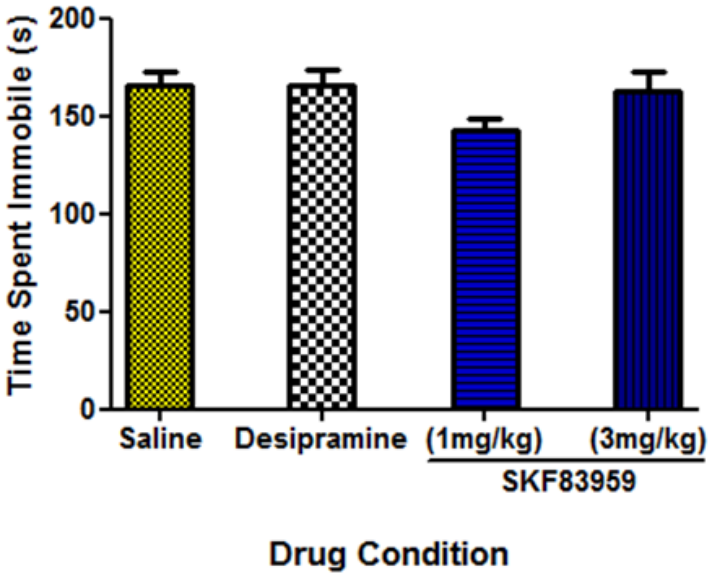


**Figure 27: Forced swim test.**

There were no significant differences between treatment groups in the forced swim assay. There was, however, a trend toward reduced immobility in mice that received 1 mg/kg SKF83959 (i.p.) prior to testing ($F(3, 23) = 2.0$, $p = 0.14$). $n = 6$ for each treatment group.

In the tail suspension test, a second assessment of behavioral despair, SKF83959 (1 mg/kg; i.p.) significantly increased mobility in wildtype mice compared to those that received saline (Figure 28; $p < 0.05$ by Two-Way ANOVA

with Bonferroni *post-hoc* comparison test). Additionally, this response was conserved and more robust in $D_2$ receptor knockout mice where SKF83959 increased mobility by more than 60 sec compared to saline and fluoxetine-treated mice of the same genotype (Figure 28; $p < 0.001$). Furthermore, $D_2$ receptor null mice spent less time immobile compared to wildtype in all three treatment groups; a difference that was most pronounced between the SKF83959-treated wildtype and null groups ($p < 0.05$ for saline and fluoxetine treatment groups and $p < 0.001$ for SKF83959 treatment groups). We did not observe any significant effects of fluoxetine treatment compared to saline in either of the genotypes tested.

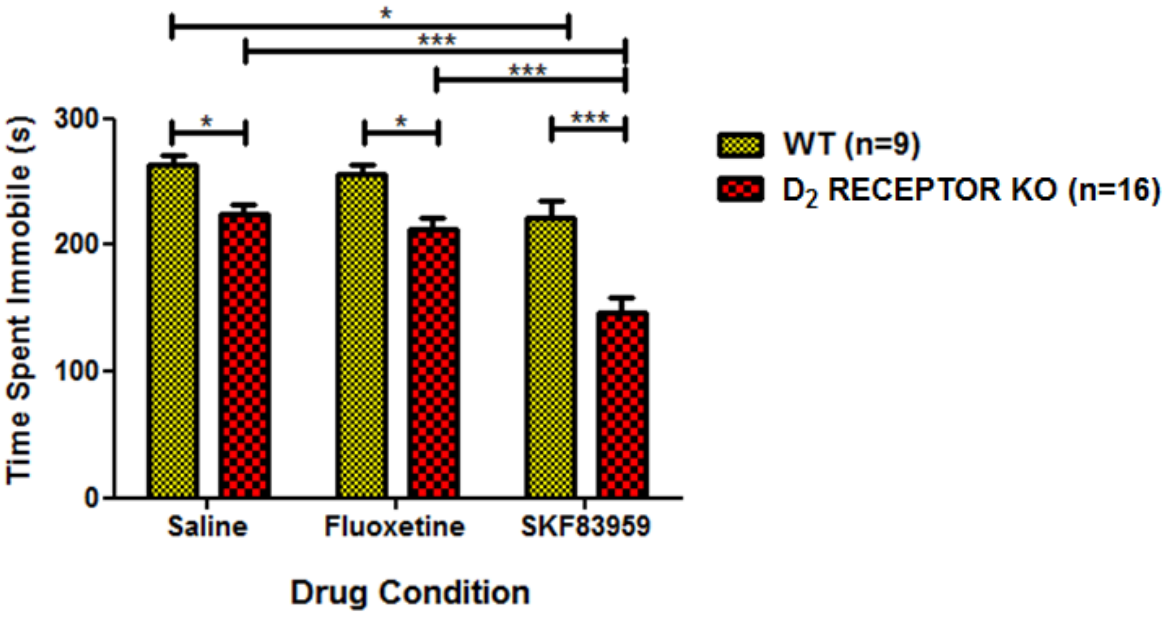


**Figure 28: Tail suspension test.**

SKF83959 significantly reduced time spent immobile in wildtype and $D_2$ receptor knockout mice ($p < 0.05$ for wildtype and $p < 0.001$ for $D_2$ null mice between saline and SKF83959-treatment groups by Two-Way ANOVA with Bonferroni *post-hoc* comparison test).

***Chronic SKF83959 appears to possess anti-anxiety/depressant potential***

In the novelty-induced food suppression paradigm, mice received daily injections of SKF83959 (0.5 mg/kg; i.p.), fluoxetine (10 mg/kg; i.p.) or saline for 21 days before assessment of their latency to consume Ensure and the amount consumed in two environments: the home cage, a familiar environment, and a novel cage with bright light, a potentially anxiety-producing environment. In the home cage, SKF83959-treated mice consumed significantly more Ensure than fluoxetine treated mice (Figure 29A; $F(2, 25) = 4.2$, $p < 0.05$) and fluoxetine-treated mice had a significantly greater latency to consume compared to animals that received saline (Figure 29C; $F(2, 26) = 3.6$, $p < 0.05$). In the novel environment, on the other hand, mice that received chronic injections of SKF83959 consumed significantly more Ensure than mice that received saline or fluoxetine (Figure 29B; $F(2, 26) = 4.8$, $p < 0.05$). Additionally, mice that were treated with fluoxetine or SKF83959 tended to approach the sipper with reduced latencies than the saline-treated controls; although not significant by comparison with One-Way ANOVA (Figure 29D; $F(2, 25) = 1.9$, $p = 0.16$). If the behavior of the animals in the home cage was taken into account in the analysis, then we reveal no significant differences between the treatment groups when assessed for differences in the amount consumed (Figure 29E; $F(2, 24) = 1.4$), however, a statistically significant difference between the two drug-treatment groups compared to saline is revealed in the latency to consume (Figure 29F; $F(2, 25) = 4.6$, $p < 0.05$).

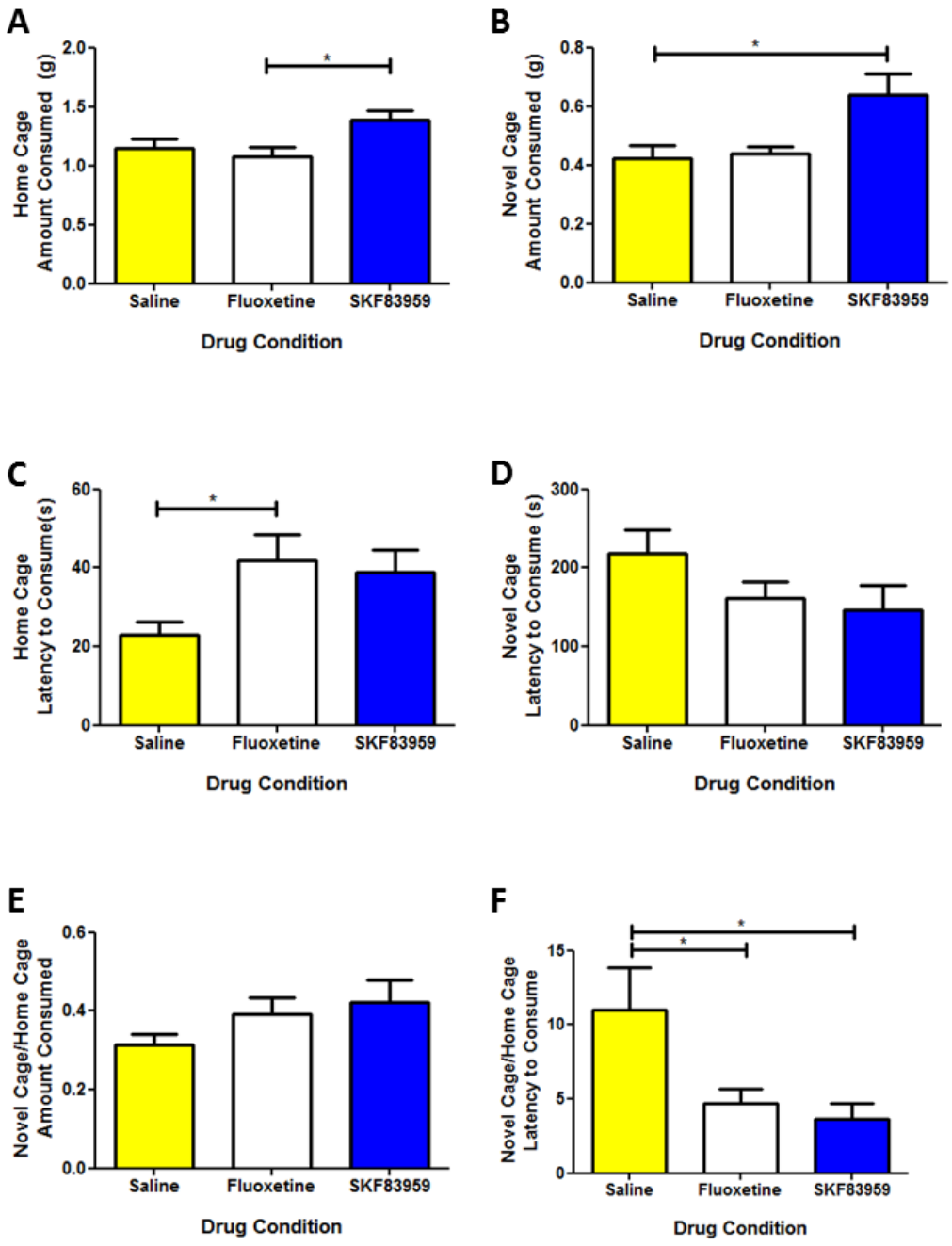


**Figure 29: Novelty-induced food suppression test.**

Chronic injections of SKF83959 (0.5 mg/kg; i.p.) increased food consumption in a novel environment (**B**; $F(2, 26) = 4.8$, $p < 0.05$) and decreased latency to sip (**D** and **F**; $F(2, 25) = 4.6$, $p < 0.05$) in wildtype mice. Chronic fluoxetine (10 mg/kg; i.p.) also decreased latency (**D** and **F**; $F(2, 25) = 4.6$, $p < 0.05$) but no effects were observed with consumption. Behaviors in the home cage are shown in (**A** and **C**) and (**E**) shows the amount consumed as a ratio for behavior in the novel as a ratio of the home cage data. $n = 10$ for each treatment group in these experiments.

**Discussion:**

We assessed the dopamine $D_1$ receptor agonist SKF83959 for behavioral efficacy in rodent models of anxiety and depression. SKF83959 produced no significant effects in an elevated zero maze; indicating lack of SKF83959-induced modulation of anxiety-related phenotypes at the dose tested (1 mg/kg; i.p). As a caveat, however, we did not conduct full dose response curves to determine if other doses of SKF83959 might be effective in this assay.

In the forced swim test, we observed a small reduction in immobility in SKF83959-treated mice, suggesting reduced behavioral despair. This effect, however, was not significant as we did not have enough power in this preliminary experiment. We would therefore need to repeat these experiments with larger group sizes for validation in this paradigm. Our observations were confirmed, however, in a second measure of behavioral despair, the tail suspension test. In this assessment, SKF83959 (1 mg/kg; i.p.) reduced time spent immobile in wildtype and $D_2$ receptor knockout mice; further evidence suggesting that $D_2$ receptor signaling is not involved in mediating SKF83959-induced actions (See Chapter III for reference).

We did not observe any effects from acute injection of desipramine (a tricyclic antidepressant) or fluoxetine (a selective serotonin reuptake inhibitor commonly referred to as Prozac) at 20 mg/kg (i.p.) in the forced swim test or the tail suspension test. These drugs were included in the studies as positive controls as they are both used clinically for the treatment of depression. The majority of studies in the literature using these drugs, or other clinically active

ligands, report antidepressant effects of these compounds in these behavioral paradigms at a wide range of doses (Bourin et al., 1998, Skrebuhhova et al., 1999, David et al., 2003, Cryan et al., 2005, Pollak et al., 2010). Some studies, however, find these drugs to be ineffective following acute administration and report that chronic exposure is necessary to observe behavioral effects (Detke et al., 1997, David et al., 2003, Dulawa et al., 2004, Pollak et al., 2010); results that might be more reflective of the clinical time-course of antidepressant efficacy in humans which may take weeks to produce any benefit. In fact, the forced swim and tail suspension tests have been criticized as behavioral tests of depression due to their acute nature and discordance with the persistent features of the human disorder. They are beneficial, however, as screens for novel compounds that may possess antidepressant efficacy as the majority of clinically therapeutic compounds do show efficacy in these models (Cryan et al., 2005, Petit-Demouliere et al., 2005). Our inability to observe effects with desipramine or fluoxetine after acute treatment could be reflective of a number of factors in the experimental design; most likely the dose administered or the length of time between drug exposure and testing.

Lastly, we evaluated chronic SKF83959 (0.5 mg/kg for 21 days) in the novelty-induced food suppression test, also commonly referred to as novelty-induced hypophagia. We observed a significant reduction in the inhibition of the fluoxetine and SKF83959-treated groups to feed in a novel environment compared to the saline group; a result likely reflective of the antidepressant potential of the compounds. It has been previously demonstrated that

assessment of feeding behavior in a novel environment in this paradigm constitutes a valid measure of the anxiety-related components of depression; one which has been shown to be sensitive to chronic, but not acute antidepressant treatment (Dulawa et al., 2004, Dulawa and Hen, 2005). Additionally, this paradigm is a useful tool to investigate anxiety-behaviors that are often expressed comorbid with depression (Weissman et al., 1996, Kessler et al., 2005, Aina and Susman, 2006).

**Conclusions:**

Taken together, these studies indicate that SKF83959 could have potential therapeutic efficacy as an antidepressant agent and could potentially define a novel class of antidepressants targeting the dopamine system. The results presented here are very preliminary, however, and would need to be replicated before any strong conclusions could be drawn. Additionally, further studies in other animal models of depression and anxiety would be necessary to characterize SKF83959 more fully before moving into trials with clinical populations.

# CHAPTER V

## SUMMARY AND DISCUSSION

As a result of the work defined here within this thesis, we have a) more precisely defined the neurobehavioral phenotype of $G_{\alpha q}$ knockout mice and their responsiveness to locomotor agents, b) used a genetic approach to demonstrate that the current dopamine $D_1/D_2$ receptor heteromer model of signaling does not explain the actions of SKF83959 and c) investigated the potential therapeutic efficacy of SKF83959 as a treatment for depression.

In Chapter II, we reported that we replicated and extended findings showing motor deficits in $G_{\alpha q}$ knockout mice in a number of behavioral tasks: the accelerating rotarod, inverted screen test, Y-maze, elevated zero maze and the open field (Frederick et al., 2012). Additionally, we demonstrated that drug-induced locomotor responses were largely intact in these mutant mice; data indicating that basal ganglia locomotor circuits are largely functional in the absence of $G_{\alpha q}$. Furthermore, we observed normal phenotypes in both the elevated zero maze and the forced swim test, but not in the Y-maze; revealing deficits in spatial working memory in the knockout mice (Frederick et al., 2012).

Working memory deficits in $G_{\alpha q}$ knockout mice are interesting with respect to the extensive literature detailing the importance of catecholamine signaling, in particular the role of dopamine, in mediating prefrontal cortex function and working memory (Vijayraghavan et al., 2007, Arnsten, 2011). Since there is

evidence in the literature suggesting that dopamine $D_1$ receptors in the prefrontal cortex are able to couple to $G_{\alpha q}$ (Wang et al., 1995, Jin et al., 2001, Mannoury la Cour et al., 2007), the altered performance of $G_{\alpha q}$ null mice in the Y-maze could be explained by lack of dopamine signaling in this pathway. We do not have any selective pharmacologic tools at this time, however, to selectively probe the role of dopamine $D_1$ receptors signaling in this pathway (See Chapter III) in working memory functions. Alternatively, we could test the contribution of $G_{\alpha q}$ coupled $D_1$ receptors by selectively knocking out $G_{\alpha q}$ in cells that express dopamine $D_1$ receptors using the Cre-lox system of site-specific recombination (Sauer, 1987, Orban et al., 1992). These experiments would be very feasible to conduct as we currently maintain a transgenic floxed $G_{\alpha q}$ colony in our laboratory (Wettschureck et al., 2005, Wettschureck et al., 2006) and $D_1$ receptor-Cre lines have already been created (Gantois et al., 2007, Lemberger et al., 2007) and are available from mouse repositories. Furthermore, we can address the possible contribution of $G_{\alpha 11}$ signaling in these studies (which may be compensating in some aspects in the absence of $G_{\alpha q)}$ by using a conditional $G_{\alpha q}$ mutant line with a constitutive deletion of *Gna11* to produce progeny that have tissue specific inactivation of $G_{\alpha q}$ signaling in a constitutively deficient $G_{\alpha 11}$ background (Wettschureck et al., 2005, Wettschureck et al., 2006). A caveat here, however, is that other receptors that couple to $G_{\alpha q}$ are co-expressed in cells that express $D_1$ receptors, so we would also be losing signaling in those pathways by deleting $G_{\alpha q}$ in $D_1$ receptor-expressing cells. Although we do not currently know what other $G_{\alpha q}$-coupled receptors or transmitter systems in the cerebral cortex may be contributing to the

observed phenotype, norepinephrine is a likely candidate. Norepinephrine, a precursor of dopamine, is highly synthesized in the cortex and has been shown to play a role in working memory (Levy, 2009, Gibbs et al., 2010, Arnsten, 2011). Furthermore, $\alpha_1$ adrenergic receptors are $G_{\alpha q}$ coupled receptors, so use of a global $G_{\alpha q}$ knockout in our studies would have blocked signaling through this pathway, potentially contributing to the phenotype as well (Bylund, 1992, Civantos Calzada and Aleixandre de Artinano, 2001).

In Chapter III, we reported that dopamine $D_1$ receptors, but not $D_2$ receptors are necessary for mediating the behavioral responses of the reported biased agonist, SKF83959. These data are contradictory to the $D_1/D_2$ receptor heteromer model that is currently described in the literature as the signaling mechanism underlying SKF83959's actions. Furthermore, we reported that SKF83959-induced locomotor and grooming responses were conserved in $G_{\alpha q}$ knockout mice (in Chapters II and III); thereby negating the role of $G_{\alpha q}$ as the G-protein responsible for mediating the signaling. Taken together, these results suggest that SKF83959 is not exclusively activating dopamine ($D_1$ and $D_2$) receptors coupled to a $G_{\alpha q}$ signaling pathway. The most likely explanation is that SKF83959 is not as selective as thought, and may activate $D_1$ receptors coupled to $G_{\alpha s/olf}$ signaling pathways, although we have not tested this hypothesis in $G_{\alpha s/olf}$ knockout mice. To this end, constitutive $G_{\alpha s}$ knockout mice have been previously generated. These mice are not viable and die *in utero*, however, due to the essential role of $G_{\alpha s}$ in peripheral signaling as well as within the CNS. We could however, evaluate SKF83959-induced behaviors in mice with tissue-specific

inactivation of $G_{\alpha s}$ signaling using the Cre-lox system as mice in which the first exon (E1) of *Gnas*, the gene that encodes $G_{\alpha s}$, has been floxed have been previously generated (Sakamoto et al., 2005a, Sakamoto et al., 2005b). Cre recombination at *Gnas* E1 has been shown to be superior to recombination at other sites (such as at exon 2 which is common to all known transcripts of *Gnas)* since *Gnas* encodes alternative protein products that are regulated by genomic imprinting, leading to monoalleleic, parental origin-dependent expression of various transcripts (Yu et al., 2001, Weinstein et al., 2004, Sakamoto et al., 2005a, Sakamoto et al., 2005b). Recombination at exon 1, therefore, has been shown to disrupt expression of $G_{\alpha s}$ without affecting the expression of other *Gnas* products including NESP55, which is expressed exclusively from the paternal allele and $XL_{\alpha s}$, which is expressed exclusively from the maternal allele (Sakamoto et al., 2005a, Sakamoto et al., 2005b). Initial analyses would need to be conducted to assess the presence of any behavioral phenotypes in these mice which could make data difficult to interpret. Similarly, we could also address the contribution of G-proteins linked to cAMP in SKF83959-induced actions by assessing SKF83959-induced behaviors in $G_{\alpha olf}$ knockout mice (Belluscio et al., 1998, Zhuang et al., 2000). As previously mentioned, $G_{\alpha olf}$ is largely expressed in the striatum (instead of $G_{\alpha s}$) (Zhuang et al., 2000, Corvol et al., 2001, Herve et al., 2001) so use of these mice would be preferred if we believed that SKF83959-induced locomotor and grooming behaviors were mediated by this region. Furthermore, use of these knockout mice could tease apart differences in the circuitry underlying these behaviors; if, for example, the

grooming response was mediated by the dorsal striatum and therefore absent in these mice, but the locomotor response was intact, that would indicate that the locomotor phenotype was mediated by a different brain region where $G_{\alpha olf}$ is not highly expressed or that $G_{\alpha olf}$ is not involved in the signal transduction pathway for the locomotor response. If SKF83959 does signal through $D_1$ receptors coupled to $G_{\alpha s/olf}$, then that would explain the locomotor phenotype that we observed after acute administration of SKF83959 (1 mg/kg; i.p.) as it is consistent with the behavioral profile of other $D_1$ receptor ligands that activate adenylyl cyclase (Arnt et al., 1992, Waddington et al., 1995).

In fact, the critical role of adenylyl cyclase activity in motor functions has been demonstrated in adenylyl cyclase 5 (AC5) knockout mice. AC5 is the major isoform found in the striatum and mice deficient in this enzyme are greatly impaired on a rotarod test (Iwamoto et al., 2003). Additionally, these mice exhibit reduced spontaneous locomotor activity and the locomotor responses to $D_1$ and $D_2$ dopamine receptor agonists are markedly diminished, unless used at very high doses (Lee et al., 2002, Iwamoto et al., 2003). Furthermore, AC5 knockout mice increase their locomotor activity in response to administration of haloperidol (0.03 mg/kg; i.p.) or clozapine (0.6 mg/kg; i.p.), an effect not observed in wildtype mice. These effects, however, are blocked by the $D_1$ receptor antagonist SCH23390; thereby indicating involvement of $D_1$ receptor signaling. The residual signaling is likely mediated by the remaining isoforms of AC present in the striatum as only 85-90% of $D_1$ receptor-stimulated AC activity and ~80% of

forskolin-induced activity was eliminated in the knockout mice (Lee et al., 2002, Iwamoto et al., 2003).

Although the importance of cyclase activity in locomotor function has been established in the literature, initial studies assessing the cyclase-stimulating activity of SKF83959 reported little to no activation *in vitro* and *in vivo* (Arnt et al., 1992, Andringa et al., 1999, Jin et al., 2003). One possibility is that the assays used to conduct these experiments were not sensitive enough to detect any activation of adenylyl cyclase by SKF83959, which could have been particularly low, and these experiments should therefore be repeated with current technologies and methods. Alternatively, it is conceivable that even if SKF83959 does activate $D_1$-$G_{\alpha s/olf}$ coupled receptors, there could be alternative cyclase-independent pathways, mediated by $G_{\beta\gamma}$ subunits or G-protein independent signaling mechanisms, which feed into SKF83959-induced PI hydrolysis (Undie and Friedman, 1990) or $IP_3$-dependent calcium mobilization (Mahan et al., 1990, Tang and Bezprozvanny, 2004, Ming et al., 2006, Liu et al., 2009a) that would support the initial biochemical characterization of this drug as a potent modulator of PI signaling. In fact, GPCR activation of PLC has been shown to be activated by both pertussis toxin-insensitive ($G_{\alpha q}$) and pertussis toxin-sensitive ($G_{\beta\gamma}$) signaling pathways. Furthermore, it has been demonstrated that the composition of the $G_{\beta\gamma}$ subunits specifically mediates the level of activation $G_{\beta\gamma}$ signaling has on specific PLC isoforms. (Boyer et al., 1994, Runnels and Scarlata, 1999). Therefore, it may be that $G_{\beta\gamma}$ subunits (that dissociated from $G_{\alpha s}$ after $D_1$ receptor activation), mediate PI hydrolysis, $IP_3$ accumulation and calcium signaling. Of

note, the aforementioned dopamine and $D_1$ receptor agonist-induced effects observed on PI hydrolysis and intracellular calcium release were blocked by SCH23390 but not by $D_2$ receptor ligands (sulpiride or domperidone)(Mahan et al., 1990, Undie and Friedman, 1990), indicating no involvement of $D_2$ receptors in the signaling. In order to address whether SKF83959 activates PI signaling at the level of PLC (independent of $G_{\alpha q}$), SKF83959-induced locomotor and grooming behaviors could be assessed in PLC$\beta$ knockout mice. There are four isoforms of PLC$\beta$, 1-4, and knockout mice have been generated for each of the isoforms revealing isoform specific functions including developmental influences of PLC$\beta$1 signaling in patterning of the cerebral cortex (Jiang et al., 1996, Jiang et al., 1997, Kim et al., 1997, Kano et al., 1998, Hannan et al., 2001). Furthermore, PLC$\beta$4 knockout mice have been reported to demonstrate an ataxic phenotype due to the role of this isoform in the development of the cerebellum, as previously also described for $G_{\alpha q}$ knockout mice (Offermanns et al., 1997a, Kano et al., 1998).

Our studies indicate that SKF83959 does not signal through $D_1/D_2$ receptor heteromers coupled to $G_{\alpha q}$ to mediate its behavioral effects, although that does not mean that $D_1/D_2$ receptor heteromers are not a physiological entity nor does it address whether dopamine receptors are able to couple to $G_{\alpha q}$ *in vivo*. Wang *et. al.* demonstrated dopamine and SKF38393-induced coupling to both $G_{\alpha s}$ and $G_{\alpha q}$ in rat striatal membranes; an effect that was blocked by the $D_1$ receptor antagonist SCH23390 (10$\mu$M) but not by the $D_2$ receptor antagonist, sulpiride (10 $\mu$M) (Wang et al., 1995). Interactions between the $D_1$ receptor and

$G_{\alpha q}$ were additionally demonstrated in other brain regions by other groups using methods of co-immunoprecipitation, [$^{35}$S]GTPγS and [$\alpha^{32}$P]GTP-binding to select G-proteins (Jin et al., 2001, Mannoury la Cour et al., 2007). These interactions were also blocked by SCH23390 (Jin et al., 2001, Mannoury la Cour et al., 2007) but not sulpiride (Jin et al., 2001), additional evidence against the involvement of $D_1/D_2$ heteromers in $D_1$ receptor-$G_{\alpha q}$ coupling. Some studies have shown that SKF83959 can induce $D_1$ receptor coupling to $G_{\alpha q}$ (Jin et al., 2003, Mannoury la Cour et al., 2007) and $IP_3$ accumulation (Jin et al., 2003) using i.p. administration of 0.8 mg/kg SKF83959. These results are more difficult to resolve in light of our data showing that SKF83959-induced behaviors are conserved in $G_{\alpha q}$ knockout mice. One possible explanation, however, could be that SKF83959, like other $D_1$ receptor agonists, has the ability to stimulate coupling to multiple G-protein families to initiate multiple signaling cascades. Although $D_1$ receptors may retain the ability to signal through $G_{\alpha q}$ *in vivo*, it may be that signaling in this pathway does not influence behavioral output and may be involved in other cellular processes yet to be determined. Recent unpublished data from the laboratory of Dr. Jonathan Javitch (Columbia University), however, convincingly argues against the ability of dopamine $D_1$ receptors to effectively couple to $G_{\alpha q}$ *in vitro* when stimulated with dopamine or SKF83959 over a wide range of concentrations (up to log [$10^{-4}$] M). Dr. Javitch's laboratory has created a novel *in vitro* bioluminescence resonance energy transfer (BRET) assay in which a signal is detected from the conformational change of activated G-proteins (Urizar et al., 2011). Using this assay, no BRET signal was detected for $G_{\alpha q}$ (indicating

activated $G_{\alpha q}$ proteins) after addition of dopamine or SKF83959 when cells were transfected with $D_1$ receptors, $D_5$ receptors, $D_2$ receptors (of either the long or short isoform) or any combination of these receptors. Conformational changes of the cognate G-proteins, however, were verified for all receptors ($G_{\alpha s}$ for $D_1$ and $D_5$ receptors and $G_{\alpha i}$ for $D_2$ receptor isoforms) and shown to be blocked by specific receptor antagonists. Furthermore, $G_{\alpha q}$ activation was demonstrated to be detectable within the system by transfecting cells with $M_1$ muscarinic receptors (which are $G_{\alpha q}$-coupled receptors) and activating the receptors with acetylcholine. Moreover, unpublished data from our laboratory visualizing the co-localization of $D_1$ and $D_2$ receptors in images of brain sections from Drd1a-tdTomato Drd2-EGFP BAC double transgenic mice reveals that $D_1$ and $D_2$ receptor co-localization *in vivo* may be much less in some brain regions, including the nucleus accumbens and frontal cortex, than had been estimated in previous reports from other laboratories (Shuen et al., 2008, Matamales et al., 2009, Valjent et al., 2009, Perreault et al., 2010, Zhang et al., 2010).

The idea that dopamine-mediated PI signaling was not mediated by the classical dopamine $D_1$ receptor stemmed largely from studies demonstrating that a) dopamine and SKF38393-induced $IP_3$ accumulation and [$^3$H]SCH23390 binding sites co-immunoprecipitated with $G_{\alpha q}$ were retained in the striatum of $D_1$ receptor knockout mice (Friedman et al., 1997) and b) SKF83959-induced PI hydrolysis was not induced in PC12 cells expressing dopamine $D_1$ receptors (Jin et al., 2003). We know, however, that $D_5$ receptors have also been reported to couple to $G_{\alpha q}$ and to induce PI signaling so this could explain why $IP_3$

accumulation was still present in $D_1$ receptor knockout mice (Sahu et al., 2009, So et al., 2009, Hasbi et al., 2010). Additionally, we do not know if there are off-target effects of SKF83959 at the concentrations used in these experiments as it has been demonstrated that SKF83959 does have moderate affinity for adrenergic and serotonergic receptors (Andringa et al., 1999, Neumeyer et al., 2003) through which signaling could become more prominent in the absence of higher affinity $D_1$ receptors to bind. Furthermore, it is also possible that there are orphan $G_{\alpha q}$-coupled GPCRs with characteristics similar to $D_1$-like receptors that could potentially be activated by SKF83959.

With respect to the studies assessing $IP_3$ accumulation in PC12 cells transfected with $D_1$ receptors, one possibility is that the machinery necessary for PI metabolism is not entirely expressed in this particular cell line. In these experiments, the authors assessed 0, 10μM, 50μM and 250μM SKF83959 for effects on $IP_3$ accumulation after a 30 minute period. In addition to testing SKF83959, they also tested SKF38393, the prototypic dopamine $D_1$ receptor agonist that has been implicated in mediating both stimulation of adenylyl cyclase activity and PI hydrolysis (Undie and Friedman, 1990, Undie et al., 1994). The authors observed no effect on $IP_3$ using either ligand, thus indicating a failed experiment if they expected positive results (i.e. increased $IP_3$ content) from SKF38393. The authors did not include any experiments where they transfected a known $G_{\alpha q}$-coupled receptor into PC12 cells (a muscarinic receptor for example) and demonstrated that they could observe dose-dependent $IP_3$ accumulation after adding a ligand. Furthermore, previous studies demonstrating

dopamine-mediated PI signaling in transfected cells did not use $IP_3$ as the functional readout; rather they assessed PLC activation (Yu et al., 1996) or intracellular calcium release (Liu et al., 1992, Frail et al., 1993, Lin et al., 1995). Additionally, these studies evaluating $D_1$ receptor stimulation in transfected cells did not use PC12 cells, rather they used HEK 293 cells (Lin et al., 1995, Yu et al., 1996) or Ltk-fibroblast cells (Liu et al., 1992); indicating that there may be differential expression of necessary proteins in these cells lines for specific cell-signaling pathways. Furthermore in both the study by Liu *et. al.* and the one conducted by Lin *et. al*., it was demonstrated that the calcium signal was mediated by a G-protein that was sensitive to cholera toxin (potentially $G_{\alpha s}$) and that the signal was potentiated by the activity of cAMP (Liu et al., 1992, Lin et al., 1995) and PKA (Liu et al., 1992). Additionally, Lin *et. al*. demonstrated that the cAMP-dependent calcium pool was also sensitive to $IP_3$, further indicating a link between G-protein signaling (independent of $G_{\alpha q}$) and $IP_3$/calcium release (Lin et al., 1995).

The ability of other $G_{\alpha q}$-coupled receptors to “prime” dopamine $D_1$-like receptor modulation of calcium currents has also been described (Dai et al., 2008). In these studies, forskolin-induced stimulation of cAMP was not sufficient to induce calcium release on its own nor was $D_1$-like receptor stimulation in the absence of priming. Dopamine $D_1$-like receptor stimulation, however, potentiated the calcium response to mGluR stimulation through a mechanism that was dependent upon cAMP and PKA (Dai et al., 2008). Although mechanisms such as these may exist to explain how dopamine $D_1$ receptor signaling could

potentially modulate PI metabolism at points downstream of $G_{\alpha q}$, it is difficult to interpret calcium signal as an end-point measurement as many systems converge to modulate its homeostasis within the cell. Calcium regulation is subject to regulation by a number of inputs and because cross-talk between pathways is very common, calcium release in not necessarily directly translatable to receptor activation at the cell membrane. As has been discussed here, there are a number of ways by which one can get increased calcium without activating $G_{\alpha q}$ coupled receptors. Furthermore, to this end, SKF83959 signaling has been implicated in regulating calcium-dependent molecules such as CaMKII$\alpha$ (Zhen et al., 2004, Rashid et al., 2007, Ng et al., 2010). We therefore assessed the role of CaMKII$\alpha$ phosphorylation at residue $Thr^{286}$ in mediating SKF83959-induced responses using phosphorylation-deficient CaMKII$\alpha$-$Thr^{286}$Ala knockin mice. In these mutant mice, we observed intact SKF83959-induced locomotor and grooming responses, thereby negating the necessity of CaMKII$\alpha$ phosphorylation at this residue in the signaling mechanism of SKF83959 as previously reported. Additionally, we were not able to reproducibly demonstrate SKF83959-induced phosphorylation of CaMKII$\alpha$-$Thr^{286}$ in a number of brain regions including the striatum (as previously reported)(Ng et al., 2010), medial frontal cortex or nucleus accumbens (data not shown); thereby further supporting our behavioral findings. These data indicate that although previous reports detected increased activity (Zhen et al., 2004) or phosphorylation of CaMKII$\alpha$ following administration of SKF83959 (Ng et al., 2010), the change in CaMKII$\alpha$ was not necessarily a direct

result of SKF83959 signaling. Additionally, phosphorylation of CaMKII$\alpha$ at $Thr^{286}$ does not appear necessary to produce the behavioral responses of the ligand.

The involvement of $D_2$ receptors in the signaling of SKF83959 was first suggested by Rashid *et. al.* when they demonstrated that $D_1$ receptor agonist-induced calcium currents were potentiated by the $D_2$ receptor agonist, quinpirole, and blocked by the $D_2$ receptor antagonist raclopride. Additionally, they used radioligand binding studies to demonstrate that SKF83959 binds with high affinity to pertussis toxin-resistant $D_2$ receptors only (non- $G_{\alpha i/o}$ coupled) in the presence of $D_1$ receptors, indicating the presence of a $D_1/D_2$ receptor heteromer (Rashid et al., 2007). The data in this paper demonstrated the ability of $D_1$ receptors to couple to $G_{\alpha q}$; however the idea that $D_2$ receptors and SKF83959 are specifically involved was pieced together from many experiments using a variety of pharmacologic manipulations. Clearly, even if the $D_1/D_2$ receptor heteromer model can be supported *in vitro* as was demonstrated by Rashid *et. al*., it is not supported by systematic *in vivo* analyses using specific knockout mice (Chapter III). Moreover, the pitfalls of using calcium signal as a readout have already been discussed.

Additionally, we assessed the involvement of the dopamine $D_5$ and $D_3$ receptor subtypes and other non-dopaminergic receptors in the signaling of SKF83959. Regarding the role of the $D_5$ receptor, its localization on cholinergic cells allows for direct dopaminergic modulation of acetylcholine levels in the striatum. Furthermore, $D_5$ receptor activation of striatal cholinergic interneurons has been shown to enhance $Zn^{2+}$ sensitive $GABA_A$ currents thereby modulating

GABA neurotransmission in the striatum (Yan and Surmeier, 1997). One potential mechanism by which SKF83959-induced actions could have been blunted in $D_5$ receptor knockout mice, therefore, could be through a reduction in $GABA_A$ currents facilitated through $D_5$ receptors on cholinergic cells altering maximal striatal output. Additionally, cholinergic interneurons have been shown to modulate striatal signaling and synaptic plasticity via activation of muscarinic receptors on MSNs and modulation of long-term potentiation at striatal synapses (Howe and Surmeier, 1995, Calabresi et al., 1999, Alcantara et al., 2001); mechanisms which could have also altered the locomotor output in the $D_5$ receptor knockout mice. Furthermore, the BRET data from the Javitch laboratory suggests that SKF83959 activation of $D_5$ receptors does not activate $G_{\alpha q}$ suggesting that this receptor is mediating signaling initiated by SKF83959 through an alternative pathway, perhaps through $G_{\alpha s}$. The BRET *in vitro* system could be used to test this hypothesis by transfecting cells with $D_5$ receptors and testing for activation of $G_{\alpha s}$ (resulting in a BRET signal) when activated by SKF83959.

In our assessments evaluating the involvement of $D_3$ receptors in mediating SKF83959-induced actions, our results proved inconclusive. We were not able to successfully inhibit locomotor activity with pharmacological blockade of $D_3$ receptors with a selective antagonist PG 01037 in wildtype or $D_2$ receptor knockout mice. Furthermore, we observed a potentiated phenotype in the $D_2$ receptor knockouts when they were administered PG 01037 or raclopride, hinting at altered $D_3$ receptor expression in these mice which confounds the results. In

order to further explore the potential role of the $D_3$ receptor subtype in SKF83959 signaling using a behavioral approach, we would need to investigate the SKF83959-induced behaviors in $D_3$ receptor knockouts and $D_2/D_3$ receptor double knockout mice. Currently, we do not maintain a $D_3$ receptor mutant colony in the laboratory that would be necessary for addressing this question. Here, we could again utilize the BRET *in vitro* system by transfecting cells with $D_3$ receptors (alone or in combination with $D_1$ receptors) and testing for activation of specific G-proteins by SKF83959.

With respect to the contribution of non-dopaminergic signaling systems, we observed an attenuation of SKF83959-induced behavior by both the mGluR5 receptor antagonist MTEP (10 mg/kg; i.p.) and by the $\alpha_2$ adrenergic receptor antagonist atipamezole (0.25 mg/kg; i.p) suggesting that signaling through non-dopaminergic receptors may contribute to producing a maximal SKF83959-mediated response. These receptors, however, are not required for SKF83959-induced locomotor activity and further support the idea that SKF83959 mainly exerts its actions by activating dopamine $D_1$ receptors.

When taken together, these data suggest that SKF83959 does not activate $D_1/D_2$ receptors heteromers coupled to $G_{\alpha q}$ as has been previously claimed in the literature and alternative signaling mechanisms need to be explored (see Figure 30 for summary). Furthermore, as a number of studies in the literature have utilized this ligand based on its reported specificity, we therefore need to re-evaluate the literature with respect to these findings.

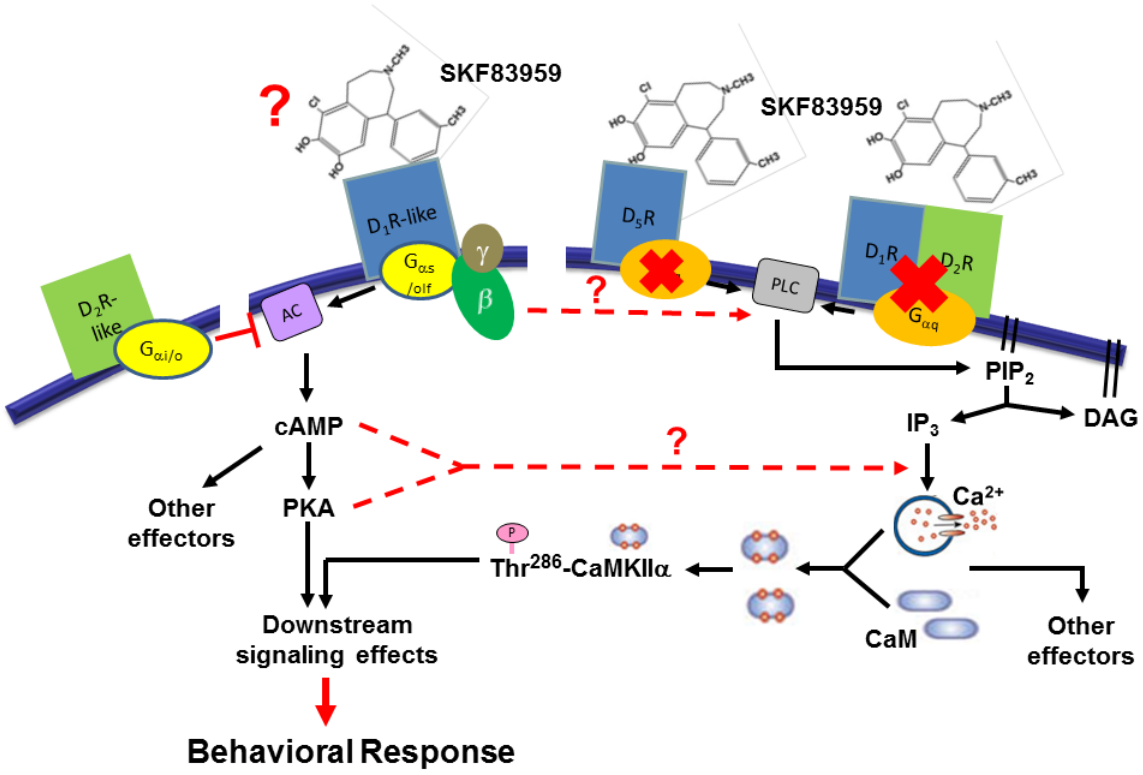


**Figure 30**

Schematic depicting some proposed mechanisms of signaling for SKF83959. On the right we have the mechanism of the $D_1/D_2$ receptor heteromer coupled to $G_{\alpha q}$, which is the current model in the literature. The red "X" denotes that we have tested the hypothesis and demonstrated that it is insufficient to explain the actions of SKF83959. On the left side, we have depicted other potential signaling mechanism for SKF83959 and potential ways in which SKF83959 could produce PI hydrolysis, $IP_3$ accumulation or intracellular calcium release without signaling through $G_{\alpha q}$.

In Chapter IV, we report our very preliminary findings assessing the therapeutic efficacy of SKF83959 in animal models of anxiety and depression. Although we did not observe any significant effects on anxiety behavior in the elevated zero maze, we did observe acute effects of SKF83959 in two tests of behavioral despair: the forced swim test and the tail suspension test. In the tail suspension test, we assessed both wildtype and $D_2$ receptor knockout mice for SKF83959-induced effects and observed interesting effects. Not only did SKF83959 reduce time spent immobile in both genotypes, but it actually had a larger response in the $D_2$ receptor mutant mice; further confirming that $D_2$ receptors do not mediate SKF83959 signaling.

The results observed from acute SKF83959 administration in the tests of behavioral despair were intriguing to us; however the forced swim and tail suspension tests have been criticized as behavioral tests of depression due to their acute nature and discordance with the persistent features of the human disorder. We therefore decided to test for antidepressant efficacy in the novelty-induced food suppression paradigm; a paradigm that has been validated to measure the anxiety-related components of depression. In particular, this paradigm is sensitive to chronic, but not acute antidepressant treatment (Dulawa et al., 2004, Dulawa and Hen, 2005). Assessment of SKF83959 in the novelty-induced food suppression paradigm revealed promising results; reducing latency to feed and food consumption in a novel environment. Although very preliminary, these studies indicate that SKF83959 could have potential therapeutic efficacy as

an antidepressant agent and could potentially define a novel class of antidepressants targeting the dopamine system.

**Summary:**

We have tested the hypothesis that SKF83959 exerts its actions by activating dopamine $D_1/D_2$ receptor heteromeric complexes coupled to $G_{\alpha q}$ and downstream CaMKII$\alpha$-Thr$^{286}$ phosphorylation using pharmacologic approaches and genetic models. Our results show that SKF83959 requires functional dopamine $D_1$ receptors to elicit drug-induced locomotor and grooming responses (and $D_5$ receptors are involved to some degree), but $D_2$ receptor signaling, $G_{\alpha q}$ expression and CaMKII$\alpha$-Thr$^{286}$ phosphorylation are not necessary to produce these behaviors. These data go against the current model in the literature and brings up many more questions surrounding the mechanism of this ligand that are yet to be addressed. Furthermore, although we do not completely understand the mechanism of SKF83959, our data suggests that the ligand may be promising as a therapeutic agent for depression and that lines of inquiry around this ligand should still be pursued in the field.

# CHAPTER VI

# APPENDIX

In Chapter III we reported data assessing SKF83959-induced behaviors in various mutant lines. Each of the studies was conducted independent of the others and multiple experiments were conducted in each mutant line examined. We therefore were interested in making sure that we were activating SK83959-induced locomotor activity to reproducible levels across experiments. In order to address this question, we assessed the SKF83959-induced locomotor response from every wildtype mouse in each mutant line tested and examined the wildtype response across experiments. Figure 31A shows the average baseline locomotor response of all wildtype mice (from all mutant lines) across experiments and the vehicle and SKF83959-induced locomotor responses. Figure 31B shows this data as a percentage change from baseline and reveals an approximate 2-3 fold increase in locomotor activity in the SKF83959-treated mice compared to those that received saline. These results are consistent with those observed in the C57/Bl6J mice that received the 1 mg/kg dose in the dose-response experiments (Chapter III, Figure 16) and demonstrates that SKF83959-induced locomotor activation is highly reproducible.

Additionally, we used an extended 3-day protocol in our experiments requiring two days of habituation to the open field chambers with saline injections and injection of SKF83959 on the third day. In some of the mutant lines,

however, we observed varied responses in basal locomotor activity between the different genotypes on the third day. We therefore were interested in knowing whether the basal level of activity (or level of habitation) would vary the SKF83959-induced locomotor response. In order to address this question, we injected C57/Bl6J mice with vehicle or SKF83959 (1 mg/kg; i.p.) on what would be Day 1 or our usual protocol. These mice, therefore, were not extensively habituated to the open field and only had the 30 minutes prior to the injection to be exposed to the chambers. We observed in these experiments that, although the baseline activity was a little higher than what we previously observed, SKF83959 still increased locomotor activity by 2-3 fold (Figure 32A – C). These results indicate that SKF83959 increases locomotor activity in the open field regardless of the basal level of activity.

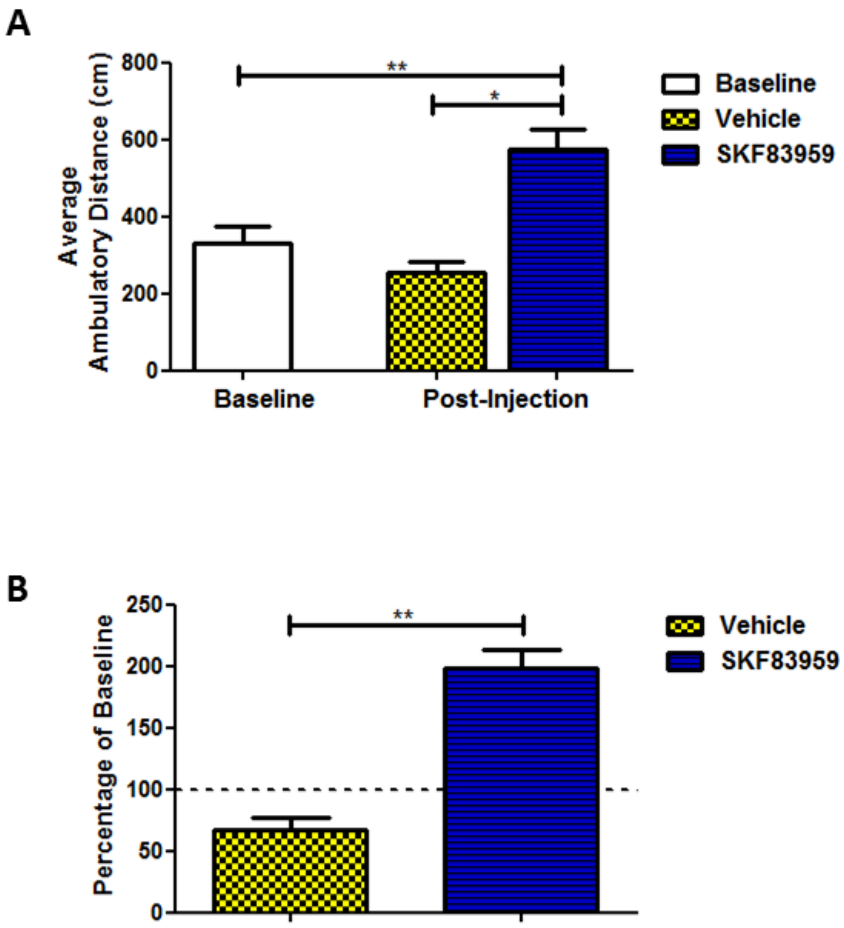


**Figure 31**

SKF83959 (1 mg/kg; i.p) significantly and reproducibly increases locomotor activity in wildtype mice from all mutant lines tested (**A and B**). The SKF83959-induced locomotor response is consistently found to be a 2-3 fold increase in locomotor activity compared to vehicle controls (**B**).

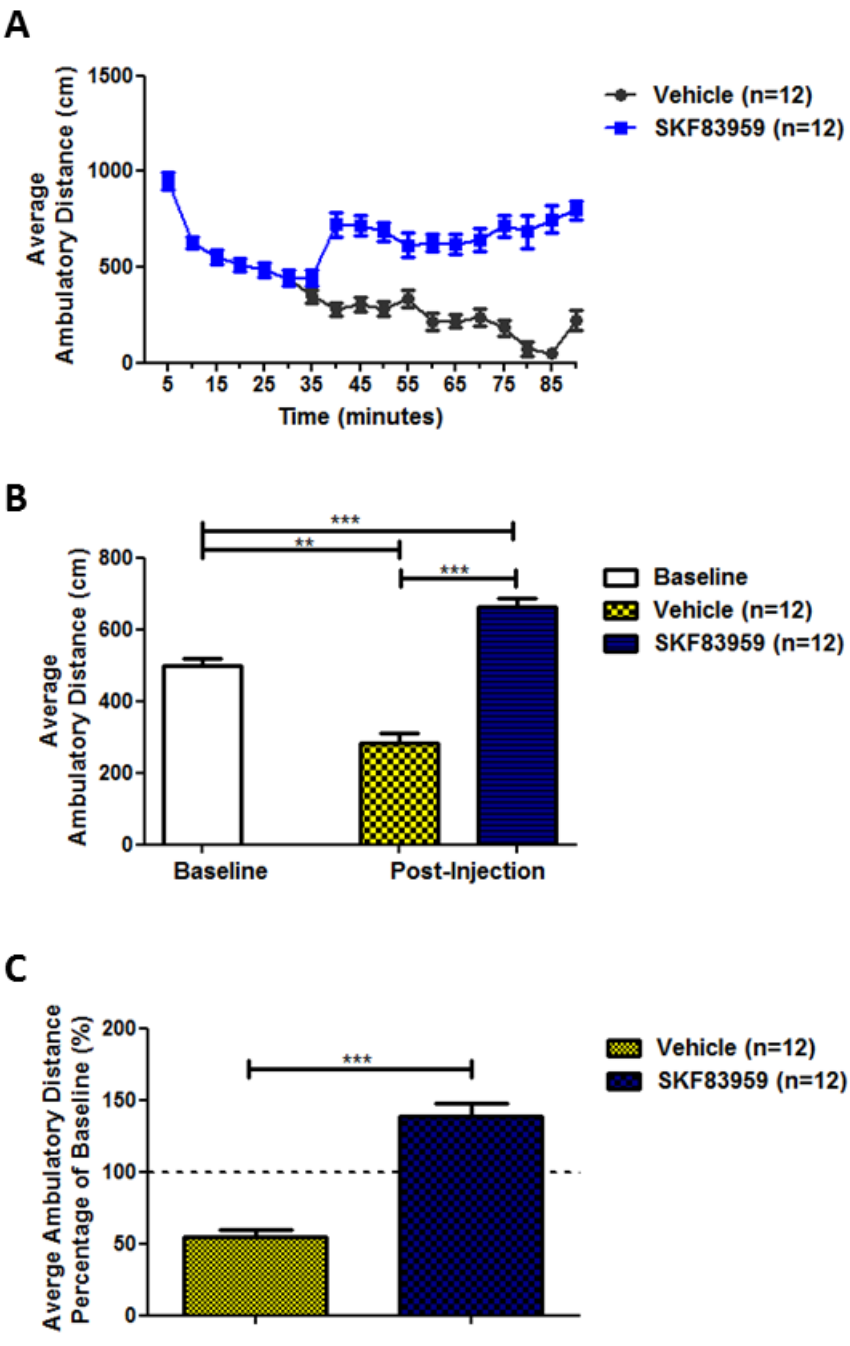


**Figure 32**

SKF83959 (1 mg/kg; i.p) significantly increases locomotor activity in C57Bl/6J mice when administered after only 30 minutes of habituation to the open field (**A and B**). The SKF83959-induced locomotor response is a 2-3 fold increase in activity compared to vehicle (**B**) regardless of extensive habituation or basal level of activity.

In Chapter III, we assessed the involvement of receptor signaling in SKF83959-induced actions using pharmacologic manipulations in addition to evaluation of the mutant mouse lines. For some of the ligands, however, we did not have good data available to determine what doses would be good to use for behavioral assessments. We therefore conducted dose-response curves for the $D_1$ receptor antagonist SCH23390 and the $\alpha_2$ adrenergic receptor atipamezole in order to find doses that did not have effects on locomotor responses when administered alone. At concentrations of 0.1 mg/kg and 0.05 mg/kg, SCH23390 had significant inhibitory effects on locomotor responses (Figure 33A and B). A dose of 0.01 mg/kg, however, was not significant from vehicle; therefore this was the dose we used in some our studies with a low-dose of SCH23390. We also used a higher dose of SCH23390 (0.25 mg/kg) is some studies and although this dose blocked SKF83959-inducd locomotor behavior, it also induced profound inhibition of locomotor activity when administered alone (Chapter III, Figure 18). Additionally, with atipamezole, we tested a large range of doses (Figure 34A and B) and decided on a dose of 0.25mg/kg for use in our experiments as we wanted a dose that was high enough that if we didn't observe an effect, it wouldn't be due to the dose being too low to exert an effect.

Lastly, in Chapter IV, we conducted studies with chronic administration of SKF83959 (0.5 mg; i.p. for 21 days). We wanted to save on costs associated with preparing the drug fresh every day; however, we did not know whether SKF83959 would be stable in solution for more than 24 hours. In all previous experiments, SKF83959 had been made up fresh in solution on the day of the

experiment. We therefore tested SKF83959 in solution over a 3-day period and observed its effect on locomotor activation. We found that the percent change in locomotor activation compared to baseline was reduced with each day the ligand was in solution (Figure 35A – G). We therefore decided to prepare SKF83959 fresh every 2 days for use in the novelty-induced food suppression experiment.

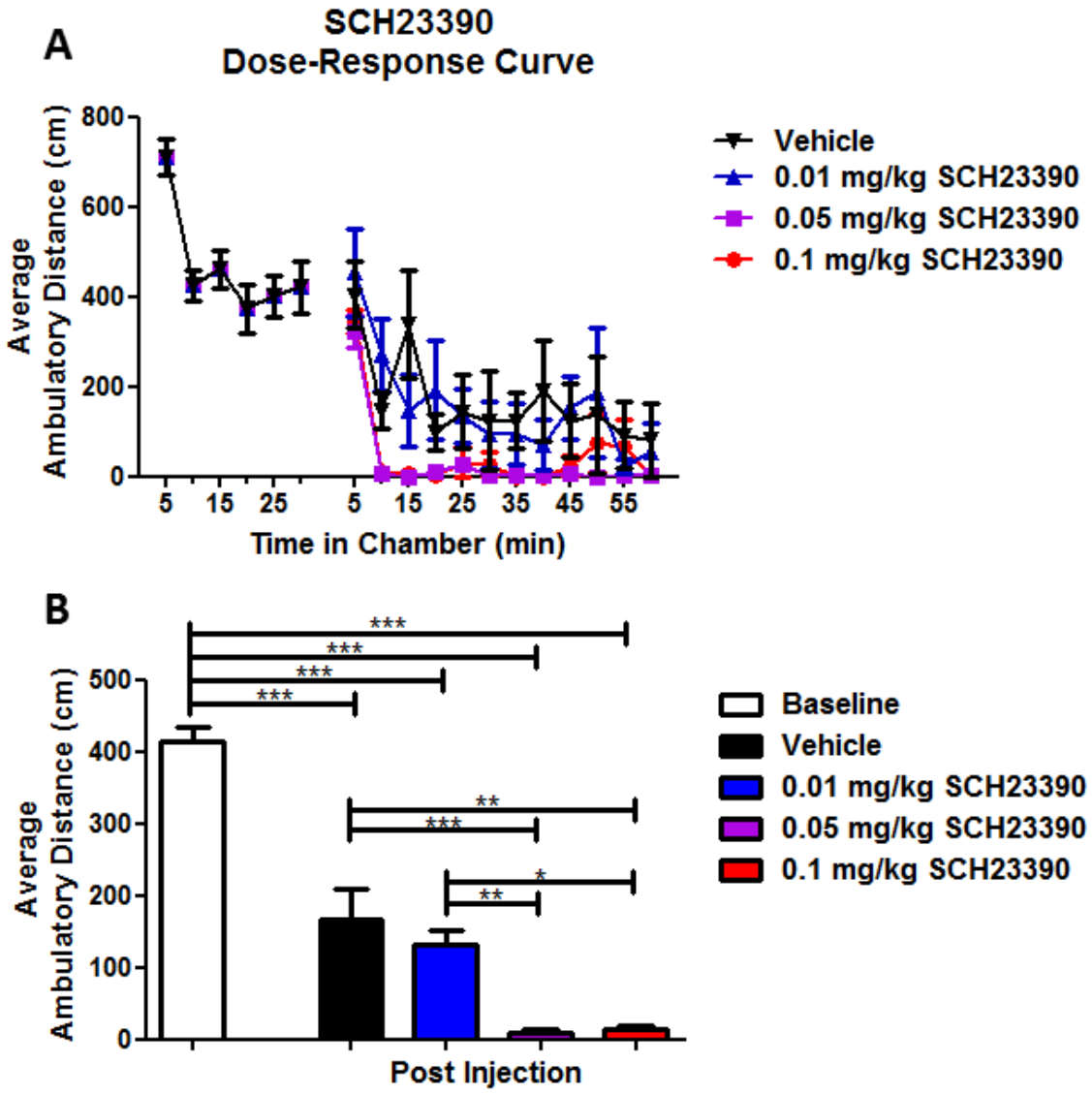


**Figure 33**

The $D_1$ receptor antagonist SCH23390 significantly inhibits locomotor activity at doses of 0.1 mg/kg and 0.05 mg/kg; however, the 0.01 mg/kg dose is not significant from vehicle (**A** and **B**).

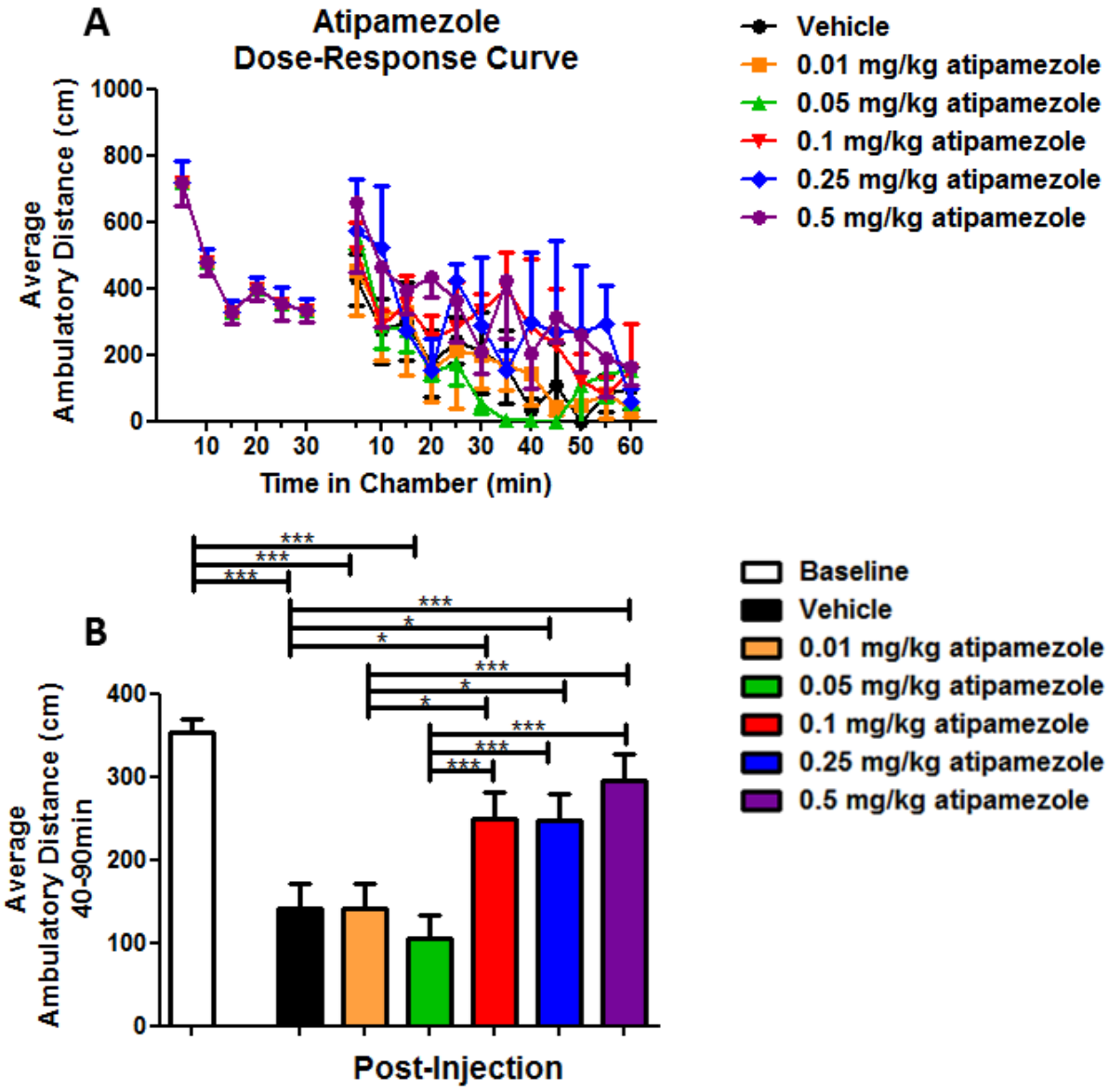


**Figure 34**

The $\alpha_2$ adrenergic receptor antagonist atipamezole does not change locomotor activity compared to baseline at 0.1, 0.25 or 0.5 mg/kg, however locomotor responses at these doses are significantly different compared to vehicle (**A** and **B**).

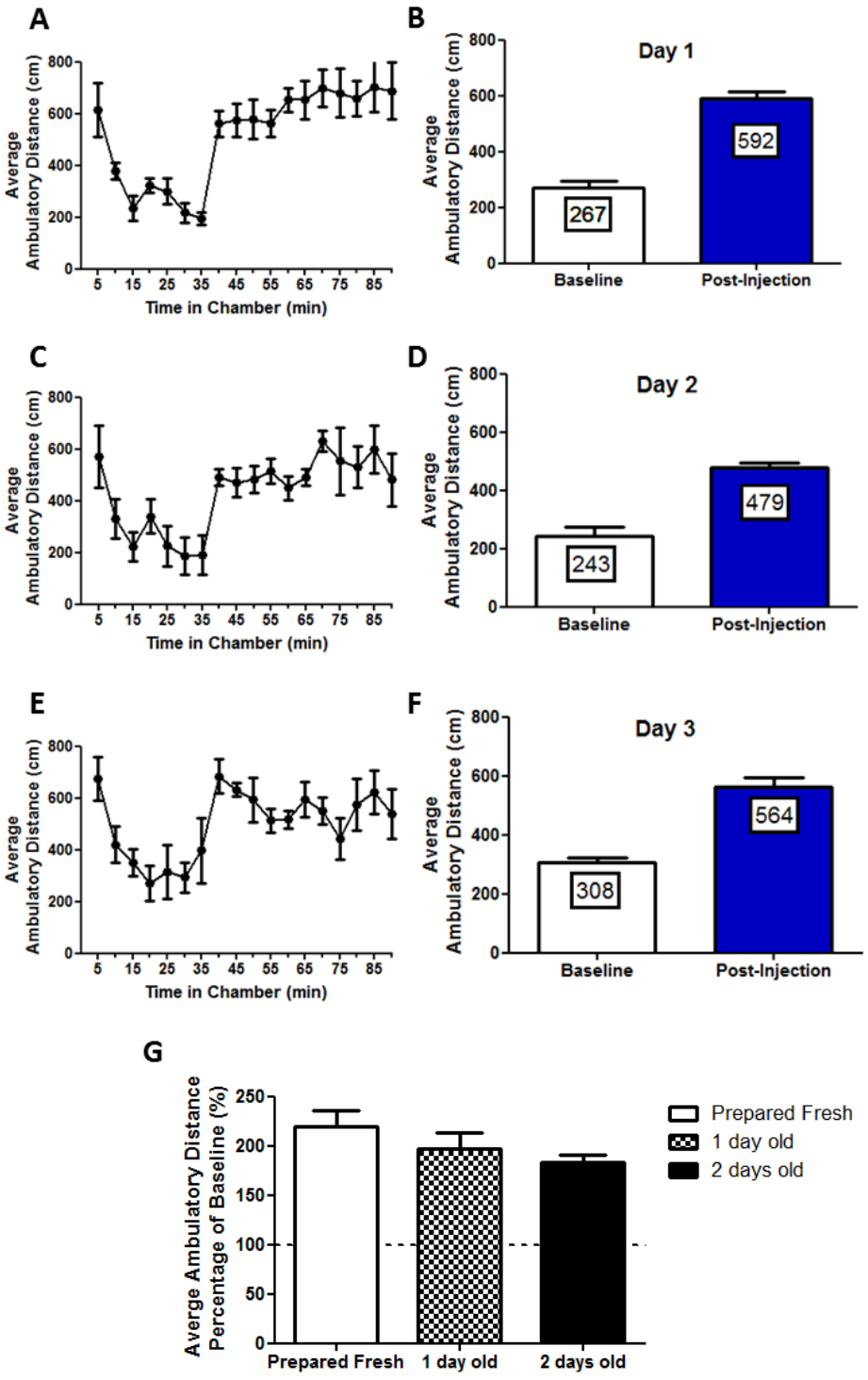


**Figure 35**

The locomotor stimulating effects of SKF83959 decline as SKF83959 is in solution over 3 days (**A** – **G**).

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