IS BRAIN CHOESTEROL THE MISSING CULPRIT IN DRUG ADDICTION?

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

24-OHC, 24(S)-hydroxycholesterol

5-HT, 5-hydroxytryptamine

ABCA1, ATP-binding cassette transporter A1

ABCG1, ATP-binding cassette transporter G1

AC, adenylyl cyclase

AMPH, amphetamine

ANOVA, analysis of variance

ApoA1, apolipoprotein A1

cAMP, 3',5'-cyclic AMP

CNS, central nervous system

CYP46A1, cholesterol-24-hydroxylase

D2R, dopamine 2 receptor

D2S, dopamine 2 receptor short form

DAT, dopamine transporter

FR1, fixed ratio one

GPCR, G-protein coupled receptors

IP1, inositol monophosphate

LRPR, low density lipoprotein receptor

MβCD, methyl-β-cyclodextrin

N2A, neuroblastoma 2A

PFC, prefrontal cortex

PLCβ, phospholipase Cβ

SCAP, SREBP cleavage-activating protein

SR1B, scavenger receptor class B type 1 transporter

SREBP2, sterol-regulatory element-binding protein

ABSTRACT

Chronic psychostimulant exposure alters the signaling of G-protein coupled receptors (GPCRs), which is associated with drug relapse. Although the molecular mechanisms remain unknown, scant evidence suggests a potential role of membrane lipids especially cholesterol in regulation of psychostimulant-mediated GPCR signaling. Plasma membrane is organized into cholesterol-enriched lipid raft and non-lipid raft microdomains. GPCRs and their interactinG-proteins are localized in lipid rafts or non-lipid rafts in a receptor-dependent manner. Depletion of cholesterol disrupts certain GPCR function. This study investigates the effects of amphetamine (AMPH) self-administration on cholesterol content, lipid raft localization of Gα subunits and effectors, and receptor-stimulated G-protein downstream signaling in rat striatum. We found that striatal cholesterol levels were significantly reduced following both 5 and 14 days of AMPH self-administration. Using sucrose density gradient fractionation, we found that more Gα subunits (Gαi1/2/3, Gαo and Gαq) were translocated into lipid rafts whereas their effectors such as adenylyl cyclase (AC) and phospholipase Cβ (PLCβ) remained in non-lipid rafts. Importantly, translocation of Gα subunit isoforms between lipid rafts and non-lipid rafts has functional consequence on receptor-mediated G-protein signaling. The ability of Gαi/o-coupled dopamine D2/D3 receptors and serotonin 5-HT1A/1B receptors to inhibit AC activity is reduced in AMPH self-administering rats. Moreover, the ability of Gαq-coupled mGluR1/5, but not serotonin 5-HT2A/2C receptors, to stimulate PLCβ activity is also decreased in AMPH self-administering rats. These data

suggest an association of cholesterol content with Gα subunit compartmentalization and signaling. Using neuroblastoma N2A cells stably expressing the short form of dopamine D2 receptors, we found that membrane cholesterol depletion with methyl-β-cyclodextrin directly caused Gαi2 movement into lipid rafts. Furthermore, the ability of dopamine D2 receptors to inhibit Gαi/o-mediated AC activity is reduced following cholesterol depletion. This is the first observation of AMPH-induced reduction in cholesterol content and G-protein movement between lipid rafts and non-lipid rafts. These findings point to cholesterol as a regulator of GPCR signaling in psychostimulant abuse.

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 AMPHETAMINE

Abuse of psychostimulants such as amphetamine (AMPH) continues to plague public health. AMPH is a substrate of dopamine transporter (DAT). AMPH exerts its action by transporting into the cell and increasing dopamine efflux through reverse transporter, resulting in an increase in extracellular dopamine concentrations (Pierce and Kalivas 1997). AMPH also acts on other monoamine transporters such as serotonin and norepinephrine transporters, causing an increase in extracellular concentrations of these neurotransmitters (Robertson et al. 2009). However, the rewarding and reinforcing properties of psychostimulants primarily result from the interaction with DAT (Chen et al. 2006). Additionally, AMPH has been shown to increase glutamate levels in many brain areas including the striatum (Del Arco et al. 1999). This glutamate release is mediated by dopamine, as increased levels are blocked by dopamine antagonists (Reid, Hsu, and Berger 1997). There have been many reports of AMPH-induced alterations in the function and signaling of G-protein coupled receptors (GPCRs) including dopamine receptors, serotonin receptors and glutamate receptors (Choe et al. 2002; Zhang et al. 1997; Jones and Kauer 1999; Calipari et al. 2014; Peterson et al. 2006). These alterations have been associated with relapse; however, the exact molecular mechanisms remain elusive.

1.2 AMPH AND GPCRS

GPCRs belong to a large and diverse class of membrane receptors

characterized by seven transmembrane domains. GPCRs induce signal transduction by activation of heterotrimeric G-proteins, which can either stimulate or inhibit the second messenger system. GPCRs couple to different G-proteins in a receptor-type dependent manner. To date, there are few studies investigating the effect of AMPH on intracellular G-protein signaling.

1.2A Heterotrimeric G-Proteins

GPCRs regulate signaling cascades through heterotrimeric G-proteins, which act as switches to turn on or off second messenger systems. G-proteins are composed of three subunits, α, β, and γ (Oldham and Hamm 2008). The Gα subunit determines the specific signaling effector. When the G-protein is in its resting conformation, GDP is bound to the Gα subunit (Oldham and Hamm 2006). When a GPCR is activated, GDP on the Gα subunit exchanges for GTP to activate the G-protein, and the G-protein complex dissociates from the receptor. The Gα subunit dissociates from the βγ subunit to activate or inhibit the downstream effectors (e.g. AC and PLCβ). Gα-GTP is then hydrolyzed to Gα-GDP and the βγ subunit can bind to the Gα subunit to reform a heterotrimeric G-protein.

The four classes of G -proteins are categorized based on the α subunit isoform as follows: Gαs, Gαi/o, Gαq/11 and Gα12/13. The Gαs class of G-proteins, including Gαs and Gαolf, activates AC to increase intracellular 3',5'-cyclic AMP (cAMP) levels, which acts as a second messenger to propagate cellular signaling

(Rasmussen et al. 2011). The Gαi/o class includes Gαi1, Gαi2, Gαi3, and Gαo, which bind to specific GPCRs with different affinities in a tissue-specific manor (Cabrera-Vera et al. 2003). In contrast to Gαs, when a GPCR coupled to Gαi/o proteins is activated, the activated Gα subunit inhibits AC to decrease intracellular cAMP levels. However, if Gi/o signaling is prolonged, heterologous sensitization of adenylyl cyclase (AC) occurs and cAMP levels increase (Brust et al. 2015). Gαq activates phospholipase Cβ (PLCβ), which in turn stimulates the production of second messengers diacyl glycerol and inositol 1,4,5-trisphosphate (Knall and Johnson 1998). Lastly, Gα12/13 stimulate RhoGTPase nucleotide exchange factors to activate RhoA (Siehler 2009).

Psychostimulant exposure has been shown to have a differential effect on expression of Gα subunit isoforms. Both AMPH and cocaine exposure (6 days intraperitoneal injection) have been shown to decrease Gαolf expression, a member of the Gαs class, while Gαs levels were unaffected in the nucleus accumbens of rats (Crawford et al. 2004). However, conflicting studies show that while mRNA levels of Gαs, Gαolf, Gαo, and Gαi are altered by binge-pattern cocaine administration in rats, they were not altered in the striatum, nucleus accumbens, and PFC (Perrine et al. 2005). Gαq/11 levels are increased in the paraventricular nucleus and amygdala but not in the PFC during cocaine withdrawal (Carrasco et al. 2003). Psychostimulant exposure can also affect G-protein activity. For example, neonatal exposure to 3,4-methylenedioxymethamphetamine increases the efficacy of 5-HT1A receptors

stimulated Gαi/o-mediate protein kinase A activity and agonist-stimulated GTPγS binding (Crawford et al. 2006). This effect appears to be receptor-dependent as cocaine administration increased membrane GTPγS binding stimulated by mu- but not kappa- or delta-opioid receptors in the striatum, nucleus accumbens, and cingulate cortex of male Fischer rats (Schroeder et al. 2003).

1.2B Dopamine Receptors

The dopamine neurotransmitter system regulates many aspects of brain functions including working memory, attention, movement and reward. dopamine neurons in the midbrain are the primary dopamine source for innervations throughout the brain (Chinta and Andersen 2005). The nigrostriatal dopamine system, which originates in the substantia nigra pars compacta and innervates the caudate-putamen plays a critical role in moderating voluntary movement (Smith and Villalba 2008). The mesocorticolimbic dopamine system originates in the ventral tegmental area to project to the nucleus accumbens, amygdala, and hippocampus and modulates emotion and reward (Arias-Carrión et al. 2010). The dopamine released from these neurons activates five subtypes of dopamine receptors, which allow for the diverse physiological actions of dopamine. D1-like receptors (D1R and D5R) are coupled to Gαs and activate AC (Vallone et al. 2000) whereas the D2-like receptor family (D2R, D3R and D4R) couple to Gαi/o and inhibit AC.

Psychostimulant abuse is associated with downregulation and desensitization of dopamine receptors. AMPH self-administration has been shown to reduce D2R function in rats (Calipari et al. 2014). Specifically, 5 days of AMPH self-administration reduced D2 autoreceptor inhibition of dopamine release in the midbrain, which may contribute to drug-taking behavior. Moreover, cocaine self-administration has been shown to influence the expression of both classes of dopamine receptors in nonhuman primates. For example, in vitro quantitative autoradiography demonstrated that D1R and D2R binding with [^{3}H]SCH-23390 or [^{3}H]raclopride, respectively, was significantly lower in the striatum of rhesus monkeys that chronically self-administered cocaine when compared to drug naive controls, indicating lower receptor density (Moore et al. 1998a; Moore et al. 1998b). Additionally, acute exposure to cocaine did not alter the availability of D1R and D2R in the striatum of rhesus monkeys, but prolonged exposure decreased D2R density and increased D1R density (Nader et al. 2002). Similar effects of cocaine on receptor binding are found in humans. Positron emission tomography demonstrated that cocaine abusers have significantly decreased D2R availability, even after 3-4 months abstinence (Volkow et al. 1993). The reduced availability and signaling of D2R has been associated with vulnerability to addiction (Wang et al. 2012; Czoty et al. 2007; Volkow and Morales 2015). Thus, it is important to investigate the molecular mechanism that underlies the regulation of D2R signaling.

1.2C: Serotonin Receptors:

Serotonin is a monoamine neurotransmitter that regulates appetite, sleep and mood. It is also implicated in neuropsychiatric disorders like depression and schizophrenia. There are 14 subtypes of 5-HT receptors across 6 families, 13 of which are GPCRs and couple to Gαi/o, Gαq/11 or Gαs (McCorvy and Roth 2015). The 5-HT1A/B/D/F and 5-HT5A/B receptors couple to Gαi/o to inhibit AC signaling (Lin et al. 2002; McCorvy and Roth 2015). The 5-HT2A/C receptors couples to Gαq/11 to activate PLCβ, but some isoforms of this family have been found to couple to Gαi/o in cultured cells (Roth et al. 1998; Garnovskaya et al. 1995). The remaining 5-HT receptor subtypes (5-HT4, 5-HT6, and 5-HT7) couple to Gαs to activate AC production of cAMP (McCorvy and Roth 2015). However, 5-HT4 receptors have been shown to increase calcium current in human atrial myocytes, indicating a possible Gαq/11-mediated signaling (Ouadid et al. 1992). The final 5-HT receptor, 5-HT3 is a ligand-gated ion channel.

Psychostimulant exposure has also been shown to alter serotonergic signaling in the brain. AMPH acts as a substrate of the serotonin transporters, which causes an increase of 5-HT levels. In vivo positron emission tomography demonstrated that chronic cocaine self-administration upregulated 5-HT2A receptors in the prefrontal cortex (Sawyer et al. 2012). Conversely, AMPH has been shown to decrease 5-HT2A receptor mRNA expression in the PFC of rat brains, which may contribute to the cognitive and emotional impairments observed during psychostimulant withdrawal (Horner et al. 2011). However, this effect is brain-

region specific, as the same rats showed higher levels of mRNA expression in the striatum and the hippocampus. Psychostimulant exposure has also been shown to alter 5-HT receptor activity. A single injection of cocaine decreases 5-HT1B-induced long term depression, as presynaptic proteins rabphilin 3A and synapsin 1 were hyperphosphorylated in the nucleus accumbens in mice (Huang et al. 2013). Serotonin receptor activation is thought to modulate drug-seeking behavior; administration of RU-24969, a 5-HT1A/1B agonist disrupts self-administration of amphetamine in rats (Fletcher and Korth 1999).

1.2D: Glutamate Receptors:

Glutamate is the primary excitatory neurotransmitter. There are two families of glutamate receptors which include ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs). mGluRs are coupled to G-proteins and further divided into 3 subfamilies (Conn and Pin 1997). The mGluR group 1 includes mGluR1 and mGluR5 which are coupled to Gαq to stimulate the PLCβ signaling pathway. The mGluR group 2 includes mGluR2 and mGluR3 and group 3 includes mGluR4, mGluR6, mGluR7, and mGluR8; both groups couple to Gαi/o to inhibit AC and reduce cAMP levels (Willard and Koochekpour 2013).

Psychostimulant exposure has been shown to alter the expression and function of mGluRs in a brain-region specific manor. AMPH increases levels of mGluR5 mRNA in pyramidal hippocampal neurons, granular cells, and ventral thalamic

nuclei (Yu et al. 2003). *In vivo,* it has been shown that a single dose of AMPH reduced mGluR5 levels in striatal synaptosomal membranes but increased levels in the medial prefrontal cortex (Shaffer et al. 2010). Additionally, cocaine self-administration in rhesus monkeys increased mGluR2/3 density in the caudate nucleus with no effect in the putamen or nucleus accumbens, relative to controls (Beveridge et al. 2011).

1.3 PSYCHOSTIMULANS AND MEMBRANE CHOLESTEROL

1.3A Membrane lipid composition and lipid rafts in the brain

The brain is a highly lipid-rich organ with different classes of brain lipids playing important roles for structural integrity and signal mediation. The three main classes of brain lipids are glycerophospholipids, sphingolipids, and cholesterol (Dietschy and Turley 2004). Glycerophospholipids are important for maintaining structural integrity of the membrane and include plasmalogens, phosphatidylcholine, and phosphatidylinositol. Sphingolipids serve a structural role in the plasma membrane and act as a binding site for extracellular proteins. Gangliosides are a specific subtype of sphingolipids characterized by sialic acids and are very abundant in the brain. Cholesterol acts as a binding partner of sphingolipids and can directly interact with membrane receptors, ion channels, and transporters (Gimpl 2016; Ferraro et al. 2016; Levitan, Singh, and Rosenhouse-Dantsker 2014).

The cell membrane is composed of a lipid bilayer of phospholipids. Sphingolipids and cholesterol form rigid lipid rafts within the membrane. Lipid rafts are less fluid than nonraft portions of the membrane and are essential for promoting or inhibiting signaling based on the compartmentalization of signaling molecules (Allen et al. 2007). There are two types of lipid rafts that share similar properties. Caveolae are invaginations of the cellular membranes characterize by caveolin proteins. Caveolae are expressed in endothelial cells, astrocytes, and oligodendrocytes. Planar lipid rafts are similar to caveolae, but they are not invaginated nor do they contain caveolin; they are characterized by flotillin, a caveolin analog. Planar lipid rafts are more common in neurons and neuroblastoma cells. In addition to high levels of sphingolipids and cholesterol, both caveolae and planar lipid rafts have glycosylphosphatidylinositol-anchored proteins and are resistant to detergent extraction.

The plasma membrane is divided into lipid raft (cholesterol-enriched) and non-lipid raft (less cholesterol content) microdomains. Lipid raft microdomains have been proposed to modulate neurotransmitter signaling through spatial organization of GPCRs and their effectors. Because GPCR signaling requires physical interaction between the receptor, the G-protein, and their effector, the rigidity of lipid rafts in the presence of cholesterol can enhance signaling by stabling these signaling complexes. However, lipid rafts can also diminish signaling by spatially separating signaling proteins. Receptor localization to lipid rafts is receptor-dependent. Studies have shown that β1-adrenergic receptor

signaling through cAMP accumulation is enhanced when it is associated with lipid rafts in rat cardiomyocytes; however, β2-receptor signaling, measured by cAMP accumulation, is diminished when it is associated with lipid rafts (Ostrom et al. 2001). The functionality of some G-proteins and G-protein effectors has also been shown to be regulated by their association with lipid rafts. It is well documented that GPCR-stimulated Gαs signaling is impaired by lipid raft association in glioma C6 cells, as raft disruption by cholesterol depletion and caveolin knockdown enhances agonist-stimulated cAMP accumulation, but the effects of raft association of Gαi/o signaling is unknown (Allen et al. 2009). The activity of AC is diminished by raft association in HEK293 cells and cardiomyocytes, as caveolin-bound AC produced less cAMP than non-caveolin bound AC (Toya et al. 1998; Rybin et al. 2000). In the present study, we examined the localization of Gαi/o and Gαq following AMPH self-administration, which has not been previously explored. This study will provide new mechanisms for understanding of changes in GPCR signaling associated with psychostimulant exposure.

1.3B Brain cholesterol synthesis, homeostasis and efflux

Brain cholesterol accounts for up to 25% of the total cholesterol content in the body (Dietschy 2009). The brain cholesterol synthesis, metabolism and efflux are detailed in Figure 1.1. Because cholesterol is unable to pass the blood brain barrier, the majority of this lipid is synthesized *de novo* in glial cells (J. Zhang and Liu 2015). Ketone bodies are transformed in a series of steps to cholesterol,

mainly in oligodendrocytes before myelination occurs (Petrov et al. 2016). There are two potential synthesis pathways that convert the precursor lanosterol to cholesterol: the Kandutsch-Russel pathway, which transforms lanosterol to 7-dehydrocholesterol and lathosterol, and the Bloch pathway, whose main intermediate is desmosterol. The two synthesis pathways are cell-type specific, with neurons primarily generating cholesterol through the Kandusch-Russel pathway and astrocytes through the Bloch pathway (Nieweg et al. 2009). After the brain matures, cholesterol synthesis occurs at low levels in neurons due to the high conservation of the lipid. Cholesterol levels are regulated by sterol-regulatory element-binding protein (SREBP-2), a transcription factor in the endoplasmic reticulum. Cholesterol is detected by SREBP cleavage-activating protein (SCAP), and the SREBP-SCAP complex is transported to the Golgi body in low cholesterol conditions (Dietschy 2009). From the Golgi, the N-terminal domain of SREBP-2 is released to translocate to the nucleus to promote transcription of cholesterol biosynthesis enzymes. Cholesterol binds to apolipoprotein E (ApoE) in the ER in the astrocyte, at which point it can be secreted by ATP-binding cassette transporters (ABCA1, ABCG1) (Hayashi 2011). ApoE-bound cholesterol is taken up by neurons through the low density lipoprotein receptors (LDLR) (Petrov et al. 2016). Niemann-Pick C (NPC) proteins regulate cholesterol trafficking within the plasma neuron. It has been shown that SREBP-2 reduction by SCAP knockout reduces cholesterol content in the brain (Eberlé et al. 2004). Thus, the present study examined SREBP-2 protein level as a measure of cholesterol synthesis in the brain.

There is a very low turnover rate of cholesterol in the brain. In the plasma membranes of glial cells and neurons, the half-life of cholesterol is 5-10 months. The cholesterol stores in myelin have a half-life of 5 years (Russell et al. 2009). The brain is unable to directly degrade cholesterol, but can export the lipid as 24(S)-hydroxycholesterol (24-OHC), an oxysterol that can pass through the blood brain barrier. Cholesterol is converted to 24-OHC through cholesterol-24-hydroxylase (CYP46A1), a brain-specific enzyme as a primary mechanism for its excretion in the brain. An additional mode of cholesterol removal from the brain is through the ABCA1/G1 transporters, which effluxes cholesterol to ApoE. At this point, cholesterol can cross through the blood brain barrier through the LDLR or scavenger receptor class B type 1 transporters (SR1B) (Gosselet et al. 2014). Upregulation of ABCA1 or CYP46A1 controls cholesterol efflux in the brain (Hayashi 2011).

Cholesterol has many proposed functions and implications for cellular signaling. Brain cholesterol levels are very high in the myelin sheath of oligodendrocytes where it helps regulate fluidity and permeability (Martin et al, 2014; Zhang and Liu, 2015). In neurons and astrocytes, cholesterol is important for morphology and synaptic transmission (Dietschy and Turley 2004). Additionally, cholesterol is needed for synaptogenesis and dendrite formation and axonal guidance (Goritz et al. 2005; de Chaves and Narayanaswami 2008). When cholesterol is depleted in neurons, vesical exocytosis and neurotransmission is impaired (Linetti et al.

2010). When cholesterol is oxidized to its biologically active oxysterol, 24S-OHC, it can impact cellular function in a concentration dependent manner. 24S-OHC can induce apoptosis though necroptosis in primarily cortical neuronal cells at high concentrations, but it exhibits cytoprotective effects through adaptive responses at lower concentrations (Noguchi et al. 2014).

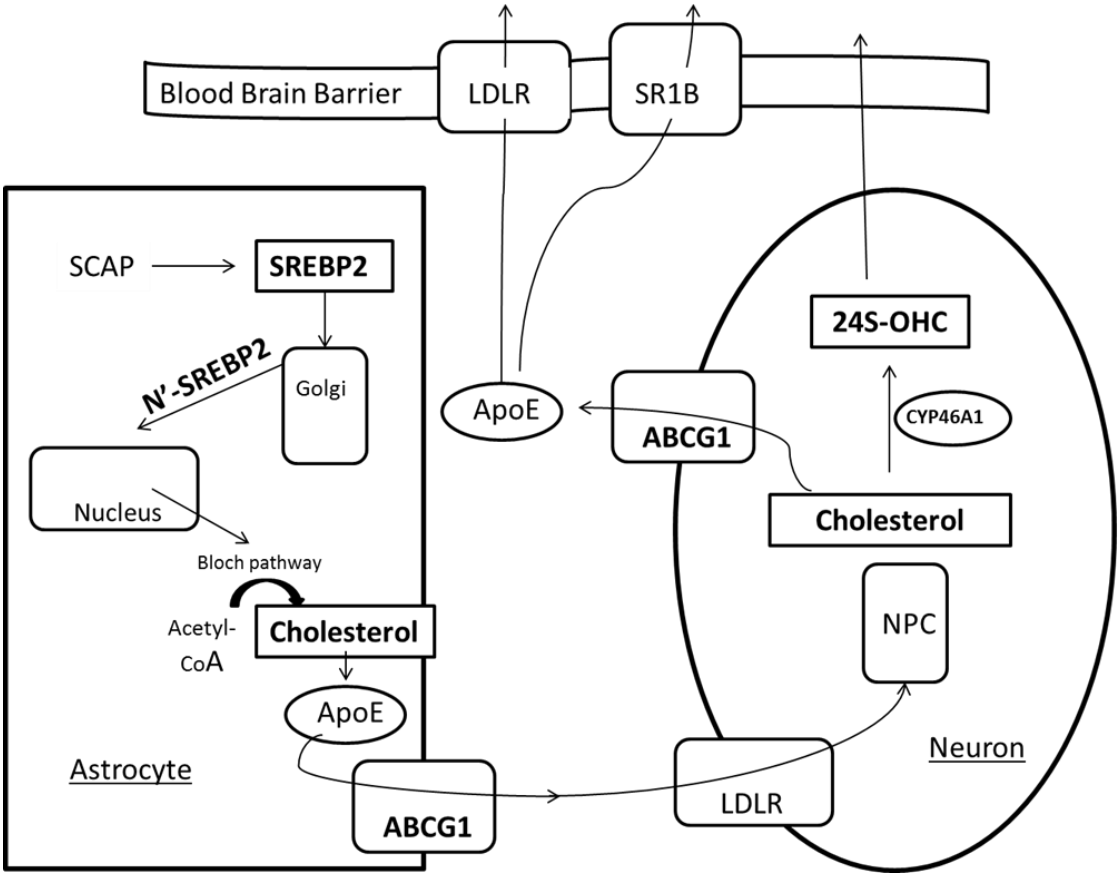

Figure 1.1. Key regulatory steps of brain cholesterol synthesis and efflux. Components evaluated in this study are in bold.

1.3C Psychostimulant-induced alterations in lipid components of the plasma membrane

There is increasing evidence for the effects of psychostimulants on brain lipids and the impact such alterations may exert for addiction (Luessen and Chen 2016). Phospholipids are the most abundant lipid on the plasma membrane in the brain. Phospholipids are particularly enriched in the cortex, a critical brain region for cognition (Svennerholm 1968). Chronic psychostimulant exposure has been shown to alter phospholipids in a region specific manner. For example, cocaine exposure in rats increases phospholipid levels in the ventral striatum and the hippocampus, but levels are decreased in the cerebellum when compared to saline controls (Cummings et al. 2015). In cocaine-dependent polydrug users, phospholipid levels are lower in central white matter compared to drug naïve human controls (MacKay et al. 1993). It is believed that the changed phospholipid levels are due to altered metabolic enzyme activity. It has been shown that human cocaine and methamphetamine users have reduced calcium-stimulated phospholipase A2 activity in the striatum, which metabolizes phospholipids by hydrolyzing the arachidonic acid bond (Ross et al. 2002).

While sphingolipids are not nearly as abundant as other lipids in the brain, they are an important component of lipid rafts and play a critical role in GPCR signaling. Previous studies have shown that cocaine treatment decreases sphingomyelinase activity and increases sphingomyelin levels in rat fibroblasts (Nassogne et al. 2004). Likewise, ganglioside levels in rats are increased following cocaine exposure in utero (Leskawa et al. 1994). Depression and

anxiety are associated with increased sphingolipid and ganglioside profiles in the hippocampus and the cortex (Müller et al. 2015). These alterations could be an underlying mechanism for the deregulated GPCR signaling observed following chronic exposure to psychostimulants which has not been investigated.

Very little is known about the effects of psychostimulant abuse on membrane cholesterol levels. One of the few studies on this subject showed that a single injection of methylphenidate in mice reduced the incorporation of [2-3H] acetate into brain cholesterol; this decrease indicated a depletion of brain cholesterol, but direct levels were not analyzed (Charach et al. 2009). Moreover, cocaine addicts with lower levels of peripheral cholesterol were more likely to relapse (Buydens-Branchey and Branchey 2003); however, the relationship between psychostimulants and cholesterol levels, the brain-specific levels were not measured. The present study examined the effect of AMPH self-administration on brain cholesterol content and its functional consequence on receptor-mediated G-protein signaling and compartmentalization.

1.4 ANIMAL MODELS OF ADDICTION

Psychostimulant addiction is often studied in animal models because they allow researchers to investigate the differential effects of acute and chronic drug abuse, similar to what is observed in human drug use. There are multiple animal model paradigms that target different stages of psychostimulant addiction. The acute model regimen can be used to determine the initial neurotoxic effects of

psychostimulants by administering a large dose of drug within a single day (Tokunaga et al. 2008). The escalating dose model attempts to mimic human chronic psychostimulant addiction by initially administering a low, nontoxic amount of drug and gradually increasing the dose over multiple days (Kobeissy et al. 2012). While these models are useful to differentiate the acute and chronic effects of psychostimulants, they do not accurately reflect the behavioral administration changes observed in humans.

The self-administration model relies on catheter implantation to allow animals, typically rats or nonhuman primates, to self-administer a psychostimulant as a positive reinforcer following an operant behavior such as nose pokes or lever presses (Niwa et al. 2008). There are many possible schedules of drug administration, but the most common are fixed-ratio and progressive-ratio schedule. Fixed-ratio schedules are most useful to determine reinforcing effects of a long-lasting drug while a progressive ratio assesses the breakpoint or how hard a subject is willing to work for the drug (Panlilio and Goldberg 2007). Self-administration also allows for choice paradigms where subjects have the option to choose between the drug or a non-drug reinforcer. Choice paradigms help model maladaptive choices associated with drug addiction. Given the added element of behavior, the self-administration model more accurately replicates human psychostimulant abuse, largely because it allows for the animals to progressively increase the amount of drug intake (Krasnova et al. 2010). When animals are self-administering drugs abused by humans, they generally have a

high responding rate (Collins et al. 1984). The many behavioral parallels between human drug abuse and animal self-administration allow this model to be the basis for preclinical abuse research.

1.5 AIMS

The primary goal of this study is to elucidate the effects of AMPH self-administration on cholesterol levels, G-protein compartmentalization and receptor-mediated G-protein signaling in rat striatal tissue. Cholesterol plays a crucial role in membrane stability and compartmentalization of signaling proteins. Altered cholesterol levels have been observed in many central nervous system (CNS) diseases including Huntington's disease and Alzheimer's disease (Shankaran et al. 2017; Leoni and Caccia 2015; Gosselet et al. 2014; Chang and Chang 2017). Additionally, chronic treatment with cholesterol-lowering and blood brain barrier permeable drugs such as statin has been linked to cognitive impairment. The effects of drugs of abuse on cholesterol are largely unknown. It has been shown that membrane cholesterol levels are increased by chronic ethanol exposure in cerebral cells (Omodeo-Salé et al. 1995). However, the only study investigating psychostimulant use and brain cholesterol assess the effect of a single methylphenidate injection, but only the levels of a precursor was measured (Charach et al. 2009). Thus, it is important to investigate the effect of psychostimulants on brain cholesterol content. Brain membrane cholesterol may be a new avenue for pharmacological interventions.

The second aim of the present study was to investigate the compartmentalization and signaling of G-proteins and their effectors in lipid rafts and non-lipid rafts. Due to the critical stabilizing role cholesterol plays in the integrity of lipid rafts, alterations in the cholesterol profile could significantly impact the localization and signaling of GPCRs, G-proteins and their effectors. It has been shown that anti-depressant administration alters G-protein membrane compartmentalization, but no study to date has investigated the effects of psychostimulants (Erb et al. 2016; Donati et al. 2015; Czysz et al. 2015). We therefore determine a potential impact of AMPH self-administration on brain cholesterol content, G-protein compartmentalization and signaling.

This study utilized rat AMPH self-administration model. To accomplish the first aim of our study, we measured striatal cholesterol levels in control and AMPH self-administration animals. After observing an AMPH-induced decrease in cholesterol levels, we evaluated potential mechanisms by measuring ABCG1 transporter levels, CYP46A1 levels, and 24-S-hydroxycholesterol activity. To address the second aim of our study, we isolated lipid raft microdomains of the striatal tissue using sucrose gradient fractionation. Raft and nonraft domains were identified using well known markers and western blotting. Fractions were pooled, subjected to SDS-page electrophoresis and evaluated for Gαo, Gαi1/3, Gαi2 and Gαq expression. Additionally, AC and PLCβ expression was measured in lipid rafts and non-lipid rafts. Receptor-mediated Gαi/o and Gαq signaling was also performed to study the functional consequence of G-protein movement

between lipid and non-lipid microdomains. To confirm a causal relationship between the cholesterol content and G-protein signaling, we depleted cholesterol levels in neuroblastoma (N2A) cells using methyl-β-cyclodextrin (MβCD) and measured D2R-stimulated Gαi/o-mediated cAMP production levels. The results from the present study strongly suggest an association of decreased brain cholesterol content induced by AMPH self-administration with altered G-protein membrane compartmentalization and signaling.

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CHAPTER 2

The Effects of Amphetamine Self-administration on Striatal Cholesterol Levels, G-protein Isoform Compartmentalization and Signaling

ABSTRACT

Chronic psychostimulant exposure alters G-protein coupled receptor (GPCR)-mediated G- protein signaling; however, the molecular mechanism remains unknown. Previous evidence suggests that brain cholesterol content regulates the stability of membrane and influences the location of GPCR and their effectors in lipid rafts and non-lipid rafts. Lipid rafts contain high cholesterol levels and depletion of cholesterol levels disrupts certain GPCR signaling. The present study examined the effect of amphetamine (AMPH) self-administration in male, Sprague-Dawley rats on striatal cholesterol levels and lipid raft localization of Gα subunit isoforms and their effectors. We found that AMPH self-administration decreased cholesterol levels in the striatum. Using sucrose density gradient centrifugation, we found that AMPH self-administration caused Gαi2, Gαi1/3, Gαo, and Gαq/11 to translocate into lipid rafts. Moreover, adenylyl cyclase (AC), the effectors of Gα proteins, are primarily expressed in non-raft microdomains under the basal condition and remained unchanged following AMPH self-administration. As a consequence, the ability of Gαi/o-coupled D2/D3 receptors and 5-HT1A/1B receptors to inhibit forskolin-stimulated cAMP production was decreased following AMPH self-administration. The increased shift of Gαi/o into lipid rafts diminishes their interaction with AC, which likely

explains decreased GPCR-mediated downstream signaling. In contrast, the ability of Gαq-coupled 5-HT2A/2C and mGluR1/5 receptors to stimulate phospholipase Cβ (PLCβ)-mediated inositol monophosphate (IP1) accumulation was unaffected and decreased, respectively. This is likely due to the observation that Gαq shifted into the lipid rafts whereas PLCβ, the Gαq effector, remained in the non-lipid rafts. These data suggest that AMPH self-administration differentially regulates the activity of Gα subunit isoforms. Taken together, these results indicate that AMPH self-administration depletes cholesterol, disrupting lipid rafts, and altering receptor-mediated Gαi/o and Gαq signaling pathways in the striatum.

Introduction

The brain is the most lipid-dense organ of the body. 15% and 23% of total cholesterol in the body is found in mouse and human brain, respectively (Dietschy 2009). Cholesterol has many proposed functions in the brain. Cholesterol is involved in synaptogenesis, dendrite formation, and axonal guidance (Goritz et al. 2005; de Chaves and Narayanaswami 2008). Cholesterol depletion impairs vesical exocytosis and neurotransmission in cultured hippocampal neurons (Linetti et al. 2010). Cholesterol also plays a critical role in the formation of lipid rafts in the plasma membrane, which are rigid membrane microdomains that promote or inhibit signaling by providing a spatial meeting point for signaling molecules (Allen et al. 2007). In addition to cholesterol enrichment, lipid rafts are characterized by detergent resisitance and marked by flotillin or caveolin. It is proposed that drugs of abuse may alter cholesterol levels, as chronic ethanol exposure is known to increase cholesterol levels in mouse synaptosomal membranes (Chin et al. 1978). Few studies have looked at the effects of psychostimulants on brain cholesterol levels. Reduced brain cholesterol levels is hypothesized following psychostimulant use, as a single injection of methylphenidate reduced the incorporation if [2-3H] acetate into brain cholesterol in mice (Charach et al. 2009). It has been shown that cocaine addicts with lower levels of circulating cholesterol are more likely to relapse (Buydens-Branchey and Branchey 2003). This study is the first to directly measure the effects of psychostimulant exposure on brain cholesterol levels.

Chronic exposure to psychostimulants causes profound changes in neurotransmitter signaling including altered G-protein coupled receptor (GPCR) function (e.g. desensitization & downregulation) and receptor-mediated intracellular signal transduction (McCreary et al. 2015). GPCRs in the brain primarily couple to Gαi/o, Gαs and Gαq/11 which interact with different downstream effectors to induce signal transduction. Previous studies suggest that the membrane localization and signaling of G-proteins is influenced by membrane raft association. For example, it has been shown that raft disruption in C6 glioma cells by cholesterol depletion or caveolin knockdown enhances Gαs lateral movement and signaling through cAMP accumulation, indicating attenuation of Gαs signaling by raft association (Allen et al. 2009). In contrast, 5-HT2A receptor-mediated Gαq signaling is enhanced when Gαq is compartmentalized to lipid rafts in C6 glioma cells (Bhatnagar et al. 2004). Specifically, raft disruption by caveolin knockdown reduced association between Gα and the 5-HT2A receptor, and attenuated receptor-mediated G-protein signaling, measured by calcium flux assays. Recent evidence indicates that CNS drugs can modulate G-protein localization. Rasenick's laboratory has reported that antidepressants accumulate in lipid rafts and decrease Gαs' association with lipid raft (Erb et al. 2016; Donati et al. 2015; Czysz et al. 2015; Chen and Rasenick 2002). This alteration may contribute to the therapeutic effect of antidepressant. However, the effects of psychostimulants on G-protein compartmentalization have yet to be determined. It is possible that altered GPCR function following chronic psychostimulant exposure is mediated by changes in

G-protein localization in lipid rafts.

The purpose of the present study was two-fold: a) investigate whether amphetamine (AMPH) self-administration altered brain cholesterol contents in the striatum; and b) determine whether AMPH self-administration caused Gα subunit isoforms and their effectors to shift between lipid raft and non-lipid raft microdomains. These experiments are important because there is a gap in understanding the mechanism underlying the addictive properties of AMPH and other psychostimulants. The present study may implicate the membrane lipid profile as an important modulator of psychostimulant-induced effects.

Materials and Methods

Animals

Male, Sprague-Dawley rats (Harlan Laboratories, Frederick, MD) were maintained in accordance with the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. Animals were kept on a 12:12h reverse light/dark cycle *ad libitum* access to food and water. The experimental protocol was approved by Wake Forest School of Medicine's Institutional Animal Care and Use Committee.

AMPH self-administration

Rats were anesthetized and implanted with chronic indwelling jugular catheters as previously described (Liu et al. 2007). Animals were singly housed and all sessions occurred during the active/dark cycle (0900-1500 hours). After a 3-day recovery period, animals were trained to self-administer AMPH. Briefly, animals were given access on a fixed-ratio one (FR1) schedule to an AMPH-paired lever. Upon responding, an intravenous delivery of 0.187mg/kg per AMPH infusion in 100 µl saline over 4s was initiated. The lever was retracted and a stimulus light was illuminated for a 20s timeout period after each AMPH delivery. Training ceased when an animal responded for 20 injections in 2 hrs for three consecutive days. After training, animals were allowed to self-administer AMPH for 6h a day (maximum 40 injections over 5 days or 14 days). Rats were sacrificed 18 hours after the last self-administration session. Brain regions were dissected and stored at -80°C for further biochemical assays and cholesterol

content measurement.

Cocaine and methylphenidate self-administration

Rats were subjected to the same training paradigm and the FR1 schedule as described for AMPH, but the lever was paired with cocaine or methylphenidate. Following a lever press, animals received an intravenous delivery of 1.5 mg/kg cocaine or 0.56 mg/kg methylphenidate. Typically, rats acquired self-administration in 2-5 days. After acquisition, animals were allowed to self-administer for maximum 40 injections within 6 hrs daily for 5 days. Rats were sacrificed 18 hours after the last drug self-administration session. Drug naïve and sham surgerized animals were used as controls.

Brain cholesterol content in the striatum and the prefrontal cortex

Cholesterol content in rat striatum and the prefrontal cortex (PFC) was measured by the Amplex Red Cholesterol Assay kit (Invitrogen) according to the manufacturer's directions. Briefly, tissue homogenate was diluted with 1x cholesterol reaction buffer (0.1 M potassium phosphate, pH 7.44, 0.05 mM cholic acid, 0.1% Triton X-100). 50 µL of Amplex Red reagent (150 µM, 1 U/ml horseradish peroxidase, 1 U/ml cholesterol oxidase, and 1 U/ml cholesterol esterase) were added to 50 µL of diluted sample in 96 well plates. After 30 minutes of incubation at 37 °C, sample fluorescence was measured using the Victor3 plate reader (Perkin Elmer) at 560 nm excitation and 590nm emission wavelengths. Cholesterol levels were normalized by protein concentration in

each sample, which was determined by a BCA protein assay (Pierce). Based on the assessment from the company where the kit was purchased, there is no reported cross reaction for 22-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, or dehydroepiandrosterone.

24S-hydroxycholesterol content in the striatum

Levels of 24(S)-Hydroxycholesterol (24S-OHC) in the striatum were determined using ELISA assay (Enzo Life Sciences) according to the manufacturer's directions. Briefly, samples were added to goat anti-rabbit IgG coated wells In the presence of biotinylated 24S-OHC, which competes with endogenous 24S-OHC for the binding to the primary antibody (rabbit 24S-OHC polyclonal antibody). After 1 hour incubation, the plate was washed to leave only antibody-bound 24S-OHC. Then HRP conjugated streptavidin was added to each well to bind to the biotinylated 24S-OHC. After incubation at room temperature, the plate is washed before tetramethylbenzidine substrate and provided stop solution was added to each well. The plate was read at 450nm using the Victor3 plate reader (Perkin Elmer). The signal strength is inversely proportional to endogenous 24S-OHC levels. The levels of 24S-OHC were determined based on the standard curve of 24S-OHC. Data are presented as relative to the control animals. There was no reported cross reaction of this assay for cholesterol, 22-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and dehydroepiandrosterone.

Western blotting of CY46A1 and ABCG1

Striatal lysates from control, 5 days, and 14 days of AMPH self-administering rats were loaded onto 8% Tris-Glycine gels for SDS-polyacrylamide gel electrophoresis. Protein was transferred to PVDF membranes. Membranes were incubated overnight with primary antibodies. CYP46A1 expression was determined by mouse anti-CYP46A (sc-136148, Santa Cruz) followed by anti-mouse IgGκ (sc-216102, Santa Cruz). ABCG1 protein was probed with rabbit anti-ABCG1 (LS-C356805, LifeSpan BioSciences) followed by goat anti-rabbit (sc-2054, Santa Cruz).

Sucrose density gradient centrifugation

Lipid rafts were isolated by sucrose gradient fractionation as previously described with slight modification (Persaud-Sawin et al. 2009). Briefly, tissue dissected from the dorsal striatum was homogenized in ice-cold MBS buffer with detergent (50mM MES, 300nM NaCl, 1% Triton-X 100, protease and phosphatase inhibitor cocktail) and incubated on ice for 30 minutes. Homogenate was sheered through a 20 gauge needle with 20 complete passes before being centrifuged at 1,000 g for 20 minutes at 4 C. Portions of the homogenate were collected as total lysate. The supernatant was mixed with 80% sucrose/MBS to form a 40% sucrose solution and loaded into a 40%/30%/5% sucrose gradient. The gradient was centrifuged at 46,000 rpm for 18 h at 4 C with a Beckman Coulter Optima XE-90 Ultracentrifuge and SW55Ti rotor. A distinct band was visible between the 5% and 30% after centrifugation.

Fifteen equal fractions were removed sequentially from the top to the end of the gradient and stored at -80°C.

Western blotting of Gα subunits, AC, and PLCβ

Equal sample volumes of each fraction or pooled fractions were loaded onto 10% or 8% Tris-Glycine gels for SDS-polyacrylamide gel electrophoresis. Protein was transferred to PVDF membranes. Membranes were incubated overnight with corresponding primary antibodies against different Gα subunit isoforms and signal effectors. Gαo proteins were probed with mouse anti-Gαo antibody (sc-13532, Santa Cruz) followed by anti-mouse IgGκ (sc-216102, Santa Cruz). Gαi2 proteins were assessed with rabbit anti- Gαi2 antibody (sc-7276, Santa Cruz) followed by goat anti-rabbit IgG (sc-2054, Santa Cruz). Gαi1/3 proteins were detected with mouse anti- Gαi3 antibody (sc-365422, Santa Cruz) followed by anti-mouse IgGκ (sc-216102, Santa Cruz). Gαq/11 proteins were assessed with rabbit anti- Gαq/11 antibody (sc-392, Santa Cruz) followed by goat anti-rabbit IgG (sc-2054, Santa Cruz). AC proteins were probed with rabbit anti-AC5 (ABS573, Millipore) followed by goat anti-rabbit IgG (sc-2054, Santa Cruz). PLCβ proteins were analyzed with rabbit anti-PLCβ (sc-205, Santa Cruz) followed by goat anti-rabbit secondary (sc-2054, Santa Cruz). Flotillin expression was probed with mouse anti-flotillin (BDB610821, BD Biosciences) followed by anti-mouse IgG (sc-2005, Santa Cruz). Transferrin expression was probed with mouse anti-transferrin (sc-52256, Santa Cruz) followed by anti-mouse IgG (sc-2005, Santa Cruz).

Membrane preparation

Receptor-mediated G-protein signaling was assessed on membrane as described previously (Calipari et al. 2014). Briefly, striatal from the control and 5 days of AMPH self-administering rats were homogenized in Tris lysate buffer with protease inhibitor cocktail. Homogenate was sheered through a 20 gauge needle with 20 complete passes. The homogenate was centrifuged at 16,000xg for 15 minutes at 4°C three times, with supernatant being collected and pellet resuspension in Tris buffer after each centrifugation. Supernatants were pooled and centrifuged at 80,000xg for 45 minutes at 4°C. The supernatant was removed and the pellet was resuspended, passed through a 20 gauge needle for 20 complete passes, and stored at -80°C.

cAMP accumulation assay

Cyclic AMP (cAMP) levels were measured using the LANCE Ultra cAMP assay (PerkinElmer) according to the manufacturer's directions. Briefly, prepared membrane was added to 384-well white, opaque OptiPlates (PerkinElmer). Forskolin (10 µM, 30 minutes) and either D2/3R agonist quinpirole (0.1 nM-1 µM) or 5-HT1A/1B receptor agonist 8-OH-DPAT (0.1 nM-1 µM) were added to the wells and incubated for 30 minutes. Eu-cAMP tracer and ULight-anti-cAMP were added to the wells and the plate was incubated at room temperature for 1 hour. TR-FRET signal was read with a 340 nm wavelength excitation and 665 nm wavelength emission with the Victor3 plate reader (Perkin Elmer). The values of

IC50 for quinpirole and 8-OH-DPAT were extrapolated from the dose-response curve.

IP-One Gq assay

Inositol monophosphate (IP1) levels were measured using the IP-One Gq assay (Cisbio) according to the manufacturer's directions. Briefly, membrane was added to 384-well white, opaque OptiPlates (PerkinElmer). 5HT2A/2C receptor agonist 1-(2,5-dimehoxy-4-iodophenyl)-aminopropane (DOI, 0.1 nM-1 µM) or mGluR1/5 receptor agonist (S)-3,5-dihydroxyphenylglycine (DHPG, 0.1 nM-1 µM) prepared in provided stimulation buffer was added to wells. Following 30 minutes of incubation, d2-labeled IP1 (FRET acceptor) and anti IP1-cryptate (FRET donor) working solution was added to all wells. The plate was incubated at room temperature for 1 hour. The endogenously produced IP1 competes with D2-labeled IP1, generating an inverse relationship between FRET signaling and endogenous IP1 levels. TR-FRET signal was read with 620nm wavelength excitation and 665nm wavelength emission with the Victor3 plate reader (Perkin Elmer). EC50 and Emax values were determined from the dose-response curve.

Data analysis

Graph Pad Prism 5 (La Jolla, CA, USA) was employed for statistical analysis. All data are presented as mean ± SEM. A one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis was performed. Student T-test was conducted to determine the difference in the values of IC50 and Emax

between control and 5 days of AMPH self-administration group. A value of $p \leq 0.05$ was considered statistically significant.

Results

Escalation and Plateau of AMPH Self-Administration over Sessions

Rats self-administered AMPH (0.187 mg/kg/infusion) for five consecutive days in daily 6-h session with a maximum of 40 total injections. Rats showed an escalation in the rate of intake across the first five sessions, indicating that rats gradually take less time to achieve 40 injections of AMPH across the first 5 days of the self-administration session (Figure 2.1A-B). After 5 days of AMPH self-administration, rate of intake plateaued. (Figure 2.1C-D).

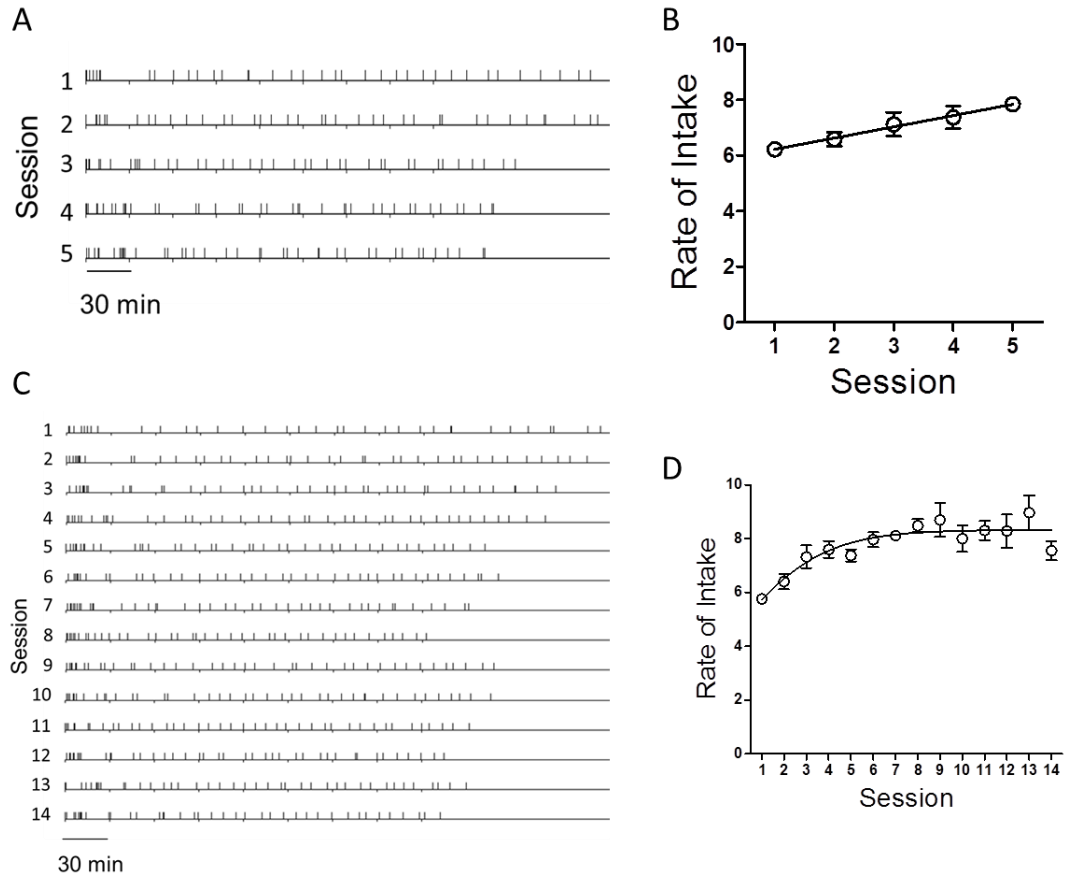


Figure 2.1. AMPH self-administration results in an escalation of intake over 5 sessions, but plateaus over sessions 6-14. Rats received AMPH (0.187 mg/kg/infusion) on a fixed-ratio one schedule of reinforcement for five or fourteen consecutive days with a maximum of 40 infusions per day. (A,C) Representative operant behavior from a rat undergoing 5 or 14 days of AMPH self-administration. Tick marks represent the events that AMPH infusions were delivered. (B) The rates of AMPH intake (infusion/hr/day) from the AMPH self-administering rats were averaged. Rats escalate their rate of intake over the first five days, but plateau over days 6-14 (N=5). Data are presented as mean±SEM.

AMPH self-administration Reduces Total Cholesterol Levels in the PFC and the Striatum

To examine whether psychostimulants altered brain cholesterol content, the PFC tissues were dissected from saline control, 5 days of AMPH, cocaine, and methylphenidate self-administrating rats. Total lysates were prepared and the total cholesterol levels were measured using Amplex Red cholesterol assay kit. A one-way ANOVA revealed that psychostimulant self-administration had a significant effect on cholesterol levels in the PFC, $F(3,19)=4.325$, $p<0.05$ (Figure 2.2A). Post hoc Bonferroni's multiple comparison test revealed significantly decreased cholesterol content following AMPH (56.7±0.2%) and cocaine (42.9±0.1%) self-administration compared to saline control. However, there was no significant change in the cholesterol levels following methylphenidate self-administration. These data suggest that psychostimulants differentially regulate brain cholesterol content.

We further determined whether there was a brain region- and time-dependent effect on brain cholesterol by focusing on AMPH. We examined the brain cholesterol content in the striatum of rats self-administering AMPH for 5 days and 14 days. Total striatal lysate was prepared from control, 5 days and 14 days of AMPH self-administration animals. Cholesterol levels were measured using Amplex Red cholesterol assay kit and normalized by the total protein. A one-way ANOVA revealed that AMPH self-administration had a significant effect

on cholesterol levels in the striatum, $F(2,15)=8.904$, $p<0.01$ (Figure 2.2B). Post hoc Bonferroni's multiple comparison test revealed that both 5 days and 14 days of AMPH self-administration reduced cholesterol levels by 51.5±0.1% and 35.6±0.1%, respectively, when compared to saline controls.

AMPH self-administration increased cholesterol conversion to 24S-OHC

To determine whether reduced brain cholesterol content was due to rapid conversion to 24S-OHC, striatal tissue was dissected from saline control, 5 days, and 14 days of AMPH self-administrating rats. Total lysates were prepared and the total levels of 24S-OHC were measured using an Enzo Life Sciences ELISA assay. 24S-OHC levels were normalized by total protein concentration. Compared to the control group, 24S-OHC levels in the striatum are show a trend towards increased levels by 1.08±.78 fold and 0.42±.44 fold in 5 days and 14 days of AMPH self-administering rats, respectively (Figure 2.3A).

We compared the conversion of cholesterol to 24S-OHC by calculating the ratio of 24S-OHC to cholesterol for each sample. A one-way ANOVA revealed that there was a significant effect of AMPH self-administration on 24S-OHC/cholesterol levels, $F(2,11)=4.402$, $p<0.05$. 24S-OHC/cholesterol ratios were increased 1.56±.0.60 fold and 1.51±0.41 fold in 5 days and 14 days of AMPH self-administering rats, respectively (Figure 2.3B)

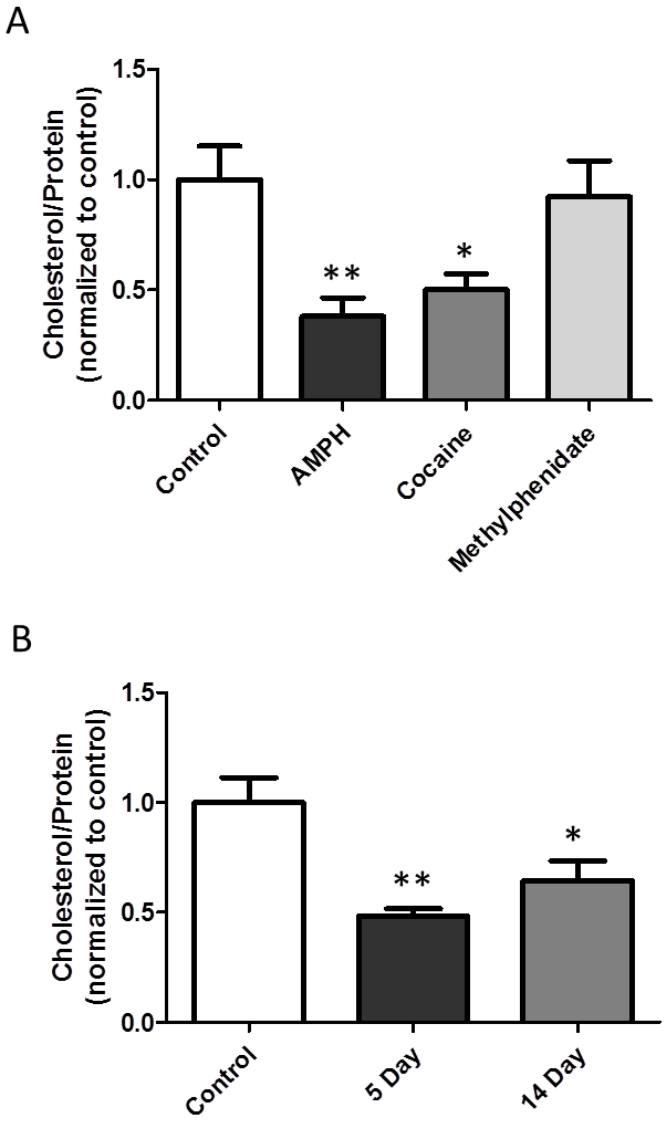


Figure 2.2. Cholesterol depletion following self-administration of psychostimulants. Male, Sprague Dawley rats were allowed to self-administer AMPH (0.187 mg/kg, 5 or 14 days), cocaine (1.5 mg/kg, 5 days), or methylphenidate (0.56 mg/kg, 5 days). The striatal or the PFC tissue was dissected 18 hrs following the last session of self-administration and total lysate was prepared. The total cholesterol levels were measured and normalized by total protein. (A) Cholesterol levels were reduced by 5 days of AMPH and cocaine self-administration in the PFC (N=5, **P<0.01, *P<0.05 vs control, a one-way ANOVA). 5 days of methylphenidate self-administration did not alter cholesterol levels in the PFC (N=5). (B) Cholesterol levels were reduced following 5 and 14 days of AMPH self-administration in the striatum (N=5, **P < 0.01, *P<0.05 vs. control, a one-way ANOVA).

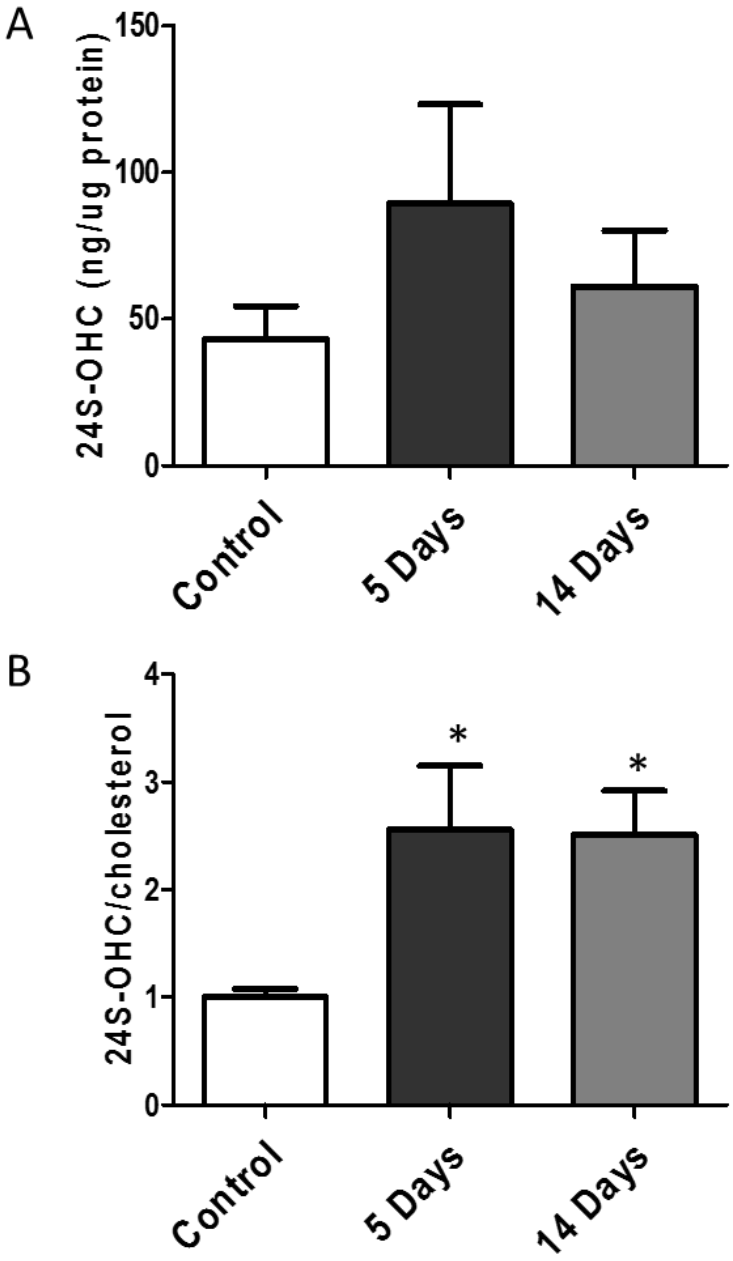


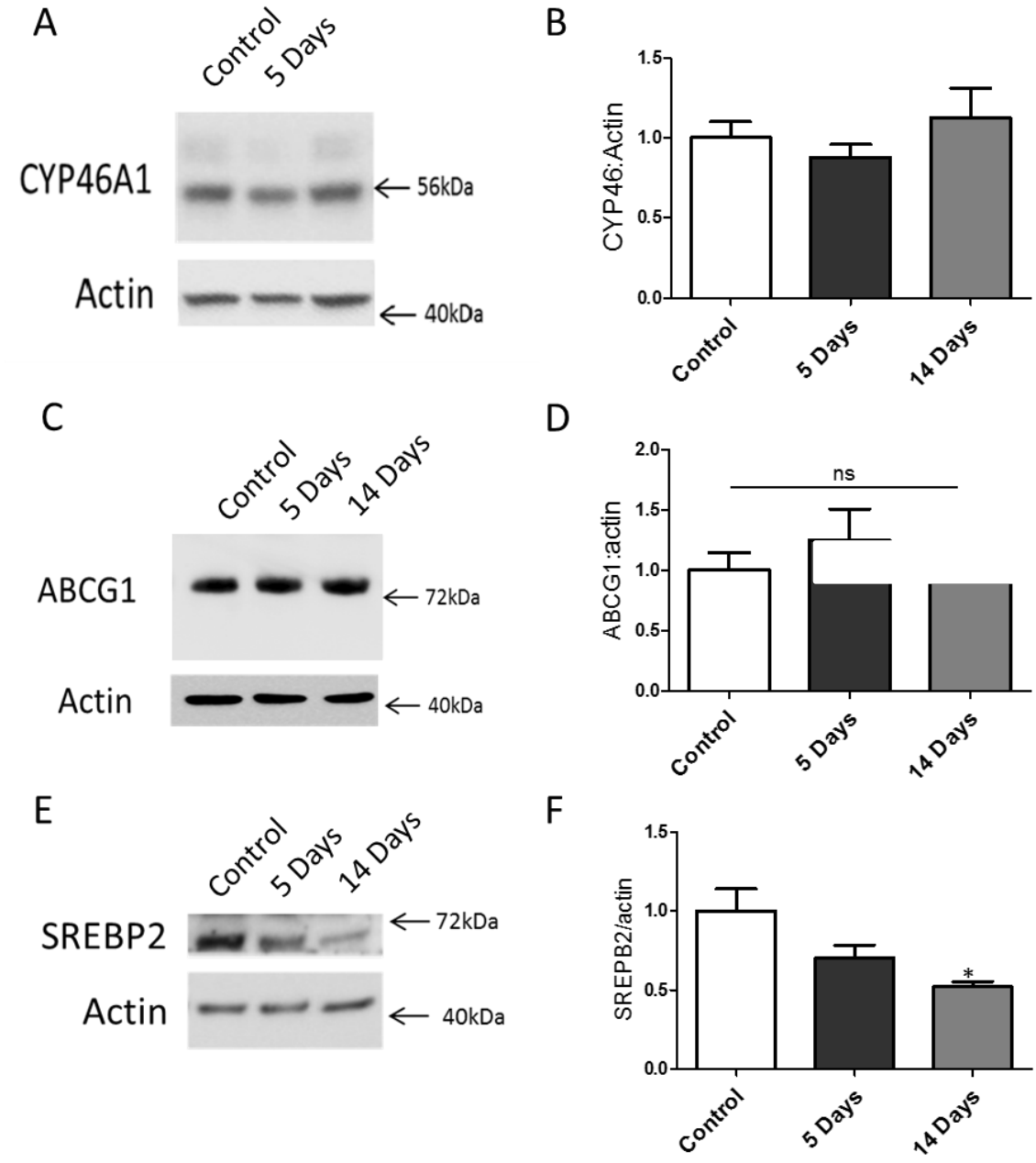
Figure 2.3. Striatal 24(S)-OHC conversion shows an increased trend following 5 days and 14 days of AMPH self-administration. (A) Levels of 24(S)-OHC were showed an increased trend after 5 days and 14 days of AMPH self-administration (N=5). (B) The ratio of cholesterol to 24S-OHC was determined for each animal and normalized to control. AMPH self-administering rats showed a decreased trend in cholesterol:24S-OHC, indicating cholesterol turnover (N=5).

AMPH self-administration impairs the synthesis of cholesterol

To assess the mechanisms for the observed cholesterol depletion, the expression of ABCG1, an isoform of cholesterol transporters in the brain, and CYP46A1, the enzyme that converts cholesterol to 24S-OHC in the brain was determined. Equal protein levels of total lysate from control, 5 days, and 14 days of AMPH self-administration striatal tissue were loaded onto the SDA-Page gel and immunoblotting for CYP46A1 and ABCG1. There was no significant difference in enzyme expression following 5 or 14 day AMPH self-administration when compared to control, $F(2,17)=0.905$ (Figure 2.4A-B). Therefore, the cholesterol depletion following AMPH self-administration is not due to increased expression of the CYP46A1 metabolic enzyme. Expression of ABCG1 transporter was also measured as a potential explanation for the depleted cholesterol levels. Equal protein levels of total lysate from control, 5 days, and 14 days of AMPH self-administration striatal tissue were analyzed by immunoblotting for ABCG1. There was no significant difference in transporter expression following 5 or 14 days of AMPH self-administration when compared to control, $F(2,20)=0.643$ (Figure 2.4C-D). Thus, the observed depleted cholesterol levels were not due to changes in ABCG1.

We next examined how AMPH self-administration altered the expression of SREBP2, a key regulator of cholesterol synthesis. Equal protein levels of total lysate from control, 5 days, and 14 days of AMPH self-administration striatal tissue were loaded onto the SDS-Page gel and immunoblotting for SREBP2. A

one-way ANOVA revealed that AMPH self-administration had a significant effect on the SREBP2 protein levels, $F(2,14)=6.383$, $p<0.05$. Post hoc Bonferroni's test showed that there was a significant decrease of SREBP2 protein after 14 days of AMPH self-administration (Figure 2.4F). Compared to control striatal tissue, 5 days of AMPH self-administration did not have a significant effect on SREBP2, as protein levels were only decreased by 0.30±.08 fold when compared to control (Figure 2.4F). However, 14 days of AMPH self-administration significantly decreased SREBP2 protein levels by 0.48±.03 fold (Figure 2.4F).


A
Control
5 Days
CYP46A1
56kDa
Actin
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B
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1.5
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ABCG1
72kDa
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SREBP2
72kDa
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0.5
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Control
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Figure 2.4. SREBP2 expression was decreased while CYP46A1 and ABCG1 expression were not affected by AMPH self-administration. (A) Representative western blot for CYP46A1 protein levels in control, 5 days, and 14 days of AMPH self-administration striatal tissue probed by mouse anti-CYP46 antibody (sc-136148, Santa Cruz). (B) Quantification of CYP46 protein levels. Protein expression was not significantly different follow 5 or 14 days of AMPH self-administration compared to control (n=6, *P < 0.05 vs. control, a one-way ANOVA). (C) Representative western blot for ABCG1 protein levels in control, 5 days, and 14 days of AMPH self-administration striatal tissue probed by rabbit anti-ABCG1 antibody (LS-C356805, LifeSpan BioSciences). (D) Quantification of ABCG1 protein levels. Protein expression was not significantly different follow 5 or 14 days of AMPH self-administration compared to control (n=6, *P < 0.05 vs. control, a one-way ANOVA). (E) Representative western blot for striatal SREBP2 protein levels from control, 5 days, and 14 days of AMPH self-administration rats probed by rabbit anti-SREBP2 antibody (PA1-338, ThermoFisher). (F) Quantification of SREBP2 protein levels. Protein expression was significantly decreased after 14 days of AMPH self-administration when compared to control (n=5, *P < 0.05 vs. control, a one-way ANOVA). Expression was normalized to actin expression.

AMPH self-administration differentially regulates translocation of Gα subunit isoforms between lipid rafts and non-lipid rafts

To isolate lipid raft microdomains, striatal tissues from control, 5 days and 14 days of AMPH self-administration were homogenized and fractionated by sucrose density gradient ultracentrifugation. Equal volumes fractions were collected. To determine the fractions associated with membrane lipid rafts and nonrafts domains, equal fraction volumes of control striatal tissue were subjected to western blotting. The samples were probed for flotillin and PKCα to indicate raft and nonraft fractions, respectively. Lipid raft domains were successfully isolated in the low density fractions 3-5 while nonraft domains remained in the high density fractions 11-15 (Figure 2.5A).

Following validation of lipid raft isolation, fractionated samples were pooled to combine all raft fractions and all nonraft fractions. Pooled fraction 1 represents the raft fraction while pooled fraction 4 represents the nonraft fraction. Equal volumes of the pooled fractions were analyzed with SDS-page electrophoresis and Gα subunit isoforms were probed with appropriate antibodies. A one-way ANOVA revealed that AMPH self-administration significantly increased the raft association of Gαo, $F(2,14)=3.986$, $p<0.05$. Post hoc Bonferroni's multiple comparison test showed that there was a significant increase of Gαo translocation to lipid rafts after 14 days of AMPH self-administration (Figure 2.5B). Compared to control striatal tissue, 5 days of AMPH self-administration did not have a significant effect on Gαo compartmentalization,

as the raft/nonraft ratio was only increased by .37±.20 fold when compared to control (Figure 2.5C). However, 14 days of AMPH self-administration significantly increased the ratio of Gαo protein in lipid raft/nonraft domains by 1.04±0.37 fold (Figure 2.5C).

Gαi2 proteins also showed a translocation to lipid raft microdomains following AMPH self-administration (Figure 2.5D). A one-way ANOVA revealed that AMPH self-administration significantly altered the translocation of Gαi2 into lipid rafts, $F(2,14)=5.667$, $p<0.05$. Post hoc Bonferroni's multiple comparison test revealed a significant increase in Gαi2 translocation to lipid rafts after 14 days of AMPH self-administration. Five days of AMPH self-administration caused a 2.56±.67 fold increase in raft/nonraft association, and 14 days of AMPH self-administration resulted in a 4.57±1.05 fold increase (Figure 2.5E). This effect is mirrored in Gαi1/3 proteins (Figure 2.5F). A one-way ANOVA revealed that AMPH self-administration significantly alters the translocation of Gαi1/3 into lipid rafts, $F(2,17)=28.57$, $p<0.0001$. Post hoc Bonferroni's multiple comparison test showed that there is a significant increase in Gαi1/3 raft association following 5 days and 14 days of AMPH self-administration. Gαi1/3 compartmentalization was increased by 5.58±0.40 fold following 5 days of AMPH self-administration and by 9.93±0.49 fold after 14 days of AMPH self-administration (Figure 2.5G)**.**

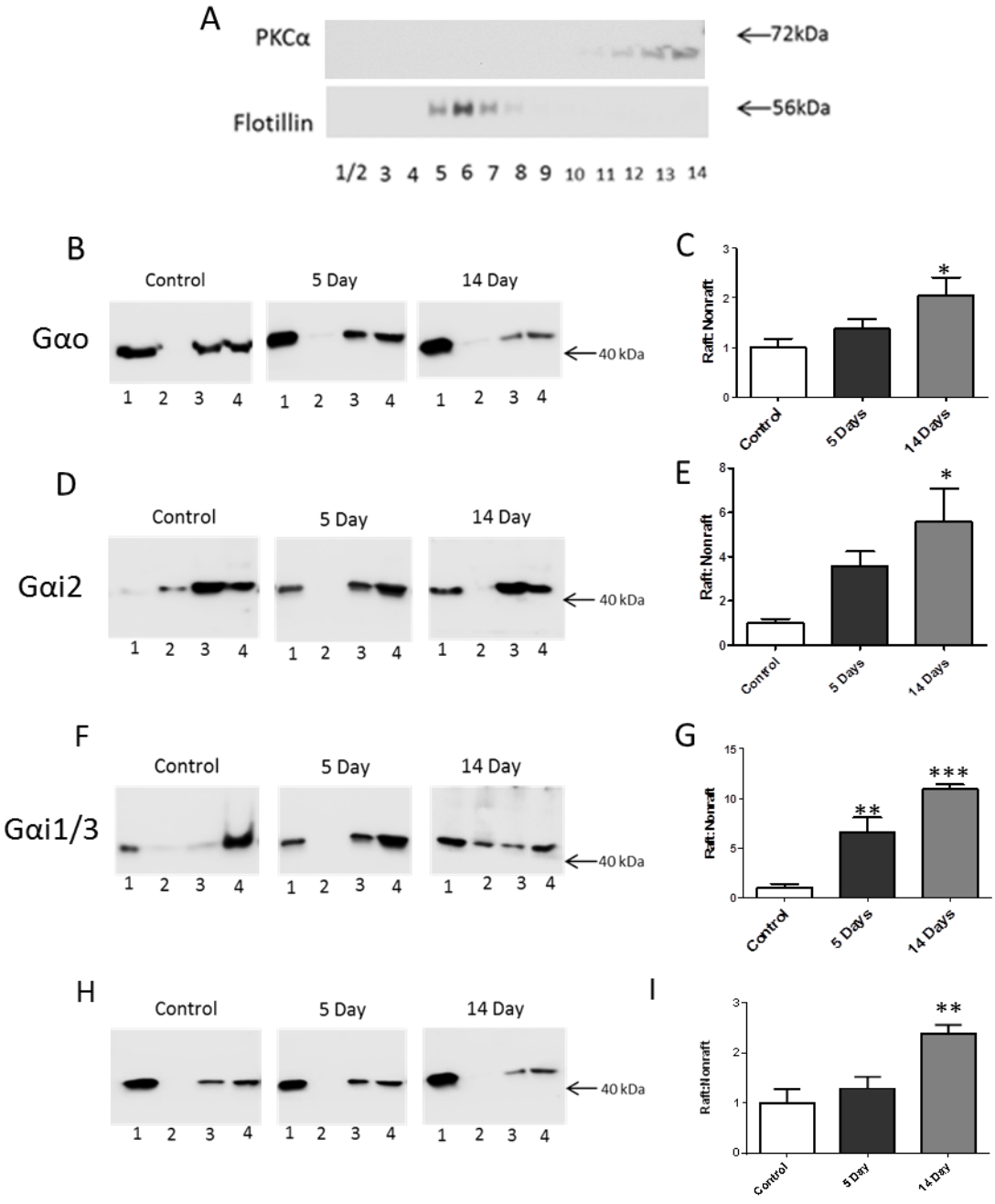
A one-way ANOVA revealed that AMPH self-administration had a significant effect on the translocation of Gαq into lipid rafts, $F(2,17)=9.136$,

$p<0.01$. Post hoc Bonferroni's multiple comparison test showed that there is a significant increase in Gαq raft association after 14 days of AMPH self-administration (Figure 2.5I).

The distribution of AC and PLCβ in lipid rafts and non-lipid rafts is not affected by AMPH self-administration.

To evaluate the effect of AMPH self-administration on downstream signaling effectors of the previously evaluated G-proteins, AC and PLCβ expression and lipid raft association were analyzed. Equal volumes of pooled fractionated samples were separated with SDS-page electrophoresis and analyzed with probed with associated antibodies.

A one-way ANOVA revealed that AMPH self-administration does not affect AC membrane compartmentalization. Neither 5 days nor 14 days of AMPH self-administration appears to have an impact on the distribution of AC in the lipid raft and nonraft membrane domains (Figure 2.6A-B). Additionally, AC appears to be almost exclusively confined to the last two pooled fractions. Likewise, a one-way ANOVA revealed that AMPH self-administration does not affect the distribution of PLCβ, as both 5 days and 14 days of AMPH self-administration do not significantly alter PLCβ nonraft compartmentalization, which is confined to the non-lipid raft fraction (Figure 2.6C-D).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PKCα
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B
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Control
5 Days
14 Days
D
Gαi2
E
F
Gαi1/3
G
H
I

Figure 2.5. Differential translocation of G-proteins to lipid raft domains following AMPH self-administration. (A) Representative Western blots for nonraft fraction identification of equal volume fractions from control striatal tissue following sucrose density gradient fractionation probed by rabbit anti-PKCα antibody (2056S, Cell Signaling) and mouse flotillin antibody (BDB610821, BD Biosciences). Lane 1 represents the raft fraction, lane 4 represents the nonraft fraction. (B) Representative western blot for Gαo protein levels in equal volume control, 5 days, and 14 days of AMPH self-administration striatal tissue probed by mouse anti-Gαo antibody (sc-13532, Santa Cruz). Fraction 1 represents the raft fraction and fraction 4 represents the nonraft fraction for quantification. (C) Quantification of Gαo protein levels. G-protein compartmentalization was significantly different following AMPH self-administration compared to control (n=6, *P < 0.05 vs. control, a one-way ANOVA). (D) Representative western blot for Gαi2 protein levels in equal volume control, 5 days, and 14 days of AMPH self-administration striatal tissue probed by rabbit anti-Gαi2 antibody (sc-7276, Santa Cruz). (E) Quantification of Gαi2 protein levels. G-protein compartmentalization was significantly different following AMPH self-administration when compared to control (n=6, *P < 0.05 vs. control, a one-way ANOVA). (F) Representative western blot for Gαi1/3 protein levels in equal volume control, 5 days, and 14 days of AMPH self-administration striatal tissue probed by mouse anti- Gαi3 antibody (sc-365422, Santa Cruz). (G) Quantification of Gαi1/3 protein levels. G-protein compartmentalization was significantly different following AMPH self-administration compared to control (n=6, ***P < 0.0001 vs. control, a one-way ANOVA). (H) Representative western blot for Gαq/11 protein levels in equal volume control, 5 days, and 14 days of AMPH self-administration striatal tissue probed by rabbit anti-Gαq/11 antibody (sc-392, Santa Cruz). (I) Quantification of Gαq/11 protein levels. G-protein compartmentalization was significantly different following AMPH self-administration compared to control (n=6, **P < 0.05 vs. control, a one-way ANOVA). Expression was normalized to control.

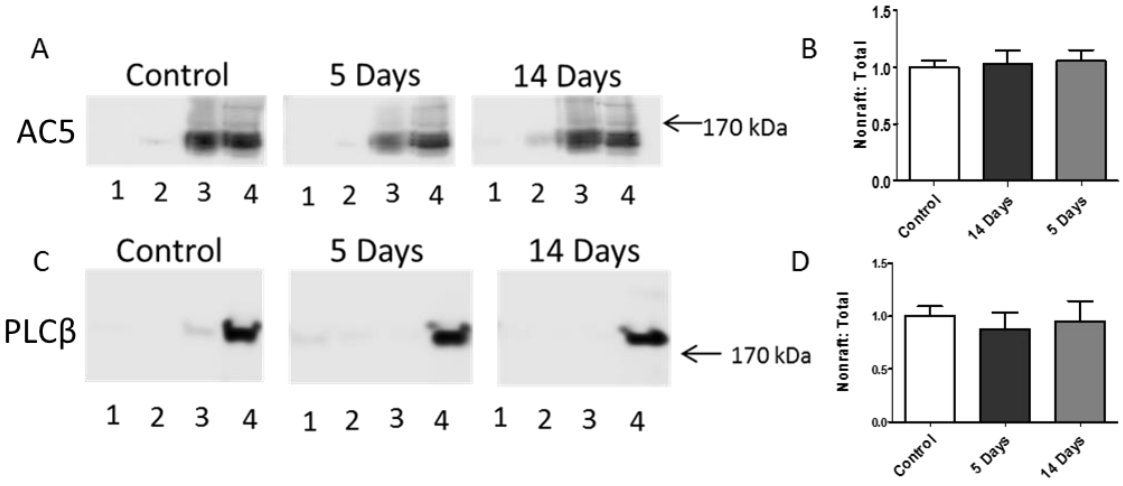

Figure 2.6. Downstream signaling effector compartmentalization is not affected by AMPH self-administration. A) Representative western blot for AC protein levels in equal volume control, 5 days, and 14 days of AMPH self-administration; striatal tissue probed with rabbit anti-AC5 antibody (ABS573, Millipore). (B) Quantification of AC5 protein levels. AC5 compartmentalization was not significantly different following AMPH self-administration compared to control (n=5, *P < 0.05 vs. control, a one-way ANOVA). (C) Representative western blot for PLCβ protein levels in equal volume control, 5 days, and 14 days of AMPH self-administration; striatal tissue probed with rabbit anti-PLCβ antibody (sc-205, Santa Cruz). (D) Quantification of PLCβ protein levels. PLCβ compartmentalization was not significantly different following AMPH self-administration when compared to control (n=3).

AMPH self-administration decreases receptor-mediated Gαi/o inhibition of cAMP production

To determine the effect of AMPH self-administration on downstream G- protein modulated signaling, we measured the ability of dopamine 2/3 receptors (D2/3Rs) and serotonin 5-HT1A/1B receptors (5-HT1AR/1BR) to inhibit forskolin-stimulated cAMP accumulation. Both D2R/D3Rs and 5-HT1A/1BRs are coupled to Gαi/o proteins which bind to AC to inhibit cAMP production. cAMP levels were measured in striatal membrane from control and 5 days of AMPH self-administration rats using LANCE cAMP accumulation assay. There was no difference in forskolin-stimulated cAMP accumulation between control (5.2±1.2 nM) and 5 days of AMPH self-administration (5.4 ±1.2 nM). However, when D2/D3Rs were stimulated by quinpirole, the inhibition of cAMP accumulation was decreased in AMPH self-administering rats. The values of IC50 for quinpirole were 0.44 ±0.10 nM and 1.77 ±0.49 nM, respectively ($t=2.65$, $p<0.05$) (Figure 2.7A-B). Similarly, when 5-HT1A/1BRs were stimulated by 8-OH-DPAT, the inhibition of cAMP accumulation was also decreased in AMPH self-administering rats. The values of IC50 for 8-OH-DPAT were 0.49 ±0.04 nM and 2.60 ±0.76 nM for control and AMPH animals, respectively ($t=2.79$, $p<0.05$) (Figure 2.7C-D).

AMPH self-administration differentially decreases receptor-mediated Gαq stimulation of IP1 production

To determine the effect of AMPH self-administration on downstream G-protein modulated signaling, we measured the ability of serotonin 2A/2C receptors

(5HT2A/2C) and metabotropic glutamate 1/5 receptors (mGluR1/5) to stimulate Gq-mediated IP1 accumulation. 5HT2A/2C receptors and mGluR1/5 receptors couple to Gαq proteins to stimulate PLCβ production of inositol 1,4,5-trisphosphate, a second messenger that is metabolized to IP1. IP1 levels were measured in striatal membrane from control and 5 days of AMPH self-administration rats using IP-One Gq assay. When 5HT2A/2C receptors were stimulated by DOI, an 5HT2A/2C receptor agonist, the values of EC50 for DOI to stimulate IP1 accumulation were not significantly different between control (2.48±0.49nM) and AMPH self-administering rats (2.47±0.21nM) Additionally, the values of Emax for DOI were not significantly different between control (85.0±4.5nM) and AMPH self-administering rats (105.6±5.2nM). In contrast, when mGluR1/5Rs were stimulated by DHPG, the stimulation of IP1 accumulation was less in AMPH self-administration (EC50=50.1 ±10.6 nM) than controls (EC50=82.6 ±4.09 nM), $t=2.862$ $p<0.05$. Additionally, a student t-test revealed that the Emax of DHPG was significantly lower in AMPH self-administration membrane (Emax=138.6±5.9nM) than control membrane (Emax=214.6±5.1nM), $t=9.622$, $p<0.01$ (Figure 2.8D-F).

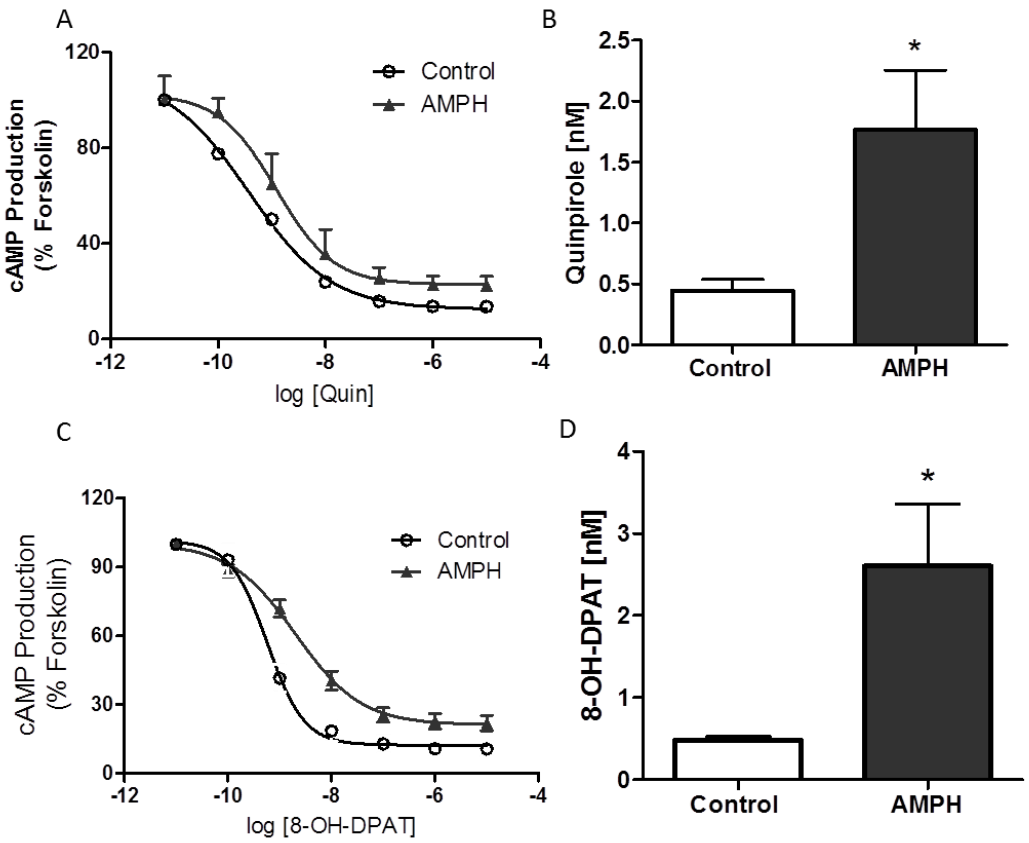

Figure 2.7. AMPH self-administration decreases quinpirole- and DPAT-stimulated cAMP inhibition. (A) Measurement of cAMP accumulation. Membrane was prepared from control and 5 days of AMPH self-administration striatal tissue. cAMP accumulation was measured using the LANCE Ultra cAMP immunoassay kit. Control and AMPH membranes were stimulated with forskolin (10 µM) and increasing doses of quinpirole (0.1 nM-10 µM) or increasing doses of 8OH-DPAT (0.1 nM-10 µM). (B)The inhibition of cAMP accumulation by quinpirole was significantly decreased in 5 days of AMPH self-administration striatal membrane (IC50=1.77 ±0.49 nM) compared to control membrane (IC50=0.44 ±0.10 nM) (*P<0.05). (C-D) The inhibition of cAMP accumulation by 8OH-DPAT was significantly decreased in 5 days of AMPH self-administration striatal membrane (IC50=2.60 ±0.76 nM) compared to control membrane (IC50=0.49 ±0.04 nM) (*P<0.05 N=4). Data were presented as percent response of forskolin treatment.

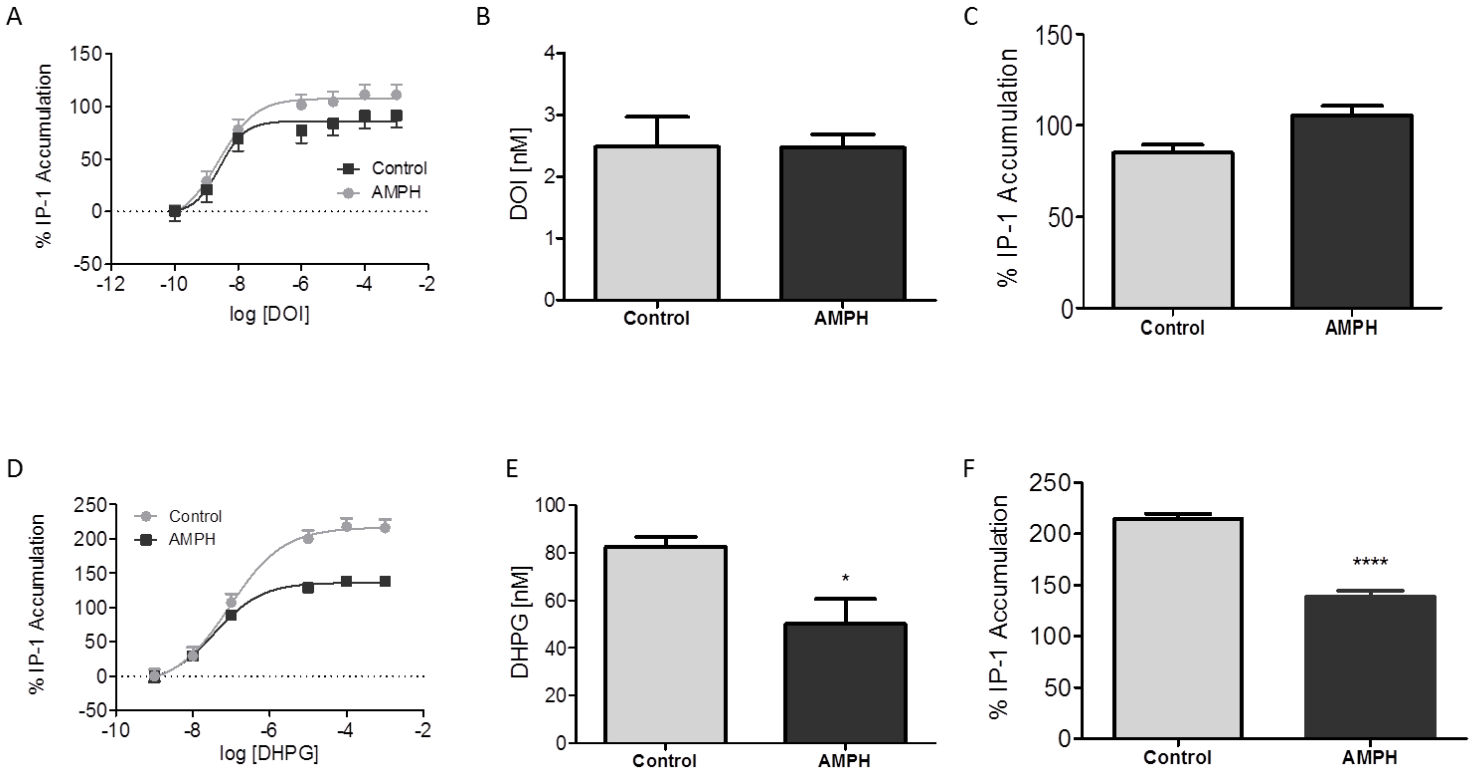

Figure 2.8. AMPH self-administration differentially affects Gαq-coupled receptor-mediated IP1 accumulation (A) Measurement of IP1 accumulation. Membrane was prepared from control and 5 days of AMPH self-administration striatal tissue. IP1 accumulation was measured using IP-One Gq assay kit. Control and AMPH membranes were stimulated with increasing doses of DOI (0.1 nM-10 µM) or increasing doses of DHPG (0.1 nM-10 µM). (A-B)The stimulation of IP1 accumulation by DIO was not significantly different in 5 days of AMPH self-administration striatal membrane (EC50=2.47 ±0.21 nM) compared to control membrane EC50=2.48 ±0.49 nM). (C) The Emax of DOI was not significantly different in AMPH self-administration membrane than control membrane.(D-E) The stimulation of IP1 accumulation by DHPG was significantly lower in 5 days of AMPH self-administration striatal membrane (EC50=50.1 ±10.6 nM) compared to control membrane (EC50=82.6 ±4.01 nM)(*P<0.05, N=4). (F) The Emax of DHPG was significantly lower in AMPH self-administration membrane than control membrane (*P<0.0001).

Conclusions

This study demonstrates the effect of AMPH self-administration on cholesterol level and associated GPCR signaling. For the first time, we showed that short- and long-term AMPH self-administration in a rat model depletes cholesterol levels in the striatum. This effect is mirrored in the PFC following 5 days of cocaine and AMPH self-administration. In contrast, methylphenidate self-administration does not impact cholesterol levels. The decreased striatal cholesterol levels appear to be the result of impaired cholesterol synthesis and increased cholesterol conversion. Cholesterol is converted to 24S-OHC by CYP46A1, at which point it can diffuse across the blood brain barrier. We found that 24S-OHC levels were higher in the AMPH self-administration rats than saline controls, but the expression of CYP46A1 were not affected. This result indicates potential hyperactivation of the enzyme to convert cholesterol to its lipid permeable metabolite. However, AMPH self-administration does not affect the expression of ABCG1, an ABCA1 transporter analog expressed in the brain. Given that neither 5 days nor 14 days of AMPH self-administration altered ABCG1 expression in the striatum, it is unlikely the depleted cholesterol is due to an increase in transport out of the cells. We focused on ABCG1 because ABCG1 is correlated with cholesterol transport in astrocytes and at the BBB (Karten et al. 2006; Kober et al. 2017); however, the expression of ABCG4 and ABCA1 should also be investigated. AMPH self-administration appears to alter cholesterol synthesis. It has previously been shown that SREBP2 cleavage-activating protein (SCAP)-knockout mice show low levels of SREBP2 and impaired synapse

function (van Deijk et al. 2017). We found that protein levels of SREBP2, a transcription factor that regulates cholesterol synthesis, were significantly decreased after AMPH self-administration, which likely contributes to the cholesterol decrease we observed.

Next we looked at the movement of protein subunit isoforms and their effectors between lipid rafts and non-lipid rafts following AMPH self-administration. We found that 5 and 14 days of AMPH self-administration caused a differential shift of Gα subunit isoforms to lipid rafts. Specifically, Gαo, Gαi2 and Gαq/11 translocate to lipid rafts following 14 days of APMH self-administration but raft-associated expression is not significantly different following 5 days of AMPH self-administration. The translocation of Gαo is in agreement with findings that show activated Gαo translocates to lipid rafts in the cerebellum (Yuyama et al. 2007). A different effect is observed in Gαi1/3 proteins- both 5 and 14 days of AMPH self-administration result in increased expression in lipid raft microdomains. As lipid rafts can promote or diminish signaling depending on the localization of signaling receptors and effectors, translocation into rafts may contribute to the altered signaling following AMPH administration. However, when we looked at the compartmentalization of AC, the effector of Gαi/o proteins, there was no difference following AMPH self-administration. Likewise, the compartmentalization of PLCβ, the effector for Gαq, was not altered by AMPH self-administration. Additionally, these effectors remained compartmentalized to high density fractions. Because G-proteins and their effectors appear to be

spatially separated following AMPH self-administration, we hypothesized that signaling would be altered.

To test the functional effects of AMPH self-administration on differential compartmentalization of Gα subunits and effectors, we measured receptor-mediated cAMP production in striatal tissues of control and 5 days of AMPH self-administration rats. Gαi/o proteins inhibit AC to decrease cAMP production. We stimulated two different GPCR classes that coupled to Gαi/o: D2R/D3R and 5-HT1A/1B. cAMP production was increased in AMPH self-administration animals when compared to control following stimulation of both GPCR classes, indicating decreased functionality of the Gαi/o-coupled receptors' ability to inhibit AC. We also examined the effects of AMPH self-administration on the ability of Gαq-coupled receptors to stimulate IP1 production. We stimulated 5HT2A/2C and mGluR1/5 receptors and observed a receptor-dependent effect. While the IP1 accumulation after 5HT2A/2C stimulation was not altered by AMPH self-administration, IP1 accumulation after mGluR1/5 stimulation was significantly decreased. These results contrast previous studies that showed enhanced Gαq activity when localized to lipid rafts (Bhatnagar et al. 2004), but is in agreement with receptor-dependent, lipid raft localization-induced signaling alterations (Nothdurfter et al. 2013)

Due to the lack of availability of reliable receptor antibodies, we were unable to directly evaluate receptor compartmentalization or the effects of AMPH

self-administration on receptor translocation. However, previous studies have characterized receptor localization and signaling in lipid rafts. In-cell biotin transfer assay in HEK293 cells demonstrated that D2R are predominately associated with detergent-insoluble microdomains, like lipid rafts (Sharma et al. 2013). 5HT1A receptors involved in receptor-mediated signaling are largely localized to lipid raft domains, and it is believed that caveolin binds to 5HT2A receptors to recruit Gαq; reduction of cholesterol by MβCD reduces 5HT1A/2A signaling (Renner et al. 2007; Bhatnagar et al. 2004). Differential effects of lipid rafts for ionotropic glutamate receptors AMPA and NMDA are known and studies have shown that cholesterol depletion by MβCD causes mGlu1α receptor translocation out of rafts and diminishes signaling (Korinek et al. 2015; Roh et al. 2014). Therefore, the possibility of receptor translocation out of lipid rafts after AMPH self-administration may contribute the receptor-dependent effects of AMPH self-administration on the downstream signaling of GPCRs.

Our study is the first of its kind to examine directly at the effects of AMPH abuse on brain cholesterol levels. Given the implications brain lipid metabolism has in many neurodegenerative diseases, including Huntington's disease and Alzheimer's disease, investigating the impact of cholesterol on drug abuse is a promising new direction for addiction research. We have shown that AMPH self-administration lowers cholesterol levels in the striatum and PFC; cocaine self-administration has the same effect in the PFC. We also showed that the same AMPH self-administration regimen alters G-protein lipid raft compartmentalization

and cellular signaling. Cholesterol and lipid metabolism may be considered a potential modulator of psychostimulant-induced altered signaling, and more research on this topic is warranted.

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CHAPTER 3

Cholesterol Regulates the compartmentalization and signaling of Gα Subunits in Neuroblastoma 2A Cells

ABSTRACT

While our findings in the previous chapter indicate a parallel effect of cholesterol depletion and altered Gα subunit signaling, a direct causal relationship could not be established from the animal model. Thus, the purpose of the present experiment was to investigate whether cholesterol content directly regulates Gα subunit compartmentalization and signaling using a neuroblastoma 2a (N2A) cells stably expressing the short form of human dopamine D2 receptors (N2A-D2S). Cells were treated with 3mM methyl-β-cyclodextrin (MβCD) for 16 hours, resulting in 50% depletion of membrane cholesterol compared to vehicle treatment. Using sucrose density gradient fractionation, we found that cholesterol depletion caused Gαi2 translocation from non-lipid rafts to lipid rafts without any effect on Gαi1/3. Moreover, altered Gαi2 localization paralleled decreased D2R – mediated Gαi/o inhibition of cAMP production. Taken together, these results indicate cholesterol is a direct mediator of Gα subunit compartmentalization in lipid microdomains and Gα-mediated downstream signaling.

Introduction

Our previous *in vivo* study showed a parallel effect of AMPH self-administration on cholesterol depletion and altered G-protein signaling. To confirm a causal relationship between these variables, we used methyl-β-cyclodextrin (MβCD) to decrease cholesterol levels in neuroblastoma 2A (N2A) cells and evaluated G-protein signaling. Thus, the purpose of this study was to determine whether cholesterol depletion directly impacted the lipid raft compartmentalization of Gα subunits and cAMP accumulation. Information learned from these experiments will help understand the functional consequence of reduced cholesterol by amphetamine (AMPH) self-administration on GPCR-mediated G-protein and downstream signaling.

Materials and Methods

Cell culture

N2A cells stably expressing the short form of human dopamine D2 receptors (N2A-D2S) were generated as previously described (Luessen et al. 2016). N2A-D2S cells were cultured in Opti-MEM media with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and selected with geneticin (400 µg/mL).

Cholesterol depletion

N2A-D2S cells were treated with vehicle or 3 mM MβCD (Sigma Aldrich) 16 hrs in the cultured medium without FBS to deplete cholesterol. Cholesterol reduction was confirmed with using Amplex Red cholesterol kit (ThermoFisher) according to manufacturer's directions.

Sucrose density gradient fractionation

Lipid rafts were isolated by sucrose gradient fractionation as previously described with slight modification (Persaud-Sawin, Lightcap, and Harry 2009). Briefly, vehicle and MβCD-treated cells were lysed in ice-cold MBS buffer with detergent (1% Triton-X 100, protease and phosphatase inhibitor cocktail) and incubated on ice for 30 minutes. Homogenate was sheered through a 20 gauge needle with 20 complete passes before being centrifuged at 1,000 g for 20 minutes at 4 C. Portions of the homogenate were collected as total lysate. The supernatant was mixed with 80% sucrose/MBS to form a 40% sucrose solution

and loaded into a 40%/30%/5% sucrose gradient. The gradient was centrifuged at 46,000 rpm for 18 h at 4 C Beckman Coulter Optima XE-90 Ultracentrifuge and SW55Ti rotor. A distinct band was visible between the 5% and 30% after centrifugation. Fifteen equal fractions were sequentially removed from the top of the gradient and stored at -80°C.

Western blotting of Gα subunit isoforms

Equal sample volumes of each fraction or pooled fractions were loaded onto 8% Tris-Glycine gels for SDS-polyacrylamide gel electrophoresis. Protein was transferred to PVDF membranes. Membranes were incubated overnight with the following primary antibodies: mouse anti-flotillin (BDB610821, BD Biosciences), mouse anti-transferrin (sc-52256, Santa Cruz), mouse anti- Gαi3 (sc-365422, Santa Cruz) and rabbit anti- Gαi2 antibody (sc-7276, Santa Cruz). Appropriate secondary antibodies were used followed by WesternBright ECL (Advansta) chemiluminescence.

Membrane preparation

The membrane was prepared as described previously (Calipari et al. 2014). Briefly, vehicle and MβCD-treated cells were homogenized in Tris lysate buffer containing protease inhibitor cocktail (Sigma Aldrich). Homogenate was sheered through a 20 gauge needle with 20 complete passes. The homogenate was centrifuged at 16,000xg for 15 minutes at 4°C three times, with supernatant being collected and pellet resuspension in Tris buffer after each centrifugation. Supernatants were pooled and centrifuged at 80,000xg for 45 minutes at 4°C.

The supernatant was removed and the pellet was resuspended, passed through a 20 gauge needle for 20 complete passes, and stored at -80°C.

cAMP Assay

Cyclic AMP (cAMP) levels were measured using the LANCE Ultra cAMP assay (PerkinElmer) according to the manufacturer's directions. Briefly, prepared membrane was added to 384-well white, opaque OptiPlates (PerkinElmer). Forskolin (10µM, 30 minutes) and quinpirole (0.1nM-1µM) were added to the wells and incubated for 30 minutes. Eu-cAMP tracer and ULight-anti-cAMP were added to the wells and the plate was incubated at room temperature for 1 hour. TR-FRET signal was read with a 340nm wavelength excitation and 665nm wavelength emission using the Victor3 plate reader (Perkin Elmer). IC50 values were extrapolated from the dose-response curve.

Statistics

Graph Pad Prism 5 (La Jolla, CA, USA) was employed for statistical analysis. All data are presented as mean ± SEM. A student T-test was used to determine the difference in cholesterol depletion, Gα isoform localization, and IC50 and Emax between vehicle and cholesterol depleted groups. A value of $p \leq 0.05$ was considered statistically significant.

Results

MβCD depletes membrane cholesterol in N2A-D2S cells

To confirm appropriate membrane cholesterol depletion in N2A-D2S cells, cells were treated overnight with vehicle or 3 mM MβCD. Cells were harvested and cholesterol content was measured in prepared membranes using Amplex Red cholesterol assay kit. A student t-test confirmed that 3 mM MBCD significantly reduced cholesterol (by 48±4%) compared to vehicle (Figure 3.1).

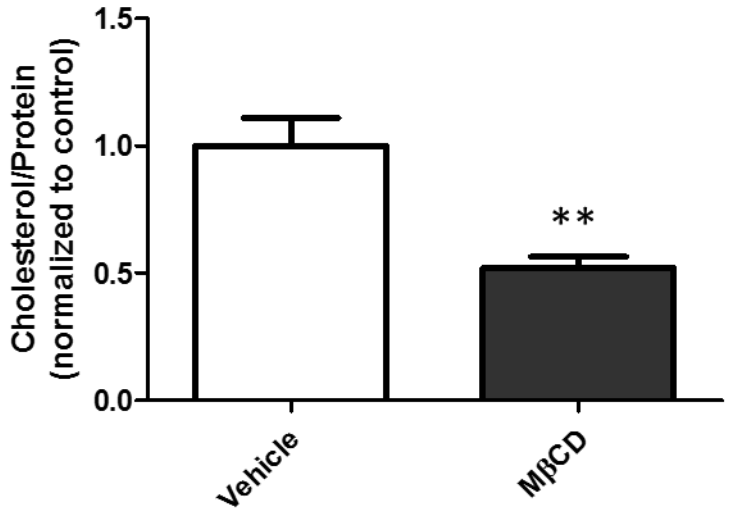

Figure 3.1. MβCD treatment significantly reduces membrane cholesterol levels in N2A cells. Membrane was prepared from N2A-D2R cells treated with vehicle or 3 mM MβCD overnight. Membrane cholesterol levels were measured with Amplex Red cholesterol kit. Membrane cholesterol was significantly reduced after MβCD treatment when compared to control (N=4, **P<0.01 vs. vehicle, student T-test).

Cholesterol depletion differentially regulates translocation of Gα subunit isoforms between lipid rafts and non-lipid rafts

To isolate lipid raft microdomains, vehicle and 3 mM MβCD treated cells were homogenized and subject to high speed sucrose density gradient fractionation as described in Chapter 2. Equal volume fractions were collected. To determine the fractions associated with membrane lipid rafts and nonraft domains, equal fraction volumes of control striatal tissue were subjected to western blotting. The samples were probed with flotillin and transferrin to indicate raft and nonraft fractions, respectively. Lipid raft domains were successfully isolated in low density fractions 4-8, while nonraft domains remained in the high density fractions 13 and 14 (Figure 3.2A). Following validation of lipid raft isolation, fractionated samples were pooled to combine all raft fractions and all nonraft fractions. Equal volumes of the pooled fractions were analyzed with SDS-page electrophoresis and Gα subunit isoforms were probed with appropriate antibodies.

A student t-test revealed that cholesterol depletion by 3 mM MβCD significantly increased the translocation of Gαi2 into lipid rafts, $t=4.244$, $p<0.05$. Compared to vehicle treated cells, cholesterol depletion significantly increased the localization of Gαi2 in lipid rafts by 3.33±0.69 fold (Figures 3.2B-C). However,

a student t-test showed that cholesterol depletion by 3 mM MβCD did not change the localization of Gαi1/3 (Figures 3.2D-E).

Cholesterol depletion by MβCD decreases receptor-mediated Gαi/o inhibition of cAMP production

To determine the effect of cholesterol depletion by MβCD in N2A-D2S cells on downstream G-protein modulated signaling, we measured the ability of D2S to inhibit forskolin-stimulated cAMP accumulation. cAMP levels were measured in N2A-D2S membrane from vehicle and 16-hour treatment of 3 mM MβCD using LANCE cAMP accumulation assay. There was no difference in forsklin-stimulated cAMP accumulation between vehicle and cholesterol depleted cells.

A student t-test showed that when the IC50 values for quinpirole inhibition of cAMP accumulation were higher in cholesterol depleted membrane (IC50=5.72 ±0.41 nM) than vehicle membrane (IC50=1.91 ±0.42 nM), $t=6.42$, $p<0.001$ (Figure 3.3A-B), suggesting that D2S activity is reduced in the presence of cholesterol depletion.

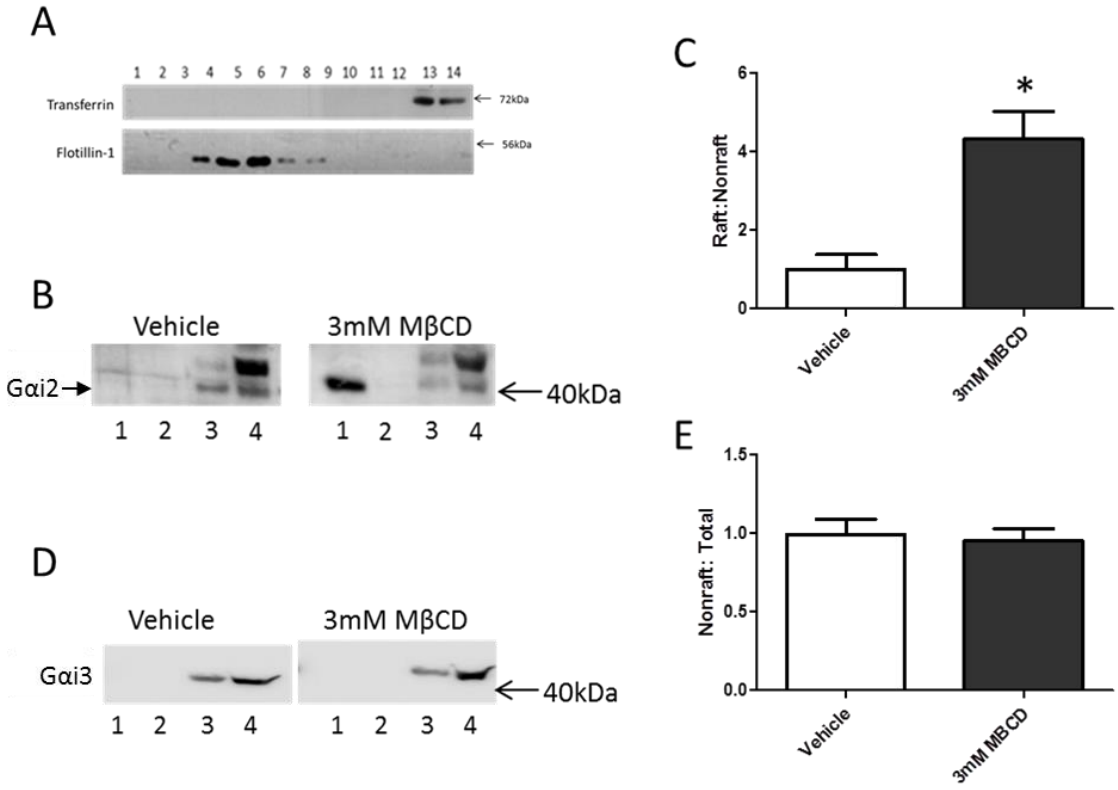

Figure 3.2. Cholesterol depletion by MβCD differentially alters Gα subunit raft association. (A) Representative Western blots for raft and nonraft fraction identification of equal volume fractions from vehicle N2A cells following sucrose density gradient fractionation probed by rabbit anti-transferrin antibody (sc-52256, Santa Cruz) and mouse flotillin antibody (BDB610821, BD Biosciences). (B) Representative Western blots for Gαi2 protein levels in vehicle and MβCD-treated N2A cells probed by rabbit anti-Gαi2 (sc-7276, Santa Cruz). (C) Quantification of Gαi2 protein levels. Gαi2 raft association was significantly different following cholesterol depletion by MβCD when compared to vehicle cells (n=3, *P<0.05 vs vehicle, student t-test). (D) Representative Western blots for Gαi1/3 protein levels in vehicle and MβCD-treated N2A cells probed by rabbit anti-Gαi2 (sc-365422, Santa Cruz). (E) Quantification of Gαi1/3 protein levels. Gα1/3 nonraft association was not significantly altered following cholesterol depletion by MβCD when compared to vehicle cells (n=3, student t-test).

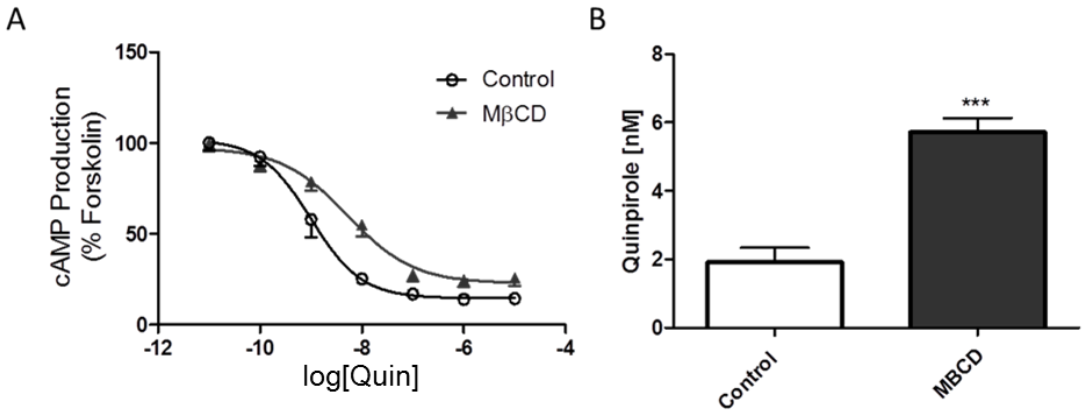

Figure 3.3. Cholesterol depletion by MβCD decreases quinpirole-stimulated cAMP inhibition by D2S. (A) Measurement of cAMP accumulation. Membrane was prepared from vehicle and 3mM MβCD-treated N2A-D2S cells. cAMP accumulation was measured using the LANCE Ultra cAMP immunoassay kit. Vehicle and MβCD membranes were stimulated with forskolin (10 µM) and increasing doses of quinpirole (0.1 nM-10 µM). (B)The inhibition of cAMP accumulation by quinpirole was significantly decreased in MβCD membrane (IC50=5.72 ±0.41 nM) compared to vehicle membrane (IC50=1.91 ±0.42 nM) (***P<0.001).

Conclusions

This study demonstrated a causal relationship between the cholesterol depletion and G-protein compartmentalization in lipid rafts and signaling in neuroblastoma N2A cells. We found that cholesterol depletion caused an increase in Gαi2 raft association when compared to vehicle conditions. However Gαi1/3 nonraft association was not significantly altered, indicating a differential modulatory role of cholesterol on Gαi subunit signaling in N2A cells. The effects of cholesterol depletion and GPCR signaling have been observed in other neurotransmitter systems. Cholesterol depletion can regulate both Gα subunits and receptors. For example, MβCD depletion reduced internalization of Gαs isoforms in C6 glioma cells (J. A. Allen et al. 2009). Activated Gαo isoforms have been shown to translocate to lipid rafts in the cerebellum (Yuyama et al. 2007). Tagged-D2Rs have been shown to compartmentalize to lipid raft microdomains under basal conditions in cultured cells (Sharma et al. 2013). While we were unable to measure D2S localization due to the lack of specific antibodies, other studies have demonstrated a receptor-dependent effect of cholesterol depletion on receptor function. MβCD-induced cholesterol depletion has been shown to increase binding efficiency of type-1 cannabinoid receptors, but decreased cholesterol diminishes Ca2+ signaling of mGlu1α receptors (Bari et al. 2005; Roh et al. 2014). Cholesterol depletion does not have a ubiquitous effect on compartmentalization and signaling of G-protein stimulated by receptors and must be investigated in a receptor-dependent manner.

Cholesterol depletion also affected adenylyl cyclase activity. cAMP levels following D2S activation were increased in MβCD-treated cells. This indicates an impaired ability of D2S and Gαi/o proteins to inhibit AC after cholesterol depletion. There is one report on the tissue-specific effects of cholesterol depletion on basal AC activity. Disruption of lipid rafts with MβCD decreased basal AC activity in dopamine 1/5 receptor-expressing HEK cells, but increased AC activity in renal proximal tubule cells expressing the same receptors (Yu et al. 2014). Our study showed spatial separation of AC and Gαi2 subunits after cholesterol depletion, which prevents the physical interaction required for AC inhibition of cAMP production. Thus, cholesterol depletion not only alters compartmentalization of G-protein and effectors in lipid rafts, but also has functional consequence on G-protein and downstream signaling. These experiments help understand the consequence of altered brain cholesterol on the physical and functional interaction among GPCCR, G-proteins and downstream effectors.

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CHAPTER 4: OVERALL DISCUSSION

The purpose of the current study was to investigate the effects of amphetamine (AMPH) self-administration on striatal cholesterol levels and membrane localization and function of Gα subunit isoforms. The major findings of this study include: a) AMPH self-administration reduced cholesterol content by increasing cholesterol metabolism and decreasing synthesis; b) AMPH self-administration differentially regulated the compartmentalization of Gα subunit isoforms between lipid rafts and non-lipid rafts and had no effect on Gα downstream effectors such as adenylyl cyclase (AC) and phospholipase Cβ (PLCβ); and c) AMPH self-administration altered G-protein-coupled receptor (GPCR)-mediated G-protein signaling. This is the first study to examine the effects of psychostimulant exposure on striatal cholesterol levels. In addition to our i*n vivo* findings, we further demonstrated a direct causal effect of depleted cholesterol levels on altered G-protein compartmentalization in lipid membrane and signal transduction in neuroblastoma 2a (N2A) cells. Collectively, these data suggest that chronic AMPH exposure reduces brain cholesterol content, which directly impacts compartmentalization and signaling of G-protein subunits.

4.1. AMPH self-administration increases cholesterol metabolism and decreases cholesterol synthesis in rat striatum

We found that AMPH self-administration reduces cholesterol content by altering cholesterol synthesis and metabolism. This is the first study suggesting the profound effect of chronic AMPH exposure on brain cholesterol homeostasis.

Because cholesterol cannot pass the blood brain barrier (BBB), brain cholesterol is produced through *de novo* synthesis (Zhang and Liu 2015). A major excretion pathway for cholesterol in the brain is conversion to 24-S-hydroxycholesterol (24-OHC) by cholesterol-24-hydroxylase (CYP46A1). This oxysterol can pass through the blood brain barrier into the circulation (Russell et al. 2009). Thus, one possible mechanism for AMPH-induced reduction in brain cholesterol content is an increase in cholesterol conversion to 24-OHC. We found that there is an increasing trend of 24-OHC levels in AMPH self-administering rats. Moreover, the cholesterol conversion (24S-OHC vs. cholesterol) showed a significant increase in both 5 and 14 days of AMPH self-administering rats, suggesting enhanced cholesterol metabolism in the striatum following AMPH treatment. We further examined the expression level of the cholesterol conversion enzyme CYP46A1 by western blot and found no difference. Thus, the greater cholesterol metabolism was likely due to increased activity of CYP46A1, not the expression level. 24S-OHC has a variety of functions in the brain and is implicated in neurodegenerative diseases like Alzheimer's Disease (Noguchi et al. 2014). For example, 24S-OHC has been shown to suppress amyloid-beta production by inducing ER chaperone protein to reduce Alzheimer's Disease progression (Urano et al. 2013). However, high levels of 24S-OHC can induce apoptosis in primary cortical neuronal cells (Yamanaka et al. 2011). Future study is warranted for investigation of the functional consequence of increased 24-OHC in the striatum following chronic psychostimulant exposure.

Cholesterol can also be excreted via ABC transporters including ABCA1, ABCG1, and ABCG4, but the specific functions of each isoform are still being investigated. They are differentially expressed in neurons and astrocytes and mediate cholesterol efflux at the plasma membrane by releasing cholesterol directly onto cerebral spinal fluid APOA1 lipoproteins (Kimet al. 2008; Zhang and Liu 2015). In primary cortical astrocytes and neurons from Sprague-Dawley rats, it has been shown that ABCA1 and ABCG1 are the primary transporters in cholesterol efflux from astrocytes, while ABCG4 is responsible for cholesterol efflux from neurons (Chen et al. 2013). ABCA1 is known to play a critical role in brain cholesterol metabolism as mice lacking ABCA1 in the CNS have lower levels of brain cholesterol content and increased brain uptake of esterified cholesterol from the plasma relative to control mice (Karasinska et al. 2009). It has been shown in porcine brain capillary endothelial cells, an *in* vitro model of the BBB, that ABCG1 is an important regulator of cholestrol metabolism at the BBB, as siRNA silencing of ABCG1 reduced cholesterol efflux (Kober et al. 2017). ABCG1 expression, but not ABCA1 expression, has been correlated with cholesterol content as cholesterol enrichment in cultured cerebellar murine astroglia increases ABCG1 expression but had no effect on ABCA1 expresion relative to control (Karten et al. 2006). Thus, we investigated the effects of AMPH self-administration on ABCG1 expression. We found that AMPH self-administration did not alter the expression level of ABCG1; however, the expression of ABCG4 and ABCA1 as well as the activity of the transporters to

remove cholesterol needs to be further examined to confirm that AMPH self-administration did not alter ABC-mediated cholesterol efflux.

To determine whether AMPH self-administration attenuated cholesterol synthesis, which may contribute to cholesterol reduction, we examined the expression level of sterol-regulatory element-binding protein 2 (SREBP-2). SREBP-2 is a membrane-bound transcription factor that regulates transcription of multiple genes in the cholesterol biosynthesis pathway including those for low density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl coenzyme A reductase, an intermediate enzyme in cholesterol synthesis (Brown and Goldstein 1997). Activation of SREBP-2 occurs when ER cholesterol levels are low and initiates a transcription cascade of enzymes required for cholesterol synthesis (Field et al. 2001). SREBP cleavage-activating protein (SCAP) knockout mice show reduction in SREBP-2 levels and cholesterol content, suggesting that SREBP-2 is involved in cholesterol synthesis (Camargo et al. 2012). Because cholesterol synthesis is a multistep process by many enzymes regulated by SREBP-2, measuring SREBP-2 levels is a useful method to indirectly measure cholesterol synthesis without investigating every enzyme involved. We found decreased SREBP2 levels following AMPH self-administration in the striatum, suggesting decreased *de novo* synthesis of cholesterol. Thus, SREBP-2 dependent cholesterol synthesis is also compromised by AMPH self-administration.

Herein, we reported for the first time that AMPH self-administration decreased cholesterol content by increasing its metabolism and decreasing its synthesis. Future research should focus on examining the activity of CYP46A1 and ABCG1. In addition to the observation that AMPH self-administration reduced cholesterol content in the striatum, we found that the cholesterol content in the prefrontal cortex was also reduced by self-administration of AMPH and cocaine. However, methylphenidate self-administration did not alter the cholesterol content. These data suggest that reduced cholesterol content may be a generalized consequence of psychostimulants with an exception of methylphenidate.

4.2. AMPH self-administration preferentially targets Gαi, Gαo and Gαq localization in lipid rafts

Cholesterol functions as a major stabilizer of membrane lipid rafts by acting as a spacer between hydrocarbon chains of sphingolipids to maintain raft integrity (Simons et al. 2002). Depletion of cholesterol in lipid rafts with methyl-β-cyclodextrin (MβCD) disrupts GPCR-mediated G-protein signaling, including angiotensin II receptor, $GABA_A$ receptor, and mu-opioid receptor (Adebiyi et al. 2014; Levitt et al. 2009; Nothdurfter et al. 2013).

GPCR association with lipid rafts has functional consequence on receptor-mediated G-protein signaling. For example, cannabinoid type 1 receptors show enhanced signaling when lipid rafts are disrupted by MβCD in rat C6 glioma cells

(Bari et al. 2005). In contrast, serotonin 2A receptor (5-HT2AR) signaling is attenuated when caveolin-1, a scaffolding protein enriched in caveolae of lipid rafts, was knocked down (Bhatnagar et al. 2004). Caveolin is hypothesized to facilitate the coupling between 5-HT2A and Gαq. These data suggest that GPCR-mediated G-protein signaling is influenced by the cholesterol content in the membrane. Additionally, Resnick's laboratory has shown that Gαs proteins translocate out of lipid raft domains following anti-depressant treatment, which may contribute to the therapeutic effects (Zhang and Rasenick 2010; Donati et al. 2015; Czysz et al. 2015). For the first time, our study has shown that AMPH self-administration differentially regulates the compartmentalization of Gα subunit isoforms into lipid rafts. AMPH self-administration caused pronounced translocation of Gαi/o subunits from non-lipid rafts to lipid rafts in both 5 and 14 days of AMPH-treated animals. The increased Gαq translocation to lipid rafts occurred only after 14 days of AMPH self-administration. These data suggest that Gαi/o subunits are more vulnerable to AMPH self-administration while Gαq subunits are more resistant to the effect. Given that many GPCRs in the striatum are coupled to Gαi/o and Gαq, the altered compartmentalization of Gα subunits would have a significant impact on the signaling of GPCRs that are coupled to Gαi/o and Gαq.

We further examined whether there is a direct causal relationship between cholesterol content and Gαi/o subunit compartmentalization in lipid rafts in neuroblastoma N2A cells. MβCD treatment resulted in 50% depletion of

cholesterol in N2A cells. Importantly, cholesterol reduction preferentially shifted Gαi2 from non-lipid rafts to lipid rafts, whereas there was no change in Gαi1/3. In AMPH self-administering animals, we found that both Gαi2 and Gαi/3 were translocated into lipid rafts. The discrepancy between N2A cells and animal brains is likely due to the difference in the model system.

4.3. AMPH self-administration causes spatial separation of Gα subunit isoforms and their effectors accompanied by altered downstream G-protein signaling

Ligand binding to GPCRs activates G-proteins, which subsequently activates an effector enzyme to induce an intracellular signaling cascade. Thus, GPCR signaling requires the physical interaction among GPCRs, G-proteins and effectors (e.g. adenylyl cyclase and phospholipase Cβ). These proteins preferentially reside in lipid rafts or non-lipid rafts at the basal condition. For example, in drug naïve animals, Gαo and Gαq are predominantly expressed in lipid rafts, whereas Gαi2 is predominantly expressed in non-lipid rafts. Thus, cholesterol depletion would alter the physical property of lipid rafts resulting in differential changes in the localization of proteins that reside in lipid rafts and non-lipid rafts. We found that adenylyl cyclase (AC) and phospholipase Cβ (PLCβ), which are effectors of Gαi/o and Gαq, respectively, were not affected by AMPH self-administration. This is in contrast to our observation of a pronounced shift of Gαi/o and Gαq into lipid rafts. Thus, AMPH self-administration causes spatial separation of Gαi/o/q proteins and their effectors

We further examined the effects of 5 days of AMPH self-administration on activation or inhibition of second messenger systems. It was previously believed that components involved in AC-mediated cAMP production were uniformly distributed across the membrane for all the receptors; however, recent studies show that compartmentalization of GPCR, G-protein, and AC in lipid rats and/or non-lipid rafts contribute to cAMP production (Agarwal et al. 2014). Treatment with antidepressants has been shown to potentiate AC activity because Gαs proteins translocate out of lipid rafts, increasing their interaction with AC (Zhang and Rasenick 2010). Our study showed that 5 days of AMPH self-administration impaired the inhibition of AC-mediated cAMP production following Gαi/o-coupled GPCRs activation. The ability of dopamine type 2/3 receptors (D2/3Rs) and 5-HT1A/1B receptors to inhibit cAMP production was significantly attenuated in AMPH self-administrating animals compared to saline controls. Because Gαi/o subunits translocate into lipid rafts after AMPH self-administration, while AC remains in the nonraft domains, the physical interaction between the two proteins that is required for signal transduction is prevented. The same effect was observed in cholesterol depleted N2A-D2S cells. Quinpirole stimulation of D2S in cholesterol depleted cells resulted in more cAMP, or less inhibition of AC, than vehicle-treated cells. While AC association with lipid rafts was not determined in the N2A-D2R cells, Gαi2 shows a translocation to lipid raft microdomains after cholesterol depletion. Thus, this shift of Gαi/o proteins into lipid rafts likely

decreases their physical interaction with AC, resulting in decreased GPCR-mediated inhibition of cAMP production.

We also measured the effect of AMPH self-administration on the interaction between Gαq and PLCβ. The effects of raft association on PLCβ are not well-characterized, but it has been shown that recruitment of PLCβ into lipid rafts is needed for Ca2+ wave propagation (Weerth et al. 2007). This study demonstrated that AMPH self-administration compartmentalizes Gαq to lipid raft microdomains, while PLCβ remains in non-lipid raft regions. The physical interaction of PLCβ and Gαq is needed to stimulate the production of second messengers diacyl glycerol and inositol 1,4,5-trisphosphate. By measuring inositol monophosphate (IP1) accumulation, a stable downstream metabolite of 1,4,5-trisphosphate, following 5-HT2A/2C and mGluR1/5 activity, we observed a receptor-dependent effect. Activation of 5HT2A/2C showed no significant difference between saline control and AMPH self-administrating animals. However, stimulation of mGluR1/5 resulted in less striatal IP1 accumulation in AMPH self-administrating animals compared to controls. This reduction indicates impaired Gαq signaling. The receptor-dependent effect we observed points to the possibility of differential receptor-raft association following AMPH self-administration. Further studies should be carry out to determine the receptor localization using radioligand binding assay.

While there is little evidence implicating an association of dysregulated cholesterol homeostasis and addiction, many neurodegenerative diseases have been linked to brain cholesterol. As previously mentioned, amyloid-beta production is suppressed by 24S-OHC; additionally, individuals with the ε4 ApoE variant are predisposed to develop Alzheimer's disease(Martín et al. 2014). Clinical trials have shown that treatment with statins to reduce cholesterol seems to prevent development of dementias, including Alzheimer's (Jick et al. 2000). Cholesterol is also implicated in Huntington's disease, as brain cholesterol metabolism is impaired in patients with the disease (Valenza et al. 2005). It is believed that SREBP is inhibited by mutant huntington, resulting in lower levels of ApoE available to transport cholesterol from astrocytes to neurons (Valenza et al. 2010). While clinical studies have yet to come to a consensus on the role of cholesterol in Parkinson's disease, in vitro studies show that cholesterol could contribute to progression of the disease (Martínet al.Dotti 2014). Treatment with MβCD and statins to deplete cholesterol prevents α-synuclein aggregation, a hallmark of Lewy bodies in Parkinson's patients (Bar-On et al. 2006; Koob et al. 2010). Given the overlap between symptoms and mechanisms of neurodegenerative diseases and drug addiction (Iacovelli et al. 2006), the evidence presented in this study and the pre-established role of cholesterol in brain disease provides support for cholesterol as a potential regulator of addiction.

Conclusion:

Taken together, the *in vivo* and *in vitro* aspects of this study identify cholesterol as a modulator of Gα subunits, and therefore GPCR signaling. Our animal study demonstrated a parallel effect of decreased cholesterol levels due to decreased synthesis and increased metabolism and altered GPCR signaling. Our cell culture study demonstrated a direct causal relationship between cholesterol levels and altered G-protein compartmentalization and signaling. Better understanding of the regulatory role of cholesterol in the brain could potentially provide insight into numerous brain diseases, including drug addiction and neurodegenerative diseases, as well as serve as a potential novel therapeutic target.

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CURRICULUM VITAE

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EDUCATION

2016-Present	Wake Forest School of Medicine Winston-Salem, NC M.S. in Biomedical Science Candidate Integrated Physiology and Pharmacology
2012-2016	Wake Forest University Winston-Salem, NC B.S. *cum laude* in Biology

EMPLOYMENT HISTORY

May 2016-Present	Graduate Research Assistant Department of Physiology and Pharmacology Wake Forest School of Medicine Laboratory of Rong Chen, PhD
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HONORS AND AWARDS

2012-2015	Dean's List, Wake Forest University
2015	ASPET Zannoni Summer Undergraduate Research Fellowship
2016	ASPET Undergraduate Student Travel Award
2016	STARR Travel Grant Wake Forest University
2016	1st place for Undergraduate Student Poster Presentation at ASPET Neuropharmacology Annual Meeting 2016

2017	Alumni Travel Award Wake Forest Graduate School of Arts and Sciences

SERVICE

April 2013-Present	Delivering Equal Access to Care: DEAC Clinic Wake Forest School of Medicine
Aug 2013-Present	Wake Forest Emergency Medical Services Aug 2013-Present: EMT-B May 2014-May 2015: Publicity Lieutenant May 2015-May 2016: Chief May 2016-Dec 2017: Equipment Lieutenant
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PROFESSIONAL MEMBERSHIPS

2015-Present	American Society for Pharmacology and Experimental Therapeutics

PUBLICATIONS

Luessen DJ, **Hinshaw TP**, Sun H, Howlett AC, Marrs G, McCool BA, and Chen R. RGS2 modulates the activity and internalization of dopamine D2 receptors in neuroblastoma N2A cells. Neuropharmacology (2016)

Hinshaw TP, Luessen DJ, Sun H, Yeager MM, Jones SR, Chen R. Amphetamine self-administration reduces membrane cholesterol levels and alters G protein membrane compartmentalization in rat brains. (In preparation- anticipated submission May 2017)

Hinshaw TP, Luessen DJ, Sun H, Jones SR, Chen R. Amphetamine self-administration alters dopamine transporter membrane compartmentalization in rat brains. (In preparation- anticipated submission July 2017).

ABSTRACTS

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Hinshaw TP, Yeager MM, Luessen DJ, Sun H, Jones SR, Chen R (2017). Cholesterol depletion and G Protein translocation in rat striatum following amphetamine self-administration. The Experimental Biology 2017, April 22-26, Chicago, IL.

PROFESSIONAL REFERENCES: Available upon request