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THC-MEDIATED INDUCTION OF ΔFOSB AND ITS MODULATION OF CB1R SIGNALING AND ADAPTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By
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Abbreviations

2-AG	2-arachindonoylglycerol
A2A	adenosine 2a
ABH4	α/β-hydrolase 4
ac	anterior commissure
AC	adenylyl cyclase
AEA	arachidonoylethanolamine
AKT	thymoma viral proto-oncogene
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMYG	Amygdala
ANOVA	analysis of variance
BLA	basolateral amygdala
CAMKII	calmodulin-dependent protein kinase II
CB_1R	cannabinoid type 1 receptor
CBLM	Cerebellum
CCK	Cholecystokinin
CDK5	cyclin dependent kinase 5
CNS	central nervous system
CP55,940	(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol
CPU	caudate-putamen
CREB	cAMP response element binding protein
D1R	dopamine type 1 receptor
D2R	dopamine type 2 receptor
DAG	*sn*-1-acyl-2-arachidonoylglycerol
DAGL	*sn*-1-acyl-2-arachidonoylglycerol lipase
DARPP-32	dopamine- and cAMP-regulated phosphoprotein of Mr32 kDA
DOR	delta-opioid receptor
DSE	depolarization-induced depression of excitation
DSI	depolarization-induced depression of inhibition
ELK	ETS domain-containing protein
ERK	extracellular signal-regulated kinase
FAAH	fatty acid amide hydrolase
FAN	factor associated with neutral sphingomyelinase
Fra	fos related antigen
FRET	fluorescence resonance energy transfer
GABA	gamma amino-butyric acid
GASP	G-protein-associated sorting protein
GDE1	glycerophosphodiesterase
GDP	guanosine diphosphate
GSK3β	glycogen synthase kinase-3β

GP	globus pallidus
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
GTPyS	guanosine 5'-O-[gamma-thio]triphosphate
EGR	early growth response protein
HDAC	histone deacetylase
HIP	hippocampus
IEG	immediate early gene
ICSS	intra-cranial self-stimulation
ir	immunoreactivity
JNK	c-Jun N-terminal kinase
KOR	kapp-opioid receptor
LA	lateral amygdala
MAGL	monacylglycerol lipase
MAPK	mitogen activated protein kinase
MOR	mu-opioid receptor
MPE	maximal percent effect
NAC	nucleus accumbens
NAPE	N-acyl-phosphatidylethanolamines
NMDA	N-methyl-D-aspartate
GLUR	(NMDA) receptor 1 glutamate receptor subunit
PAG	periaqueductal gray
PET	positron emission topography
PKA	protein kinase A
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
RAF-1	v-raf-1 murine leukemia viral oncogene homolog 1
SCH23390	(*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride
SCH39166	(6a*S*-*trans*)-11-Chloro-6,6a,7,8,9,13b-hexahydro-7-methyl-5*H*-benzo[*d*]naphth[2,1-*b*]azepin-12-ol hydrobromide
SN	substantia nigra
SR141716A	N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide
SRF	serum response factor
STEP	striatal-enriched protein tyrosine phosphatase
THC	Δ^9-tetrahydrocannabinol
TRPV	vanilloid type

VTA	ventral tegmental area
WIN55,212-2	R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate

Abstract

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The main psychoactive and therapeutic effects of Δ^9-tetrahydrocannabinol (THC) are mediated through cannabinoid type 1 receptors (CB_1Rs). The therapeutic uses of THC are mitigated by the development of tolerance to these therapeutic effects, whereas tolerance does not readily develop to some of the side-effects of THC, like motor impairment and reward. The development of tolerance occurs through adaptations at CB_1Rs, which include desensitization (G-protein uncoupling) and downregulation (receptor degradation). Brain region-dependent differences in THC-mediated adaptations are proposed to explain the differences in tolerance to various THC-mediated effects. These studies focused on whether ΔFosB, a stable transcription factor, could regulate CB_1R adaptations since regions resistant to CB_1R adaptations, like the basal ganglia, exhibit THC-mediated ΔFosB induction. The studies in this dissertation tested the hypothesis that THC-mediated induction of ΔFosB is regulated through interactions between cannabinoid and dopamine systems and that brain region-dependent differences in ΔFosB transcriptional regulation could explain some aspects of long-term CB_1R signaling and CB_1R adaptations. Results determined that THC induced ΔFosB primarily in forebrain areas, like striatum, that are innervated by midbrain dopamine neurons. An inverse, brain region-dependent

correlation was found between CB_1R desensitization and ΔFosB induction. Studies utilizing bitransgenic mice with overexpression of ΔFosB, or its dominant negative ΔcJun, determined that ΔFosB regulates CB_1R signaling and reduces CB_1R desensitization. Based on this regional profile, studies determined the role of dopamine signaling in THC-mediated ΔFosB induction. Results showed that THC-mediated induction of ΔFosB required dopamine type 1 receptors, but not the dopamine-and cAMP-dependent phosphoprotein of Mr 32kDA. Finally, the functional consequences of THC-mediated ΔFosB induction were assessed by measuring expression of known targets of ΔFosB following both acute and repeated THC administration. Results found that, in prefrontal cortex, known targets of ΔFosB exhibited functionally different signaling expression patterns when comparing acute THC with THC-challenge in THC-experienced mice, which enhanced ΔFosB induction. These studies establish a role for ΔFosB in regulating long-term CB_1R signaling/adaptation following repeated THC administration and could have implications for changes in the effects of THC during repeated administration, including the development of differential tolerance to motor-impairing and rewarding effects of THC versus other pharmacological effects.

Introduction

0.1 History of cannabis use

Marijuana is derived from the *Cannabis sativa* plant, which provides food from its seeds, fiber from its stalks and intoxicating preparations from its flowers, leaves and resins. Marijuana was first used in making fibers, known as hemp, as early as 8000 B.C. (Kabilek, 1960). Hemp's most important uses historically were for bow strings and rope for sailing, with minor uses for paper and clothing. Although it is not clear when the marijuana plant was first used for medicine, historical records indicate that the first prescribed uses were around 2737 B.C. by Shen Neng, a Chinese emperor. He recommended the use of marijuana tea for gout, malaria, beriberi, rheumatism and poor memory (Abel, 1980). The use of marijuana for medicine migrated to India, and it was listed in the Indian text *Artharvaveda* as a holy plant that relieved stress. Pliny the Elder, a Roman philosopher, also mentioned the use of marijuana as a painkiller, although the side effect of impotency was noted. Pedacius Dioscrides, a physician in Nero's army compiled a pharmacopoeia in 70AD that listed marijuana for earaches and other medical applications. Side effects were also noted for the use of marijuana; Ibn Wahshiyah's Arabic text *On Poisons* mentioned that *hashish* produced blindness and muteness.

W. B. O'Shaunessey, an Irish physician serving in the British army, familiarized the medicinal properties of marijuana to the Western world after studying it in India and produced a treatise in 1839 describing its medicinal properties (Adams and Martin, 1996). His studies focused on the safety of marijuana in animals and determined that even high doses did not produce death (Snyder, 1971). He recommended marijuana as an anticonvulsant, analgesic, antiemetic and antianxiety agent, promoting its use in both the United Kingdom and throughout

Europe (Mechoulam and Feigenbaum, 1987). The Ohio State Medical Society listed several medicinal uses for marijuana in 1860. By the 1900s, pharmaceutical companies like the Squibb Company, Eli Lilly and Parke-Davis provided tinctures of the extract. The disuse of marijuana as medicine coincided with the Marijuana Tax Act of 1937, which resulted in the removal of marijuana from the U.S. Pharmacopoeia in 1941 and criminalization of marijuana in every state. This also ended most research into marijuana for medicinal purposes in the United States and abroad. In the 1960s, states began to decriminalize marijuana use, but criminalization of marijuana returned in the 1980s. More recently, several states have approved marijuana for medicinal and recreational uses. Marijuana is the most commonly abused illicit drug, with 46% of Americans having tried marijuana and ~9% of marijuana users considered dependent based on DSM-IV-R criteria (SAMHSA, 2010).

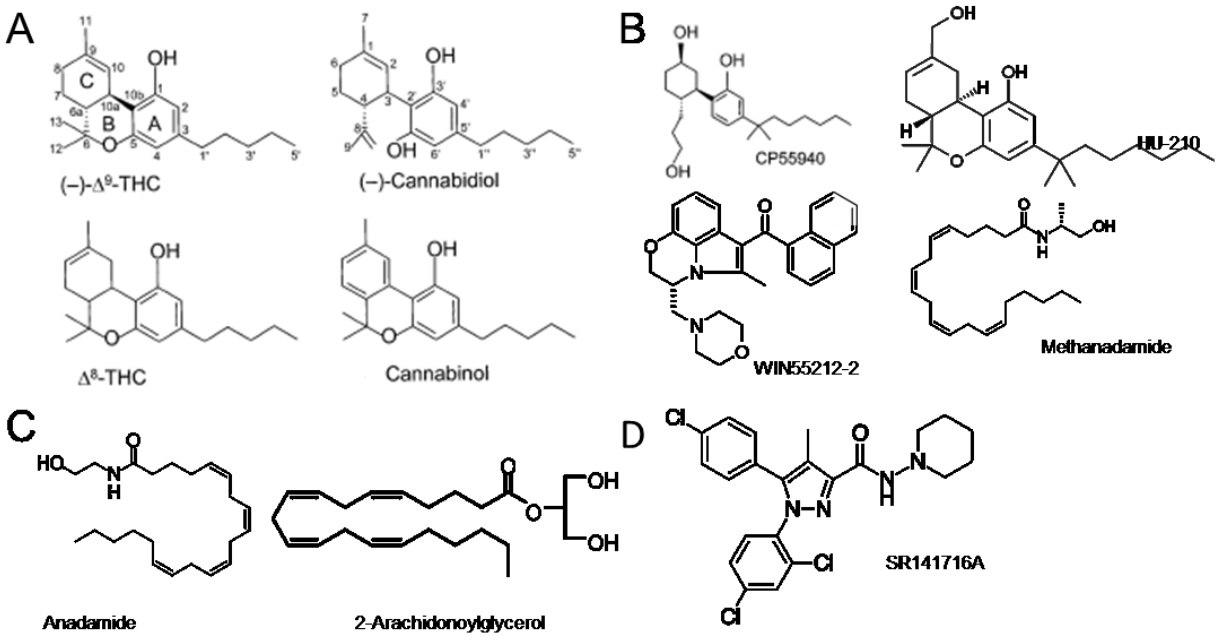


Figure 0.1. Representative chemical structures of A) phytocannabinoids B) synthetic cannabinoids C) endogenous cannabinoids and D) CB_1R inverse agonist

0.2 THC and Synthetic Cannabinoids

Although marijuana is composed of more than 60 cannabinoid constituents (Mechoulam and Parker, 2013), Δ^9-tetrahydrocannabinol (THC) is the main psychoactive constituent. Roger Adams first isolated the main constituents of marijuana in the 1940s, but these compounds did not have psychoactive properties (Adams, 1940). Raphael Mechoulam first reported the isolation of several active compounds of similar lipid structure, including the structure of THC (Gaoni, 1964; Mechoulam and Gaoni, 1965). Based on this structure, several synthetic cannabinoid ligands have been produced and are grouped by structure (Figure 0.1). Synthetic compounds used in research include HU-210, an ABC-tricyclic dibenzopyrans, that was synthesized by Mechoulam in 1988 (Mechoulam et al., 1988), the AC-bicyclic, ((-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol)(CP55,940) and the aminoalkylindole (R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate) (WIN55,212-2), which has a very different structure from other cannabinoids (Howlett et al., 2002). From the structure of these compounds, the antagonist N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A) was also created by Sanofi Aventis (Rinaldi-Carmona et al., 1994). CP55,940 is a high efficacy partial agonist at the cannabinoid type 1 receptor (CB_1R) and is a full agonist at the cannabinoid type 2 receptor (CB_2R), with similar binding affinities for both CB_1Rs and CB_2Rs (Howlett et al., 2002). [^{3}H]CP55,940 is one widely used radiolabeled cannabinoid ligands and has historical significance, as it was first used to demonstrate a specific cannabinoid binding site (Devane et al., 1988) and to anatomically map the distribution of cannabinoid receptors in rat brain (Herkenham et al., 1991b) using autoradiography. WIN55,212-2 is a full agonist at CB_1Rs, and the prototype of the aminoalkylindole family whose

structure is not based on the structure of THC (Figure 0.1). WIN55,212-2 has also been used in autoradiographic studies (Jansen et al., 1992). Synthesis of a CB_1R-selective antagonist SR141716A, which was determined to be an inverse agonist (Gueudet et al., 1995; Landsman et al., 1997) (Gifford and Ashby, 1996), was critical in establishing the specificity of CB_1R-mediated effects, and demonstrated that the centrally-mediated *in vivo* and behavioral effects of cannabinoids are CB_1R-dependent (Rinaldi-Carmona et al., 1994). SR141716A has also been used to map CB_1Rs in rodent brain (Rinaldi-Carmona et al., 1996).

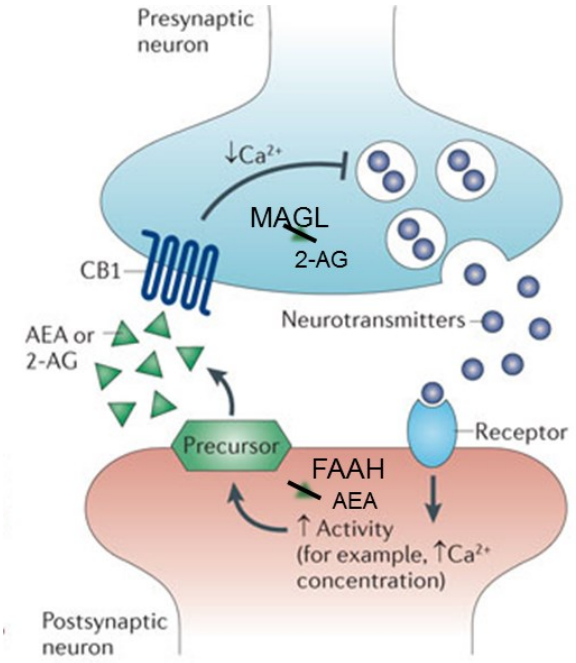


Figure 0.2. Schematic diagram of the endogenous cannabinoid system. Neurotransmitter released from the presynaptic terminal causes on-demand synthesis of 2-arachindonoylglycerol (2-AG) and arachidonoylethanolamine (AEA). 2-AG is degraded by monacylglycerol lipase (MAGL) and AEA is degraded by fatty acid amide hydrolase (FAAH). Both 2-AG and AEA are agonists at the cannabinoid type 1 receptor (CB_1R). Cannabinoid type 2 receptors (CB_2Rs), not pictured here, are found primarily on non-neuronal cells. Adapted from (Guzman, 2003)

0.3 The endogenous cannabinoid system

Based on the lipid structure, early researchers suggested that THC acted directly on the cell membrane as opposed to a specific receptor system (Martin et al., 1988). The first evidence of a specific receptor-mediated mechanism of action for THC was provided by Howlett and colleagues. They discovered that THC inhibited adenylyl cyclase (AC) activity in neuroblastoma cells under both basal and hormone-stimulated conditions (Howlett, 1984; Howlett and Fleming, 1984). This group later reported that THC required the G-protein subunit $G\alpha_i$ to produce their biological responses (Howlett et al., 1986). The role of $G\alpha_i$ was determined by using pertussis toxin, which is derived from Bordetella pertussis. Pertussis toxin ribosylates a cysteine on $G\alpha_i$ and $G\alpha_o$ subunits when they are associated with $\beta\gamma$ subunits (Locht and Antoine, 1995; Mangmool and Kurose, 2011). The creation of a tritiated form of CP55,940 led to the discovery of a specific binding site for cannabinoid compounds in the brain (Devane et al., 1988). This study also determined that the nonhydrolyzable guanosine triphosphate (GTP) analog, guanylylimidodiphosphate, displaced CP55,940 from its binding site, suggesting that CP55,940 coupled to a site that also coupled to G-proteins. Two cannabinoid receptors were subsequently cloned from cDNA libraries; the CB_1R from rat cerebral cortex (Matsuda et al., 1990) and CB_2R from spleen (Munro et al., 1993). The CB_1R gene in mice and rats encodes a 473 amino acid protein and is composed of two encoding exons and one non-encoding exon. Amino acid identity between mouse and rat CB_1Rs is 99.5% while mouse and human sequence identity approaches 97% (Abood et al., 1997). Phylogentically, CB_1Rs and their homologuesare expressed in animals of the chordate phylum, as well as invertebrates in the annelid phylum (McPartland and Glass, 2003). CB_1Rs and CB_2Rs share 44% structural homology and THC binds to both receptors with similar potency. CB_2Rs are commonly found on immune cells

(Cabral and Marciano-Cabral, 2005; Pettit et al., 1998), but may also be expressed by neurons (Onaivi et al., 2006; Van Sickle et al., 2005). CB_2Rs have also been implicated in the rewarding properties of cocaine (Xi et al., 2011), nicotine (Ignatowska-Jankowska et al., 2013) and ethanol (Ortega-Alvaro et al., 2013). Herkenham and collaborators used [^{3}H]CP55,940 autoradiography to localize CB_1R in the rodent central nervous system (CNS) (Herkenham, 1991).

The discovery of endogenous cannabinoid receptors was followed by identification of endogenous ligands. Although several putative lipid-based endogenous ligands have been discovered, arachidonoylethanolamine (anandamide, AEA) (Devane et al., 1992) and 2-arachindonoylglycerol (2-AG) (Mechoulam et al., 1995; Stella et al., 1997; Sugiura et al., 1995) are considered the only confirmed endocannabinoids (Figure 0.2). Although AEA and 2-AG have similar binding affinities, 2-AG exhibits higher efficacy than AEA at both CB_1Rs and CB_2Rs (Pertwee, 2005). Because AEA is highly susceptible to metabolism, synthetic derivatives such as (R)-(+)-methanandamide have been developed that exhibit greater metabolic stability, affinity, and CB_1R selectivity (Di Marzo et al., 2001; Lin et al., 1998). Unlike classical neurotransmitters, endocannabinoids are produced on demand (Marsicano et al., 2003) following increases in intracellular calcium (Rodriguez de Fonseca et al., 2005) and undergo retrograde transmission. Initial studies had suggested that AEA was primarily synthesized by hydrolysis of N-acyl-phosphatidylethanolamines (NAPE) by NAPE phospholipase D (NAPE-PLD) (Schmid et al., 1990). However, AEA is produced in mice with genetic deletion of NAPE-PLD (Leung et al. 2006) suggesting that alternative pathways include double-deacylation of NAPE by α/β-hydrolase 4 (ABH4) followed by phosphodiesterase-mediated cleavage by glycerophosphodiesterase 1 (GDE1) (Simon and Cravatt, 2006) and phospholipase C-catalyzed cleavage of NAPE and dephosphorylation of NAPE (Liu et al., 2006). The production of AEA

in mice with genetic deletion of both GDE1 and NAPE further suggests that multiple biosynthesis pathways exist for AEA (Leung et al., 2006; Simon and Cravatt, 2010). The synthesis of 2-AG has been more clearly defined. 2-AG is synthesized in a phospholipase C-dependent manner by the cleavage of *sn*-1-acyl-2-arachidonoylglycerols (DAGs) by DAG lipase (DAGL). Two isoforms of DAGL exist, DAGLα and DAGLβ, although DAGLα appears to predominant in the CNS (Gao et al., 2010; Tanimura et al., 2010). AEA and 2-AG are rapidly degraded following release by two separate enzymes, fatty acid amide hydrolase (FAAH) and monacylglycerol lipase (MAGL), respectively.

Although CB_1Rs and CB_2Rs are considered the accepted cannabinoid receptors and AEA and 2-AG are the accepted ligands, there is evidence to support a growing number of receptors and ligands that could be considered part of the endogenous cannabinoid system. GPR55 has been considered a putative cannabinoid binding receptor (Ross et al., 2012) while noladin ether (Fezza et al., 2002) and N-arachidonoyldopamine (Bisogno et al., 2000) have been suggested as putative endogenous ligands. Further, AEA has been suggested to be an agonist at the vanilloid type 1 (TRPV1) receptor (Di Marzo et al., 2001). More recently, studies in our laboratory have determined that WIN55,212-2 shows brain region-dependent activation of other receptors (Non CB_1R/CB_2R/GPR55) while CP55,940 appears to be specific for the CB_1Rs in all brain regions (Nguyen et al., 2010).

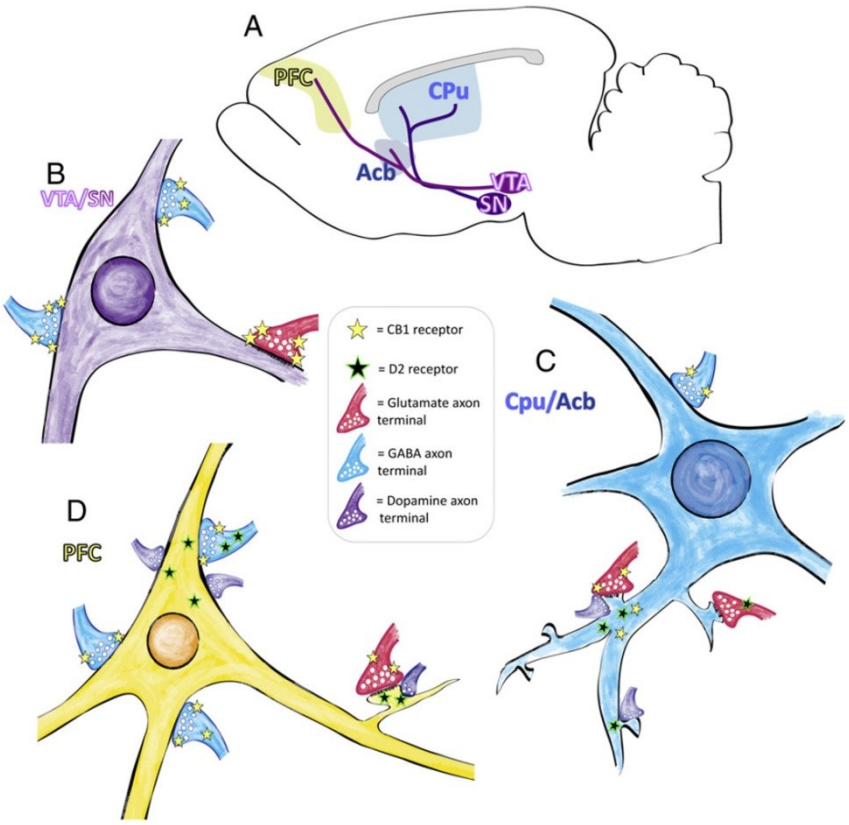


Figure 0.3 Location of CB_1Rs in forebrain and midbrain regions of the mesocorticolimbic dopaminergic system. From (Fitzgerald et al., 2012)

0.4 Neuroanatomical localization of CB_1Rs and *in vivo* effects

CB_1Rs are expressed heterogeneously throughout the CNS and are one of the most abundant G-protein coupled receptors (GPCRs) in the brain (Howlett et al., 2002) (Figure 0.3). Very high expression of CB_1Rs is found in the globus pallidus, substantia nigra pars reticulata and molecular layer of the cerebellum. Moderate expression in the hippocampus, striatum (caudate-putamen and nucleus accumbens) and lower expression occurs in the hypothalamus, periaqueductal gray (PAG), basolateral amygdala, ventral tegmental area and cortex

(Herkenham, 1991). In the human CNS, the distribution of CB_1Rs is very similar even throughout development where CB_1R densities are higher in earlier developmental stages (Glass et al., 1997). Studies utilizing CB_1R knockout mice and the CB_1R-specific inverse agonist, SR171614A, have demonstrated that these receptors mediate many of the behavioral effects of THC (Rinaldi-Carmona et al., 1994; Zimmer et al., 1999). Corresponding to the regional expression of CB_1Rs, cannabinoid agonists produce effects in rodents that include motor impairment, memory impairment, hypothermia, antinociception, anxiety-like behaviors and hyperreflexia (Compton et al., 1993; Dewey, 1986). In preclinical studies, behaviors attributed to marijuana use in humans are attributed to CB_1R activation including: increased feeding (Beardsley et al., 1986; Chambers et al., 2007), reduced emesis and nausea (Darmani, 2001a, b), a wide range of analgesia/antinociception or reductions in pain hypersensitivity (Lichtman and Martin, 1991; Martin et al., 1999). There are also impairments in several aspects of memory (Lichtman and Martin, 1996; Niyuhire et al., 2007) and reduced pressure in the aqueous humor in the eye (Chien et al., 2003; Green and Pederson, 1973); however, only some behaviors like "subjective high" and tachycardia have been verified to be CB_1Rs-dependent in humans (Huestis et al., 2001). THC has been found to increase dopamine release in the nucleus accumbens and increase activation of ventral tegmental area neurons like other drugs of abuse; however, it is not certain that acute THC is rewarding (Gardner, 2005b). THC also increases dopamine release in the human striatum (Bossong et al., 2009). In mice, place preference has been shown with low doses of THC (Lepore et al., 1995) or after priming the mouse with a single dose of THC and testing the animal after 24 hours with another single dose of THC (Valjent and Maldonado, 2000). Mice (Martellotta et al., 1998), rats (Fattore et al., 2001) and squirrel monkeys (Tanda et al., 2000) self-administer THC or WIN55,212-2, and THC microinjections into the nucleus

accumbens and ventral tegmental area of rats (Zangen et al., 2006) increase lever pressing. Intracranial self-stimulation (ICSS) paradigms with synthetic cannabinoid agonists such as WIN55,212-2 show rightward shifts in rats suggesting aversion (Vlachou et al., 2005), but other studies with THC show leftward shifts in rats suggesting reward (Gardner et al., 1988; Lepore et al., 1996). In humans, THC is reported to have both rewarding and aversive aspects and those who smoke marijuana often report that the positive effects remain stable while certain negative effects like dry mouth and lightheadedness are reduced with repeated use (Green et al., 2003). This might suggest that less tolerance develops in those brain regions involved with reward. CB_1Rs also appear to be important for mediating the rewarding properties of other drugs of abuse as CB_1R knockout mice fail to demonstrate elevated dopamine release in nucleus accumbens or substantial intake by ethanol or morphine (Hungund et al., 2003; Mascia et al., 1999).

Neocortex

The neocortex is involved with higher order functions that involve the processing of sensory stimuli (olfactory, somatosensory, visual, auditory, associational), the execution of complex movements (primary and motor cortices) and executive control/working memory (prefrontal cortex and anterior cingulate cortex). The prefrontal cortex is responsible for the planning of movements, plays a role in the consolidation of memories and may be involved with reward. CB_1 receptors are located on axon terminals of corticostriatal projections, which may contribute to the locomotor suppressant effects of Δ^9-THC. CB_1Rs are expressed throughout the neocortex with the highest expression in layers I and VI and lower levels expressed throughout layers II-V (Herkenham, 1991). The neocortex is comprised of large, glutamate-containing pyramidal neurons that are expressed in deep layer III and layer V and serve as the main

projections of the cortex to subcortical brain regions and throughout the body. Much smaller pyramidal neurons contained in layers II and III project to other cortical areas while layer VI pyramidal neurons that have axon collaterals throughout the neocortex and thalamus. The cortex is also comprised of several different GABAergic interneurons, which heavily populate layer IV (the main destination of thalamic projections to cortex), that are classified by their morphology, peptides (i.e., cholecystokinin (CCK), parvalbumin, neuropeptide Y, calretinin) and their electrophysiological characteristics (Butt et al., 2005). CB_1Rs are expressed in cholecystokinin (CCK)-positive GABAergic interneurons (Tsou et al., 1998), non-CCK GABAergic interneurons (Hill et al., 2007) and in some glutamatergic pyramidal neurons throughout the neocortex (Hill et al., 2007; Monory et al., 2006); however, they have not been found on parvalbumin interneurons (Bodor et al., 2005). In the prefrontal cortex, CB_1Rs are known to exist on adrenergic afferents (axonal projections) whose cell bodies most likely originate in the locus coeruleus (Oropeza et al., 2007). No studies to date have reported expression of CB_1Rs on dopaminergic afferents (Miner et al., 2003).

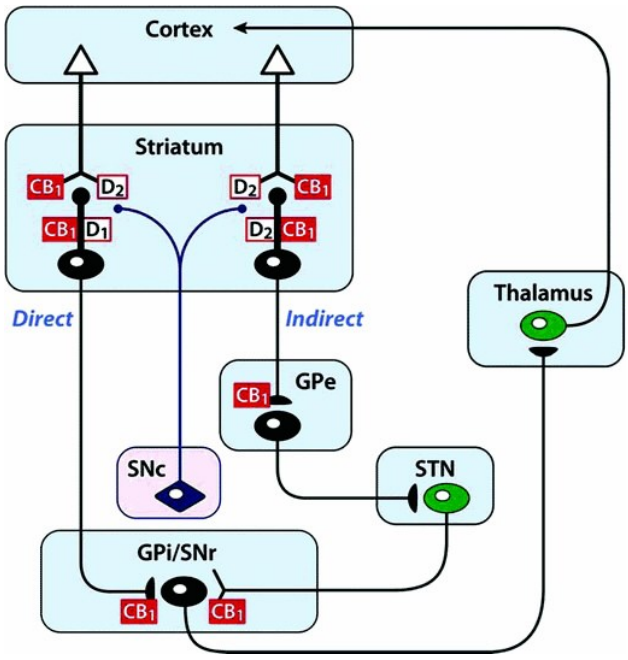


Figure 0.4 Example of direct (D_1R) and indirect (D_2R) signaling pathways of the caudate-putamen in the CNS. MSNs of the D_1R/direct pathway project primarily to the substantia nigra pars reticulata (SNr) and entopeduncular nucleus (represented here as GPi) while MSNs of the D_2R/indirect pathway project to the globus pallidus (GPe). Note that CB_1Rs are found in both populations. The primary dopaminergic innervation to the caudate-putamen is the substantia nigra pars compacta (SNc). The primary glutamatergic innervation to the caudate-putamen is from the cortex. Both pathways feed-back on the cortex through the thalamus. From (Benarroch, 2007).

Basal Ganglia

The highest expression of CB_1Rs in the CNS are found in the output regions of the caudate-putamen: substantia nigra pars reticulata, entopeduncular nucleus and globus pallidus (Herkenham, 1991) (Figure 0.4). Moderate expression is also found in the caudate-putamen and subthalamic nucleus (Herkenham, 1991). The predominate neurons in the caudate-putamen are GABAergic medium spiny neurons (MSNs), which comprise 95% of the total neuronal

population (Kemp and Powell, 1971). Remaining neurons are interneurons that are subdivided by morphology, neuropeptide/ acetylcholinergic production and based on electrophysiological properties (Kawaguchi et al., 1995). CB_1Rs are expressed in the MSN population, as well as interneurons that primarily express parvalbumin (Fusco et al., 2004). The MSN population is further subdivided into two populations: those containing dopamine type 1 (D_1 and D_5) receptors (D_1Rs)/substance P/dynorphin and those containing dopamine type 2 ($D_{2\text{-}4}$) receptors/enkephalin (D_2R)(Gerfen, 1992; Gerfen et al., 1990; Le Moine et al., 1995). CB_1Rs are located in both populations (Hohmann and Herkenham, 2000). These subpopulations also have specific axonal projection. The D_1R/dynorphin MSN population projects primarily to substantia nigra pars reticulata (the direct pathway) and the D_2R/enkephalin MSN population projects primarily to the globus pallidus (the indirect pathway) (Gerfen, 1988). In regards to motor control by caudate-putamen, these specific projections produce an opponent process system that produces increases in locomotor activity following dopamine release. Dopamine increases activity in D_1R/dynorphin MSNs and suppresses activity in D_2R/enkephalin MSNs. This is achieved through the differences in coupling of these receptors to specific G-proteins and control of ACS activity. D_1Rs couple primarily to $G\alpha_{s/olf}$ (Drinnan et al., 1991) and stimulate ACS (Kebabian et al., 1984; Kebabian et al., 1972) and D_2Rs couple primarily to $G\alpha_{i/o}$ (Kebabian et al., 1984; Senogles et al., 1990) and inhibit ACS (Stoof and Kebabian, 1981). Therefore, dopamine release differentially activates the neurons in which these receptors are located. As further illustration, direct injection of $GABA_A$ receptor agonists (which suppress neuronal activity, e.g. muscimol) into these regions produce opposing effects. Injection of muscimol into the globus pallidus produces locomotor suppression while injection of muscimol into substantia nigra produces locomotor activity (Amalric and Koob, 1989). More recent optogenetic studies have found that

selective activation of D_1R MSNs in the caudate-putamen increased ambulation while selective activation of D_2R MSNs reduced ambulation, further providing evidence for the opponent process system (Kravitz et al., 2010).

Although the projections of these neurons primarily terminate in their respective output nuclei, axon collaterals from the D_1R/dynorphin MSNs also project to the globus pallidus (Lindvall and Bjorklund, 1979) and there are GABAergic axonal projections from the globus pallidus that terminate in the substantia nigra pars reticulata (Bolam et al., 1993). The preponderance of CB_1Rs that are located in the substantia nigra pars reticulata, entopeduncular nucleus and globus pallidus originate from cell bodies in the caudate-putamen because lesion of the caudate-putamen abolishes the expression of CB_1Rs in these areas (Herkenham et al., 1991a). This study also found that lesion of the medial forebrain bundle did not affect CB_1R levels in the caudate-putamen of the lesioned side, suggesting that CB_1Rs found in the caudate-putamen do not arise from the dopaminergic axonal projections of the medial forebrain bundle. More recent studies using detailed electron microscopy corroborate the finding that CB_1Rs are not expressed in axons containing dopamine in the caudate-putamen (Fitzgerald et al., 2012). CB_1Rs are found on glutamatergic and GABAergic axon terminals in the caudate-putamen (Rodriguez et al., 2001). The glutamatergic axons arise from neocortical projections, primarily motor cortices, which also contain D_2R autoreceptors (Wang and Pickel, 2002). The GABAergic axons are primarily derived from local MSNs as well as GABAergic interneurons, which contain both CB_1Rs and D_2Rs (Bennett and Bolam, 1994). There are also glutamatergic axonal projections from the amygdala and hippocampus that terminate in the caudate-putamen (Gerfen, 1984), but it is not clear if these projections also contain CB_1Rs.

Systemic administration of THC produces primarily locomotor suppression and catalepsy (Dewey, 1986) and suppresses total neuronal activity in all regions of the basal ganglia (Shi et al., 2005). Several studies have attempted to dissect which brain regions are responsible for these effects, but the results are inconclusive. Early studies compared the effect of cannabinoids on unilateral injection of muscimol into the globus pallidus and substantia nigra. Injection of muscimol into the globus pallidus produces catalepsy while injection of THC enhances the muscimol effect, suggesting that globus pallidus could play a role in THC-mediated catalepsy (Wickens and Pertwee, 1993). Unilateral injection of muscimol into the substantia nigra produce contralateral circling, a measure of hyperactivity, and 1 µg of THC enhanced this effect while 10 µg of THC abolished this effect (Wickens and Pertwee, 1995), suggesting a dose-response. Intranigral injection of CP55,940 alone also produces contralateral turning (Sanudo-Pena et al., 1996). Intrastriatal injections also produce contralateral turning, which is blocked by the D_2R agonist quinpirole (Sanudo-Pena et al., 1998). More recently, studies using mice with genetic deletion of CB_1Rs in either glutamate-, GABA- or D_1R-containing forebrain neurons determined that cannabinoid-mediated locomotor suppression was reduced only in mice that had genetic deletion of CB_1Rs in glutamate-containing neurons (Monory et al., 2007). This study also determined that catalepsy was abolished in mice that had genetic deletion of CB_1Rs in D_1R-containing neurons. The source of glutamate-containing neurons that modulate THC-mediated locomotor suppression is not clear; however, the subthalamic nucleus, which is part of the basal ganglia, may play a part. The subthalamic nucleus receives GABAergic projections from the globus pallidus and sends glutamatergic projections to substantia nigra and globus pallidus (Deniau et al., 1978). Direct injection of CP55,940 into the subthalamic nucleus produces

locomotor suppression, suggesting the importance of this region in cannabinoid-mediated locomotor suppression (Miller et al., 1998).

Nucleus accumbens

The nucleus accumbens (ventral striatum) is similar to the caudate-putamen in that it contains D_1R/dynorphin and D_2R/enkephalin MSN populations (Curran and Watson, 1995) that project to the ventral tegmental area and ventral pallidum, respectively. There is some evidence that D_1R/dynorphin MSNs also express the D_3, D_2R subtype (Ridray et al., 1998). The nucleus accumbens is subdivided into the core and shell areas, which are differentiated mainly by calbindin staining that is strongly stained in the core but much lighter in the shell (Groenewegen et al., 1999). Functionally, the nucleus accumbens shell may play a more important role in drug reward because cocaine, morphine and amphetamine generally increase dopamine in the shell but not the core (Pontieri et al., 1995), and several drugs of abuse are self-administered when injected directly into the shell but not the core (Di Chiara et al., 2004). CB_1Rs are located predominantly on axon terminals in the shell, and are also found on both D_1R and D_2R MSNs (Pickel et al., 2004). Despite the location of CB_1Rs in nucleus accumbens and their regulation of dopamine release (Wu and French, 2000), preclinical measures of reward-related behavior, especially in rodents, have failed to provide clear results regarding THC or other cannabinoids in regards to their reward profile (Tanda and Goldberg, 2003).

Other regions

CB_1Rs are primarily expressed by GABAergic CCK-containing basket cells of both the hippocampus (Freund and Hajos, 2003; Mackie, 2005) and amygdala (Katona et al., 2001; Marsicano and Lutz, 1999; Tsou et al., 1998). The hippocampus contributes to learning behavior and cannabinoids are known to disrupt tasks such as the delayed nonmatch-to-sample task and

Morris water maze task, which involve this region (Hampson and Deadwyler, 1998; Varvel and Lichtman, 2002). The amygdala contributes to anxiety- and fear-related behaviors and the ventral hippocampus can contribute to anxiety-related behaviors (Rubino et al., 2008). Focal injection of cannabinoids into the amygdala produces anxiety-related behaviors while focal injection into ventral hippocampus produces anxiolytic-related behaviors (Rubino et al., 2008). Systemic injection of cannabinoids produces anxiolytic-related behaviors at low doses and anxiogenic-related behaviors at higher doses (Parolaro et al., 2010). In amygdala, dopamine is actually increased during stress and enhances amygdala-related behavior (Inglis and Moghaddam, 1999; Rosenkranz and Grace, 1999). Further, the amygdala has been implicated in drug reinstatement, as shown by its involvement in consolidation of drug-paired cues (e.g. associated with conditioned place preference paradigms) (Fuchs and See, 2002; Luo et al., 2013).

The hypothalamus regulates mostly autonomic, metabolic and circadian rhythm functions. The medial preoptic area controls thermoregulation and direct injection of WIN55,212-2 into this region produces hypothermia (Rawls et al., 2002); however, THC-mediated hypothermia is still present in rats with lesions to this area (Schmeling and Hosko, 1976). The hypothalamus receives inputs from the limbic system and midbrain. The thalamus is a gateway between the cortex and the rest of the CNS and mediates sensory perception as well as motor function. Although CB_1R expression and G-protein signaling is low in thalamus, there is high CB_1R expression in the lateral habenula (Tsou et al., 1998). There is low expression of CB_1Rs in periaqueductal gray and this region is partly responsible for the antinociceptive properties of cannabinoids (Herkenham, 1991; Lichtman and Martin, 1991). The spinal cord is also involved in the antinociceptive properties of cannabinoids where CB_1Rs are found in the dorsal root ganglia nociceptive neurons. Finally, very high expression of CB_1Rs is found in the

molecular layer of cerebellum, a region that is important for motor coordination. Further, some evidence suggests that cerebellum is involved in the withdrawal signs (Tzavara et al., 2000) following cannabinoid abstinence and for the hyperreflexia observed after cannabinoid administration (Patel and Hillard, 2001).

0.5 CB_1R **signaling**

CB_1Rs belong to the rhodopsin-like class A family of G-protein coupled receptors (GPCRs), which contain seven transmembrane domains with an extracellular glycosylated amino terminus and an intracellular carboxyl-terminus. G-proteins are composed of three separate subunits: Gα, Gβ and Gγ. In the inactive confirmation, guanosine diphosphate (GDP) binds to the α subunit, which forms a heterotrimeric complex with the βγ dimer that binds. Agonist binding to the receptor results in the exchange of guanosine triphosphate (GTP) with GDP. In this active state, the βγ dimer dissociates from the α subunit providing two distinct signaling mechanisms (Childers et al., 1993). The GPCR acts as a catalyst for this exchange and allows for the activation of several G-proteins, which amplifies signaling (Breivogel et al., 1997). CB_1Rs typically couple to $G\alpha_{i/o}$ subunits, although some research suggests CB_1R coupling to $G\alpha_s$ (Bonhaus et al., 1998; Glass and Felder, 1997) and $G\alpha_{q/11}$ (De Petrocellis et al., 2007; Lauckner et al., 2005). G-protein coupling can occur at the intracellular loops (Abadji et al., 1999) and the c-terminus (Howlett et al., 1998; Mukhopadhyay et al., 1999) of CB_1Rs. The activation of $G\alpha_i$ subunits typically leads to an inhibition of ACS and a decrease in accumulation of cAMP (Smigel et al., 1984); however, it should be noted that co-expression of CB_1Rs and ACS isoforms I, III, V, VI or VIII decreases the accumulation of cAMP whereas cAMP accumulation increases when CB_1Rs are co-expressed with AC isoforms II, IV, and VII (Rhee et al., 1998). CB_1Rs modulate multiple downstream signaling events via activation of $G\alpha_{i/o}$ and $G_{\beta\gamma}$ subunits,

including phosphorylation of p42/p44 mitogen activated protein kinases (MAPK), which are also known as extracellular signal-regulated kinases (ERK1/2) (Bouaboula et al., 1995; Derkinderen et al., 2001; Galve-Roperh et al., 2002), inhibition of N-type and P/Q type voltage dependent Ca^{+2} channels (Pan et al., 1996; Twitchell et al., 1997) and stimulation of inward rectifying K^+ channels (Mackie et al., 1995; Vasquez et al., 2003). CB_1Rs can also inhibit Na^+ channels (Nicholson et al., 2003), stimulate phospholipases C and A2 (PLC, PLA2) (Hunter et al., 1986), activate c-Jun N-terminal kinase (JNK) 1 and 2 (Rueda et al., 2000b), p38 MAPK (Rueda et al., 2000a), nitric oxide (Prevot et al., 1998) and protein kinase B (also known as thymoma viral proto-oncogene (AKT) (Gomez et al., 2011). CB_1Rs can also activate the factor associated with neutral sphingomyelinase (FAN), which increases ceramide production in a pertussis toxin independent manner (Sanchez et al., 2001).

GPCRs can also signal through the recruitment of scaffolding proteins, such as arrestins. Studies using channel rhodopsin led to the discovery of a 48 kDa protein that bound to phosphorylated rhodopsin that is now known as arrestin1 or visual arrestin (Wilden et al., 1986). In 1990, a similar molecule was found to inhibit function of the β2-adrenergic receptor (Lohse et al., 1990) and was termed β-arrestin1. Soon after, β-arrestin2 was discovered and shown to interact with the β_2-adrenergic receptor (Attramadal et al., 1992). The discovery of the arrestins was in part due to the isolation and purification of the G-protein receptor kinase 2 (GRK2, referred to β adrenergic receptor kinase at the time) (Benovic et al., 1987). Arrestins were initially identified as accessory proteins that promote desensitization (a reduction in G-protein activation) of GPCRs. However, more recent studies have determined that βarrestins can recruit c-Src, a nonreceptor tyrosine kinase that activates ERK 1/2, (DeFea et al., 2000; Luttrell et al., 1999) through a βarrestin2, v-raf-1 murine leukemia viral oncogene homolog 1 (Raf-1), mitogen

activated protein kinase kinase 1 (MEK1) and ERK1/2 scaffolding complex (Luttrell et al., 2001). βarrestins can also recruit JNK3 into a scaffolding complex (McDonald et al., 2000) and activate PI3K through an AKT scaffolding complex (Povsic et al., 2003) that can also recruit protein phosphatase 2A (PP2A) in the brain (Beaulieu et al., 2005). CB_1Rs are desensitized by mechanisms that involve GRK3 and βarrestin2 (Jin et al., 1999), therefore CB_1Rs might also activate these signaling proteins through βarrestin scaffolding, as shown for the β_2-adrenergic receptor.

Homo- and hetero-dimerization of GPCRs also provides a novel mechanism of GPCR signaling. Evidence for GPCR dimerization was initially provided by studies showing that $GABA_B$ receptors form obligatory homodimers (Kubo and Tateyama, 2005). Histological techniques, such as electron microscopy and fluorescence resonance energy transfer (FRET), showed that the CB_1Rs form homodimers and heterodimers with mu, kappa, and delta-opioid receptors (MOR, KOR, DOR), orexin 1, adenosine type 2A (A_{2A}), β_2-adrenergic receptors and D_2Rs (Hudson et al., 2010; Wager-Miller et al., 2002). Electron microscopy studies have supported dimerization between CB_1Rs and MORs in the nucleus accumbens (Pickel et al., 2004). Likewise, functional studies conducted in striatal cell membrane homogenates showed that the MOR-selective agonist DAMGO reduced WIN55,212-2 stimulated [^{35}S]GTPγS activation (Rios et al., 2006). In contrast, a study using transfected *Xenopus* oocytes showed a cooperative effect between CB_1Rs and MORs (Hojo et al., 2008). Heterodimerization of D_2Rs and CB_1Rs has been shown functionally in cell culture and in vitro with striatal cultures (Glass and Felder, 1997; Jarrahian et al., 2004; Kearn et al., 2005; Marcellino et al., 2008), in which agonist stimulation of CB_1Rs increased AC activity, perhaps through $G\alpha_{s/olf}$ activation. CB_1R and D_2R agonists alone inhibited cAMP production but simultaneous introduction of agonists for

both receptors led to cAMP accumulation. A similar study showed that A_{2A} receptors co-localized with CB_1 Rs in vitro and that A_{2A} receptor antagonist administration in rats abolished the inhibitory motor effects of WIN55,212-2 (Carriba et al., 2007). Indirect evidence has also shown that that D_2R and $A2_A$ receptors promote cannabinoid-mediated increases in AC activity in the striatum. Administration of CP55,940 in mice with genetic deletion of either D_2R or A_{2A} receptors abolished phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of Mr32 kDA (DARPP-32) at threonine 34 (Andersson et al., 2005) (Figure 0.5). Phosphorylation of DARPP-32 at this site was also abolished in A_{2A} knockout mice following THC administration (Borgkvist et al., 2008). DARPP-32 (Hemmings et al., 1984b; Ouimet et al., 1984; Walaas et al., 1983; Walaas and Greengard, 1984) is highly expressed in dopaminoceptive neurons of striatum and is expressed in all neuronal compartments. DARPP-32 is phosphorylated by protein kinase A (PKA) at threonine 34 and becomes an inhibitor of protein phosphatase 1 (PP1) (Hemmings et al., 1984a; Huang et al., 1999). Therefore, it is possible that dimerization of CB_1Rs with either D_2Rs or A_{2A} receptors increases ACS activity, which increases PKA activity (Walsh et al., 1968) and leads to phosphorylation of DARPP-32 at threonine 34.

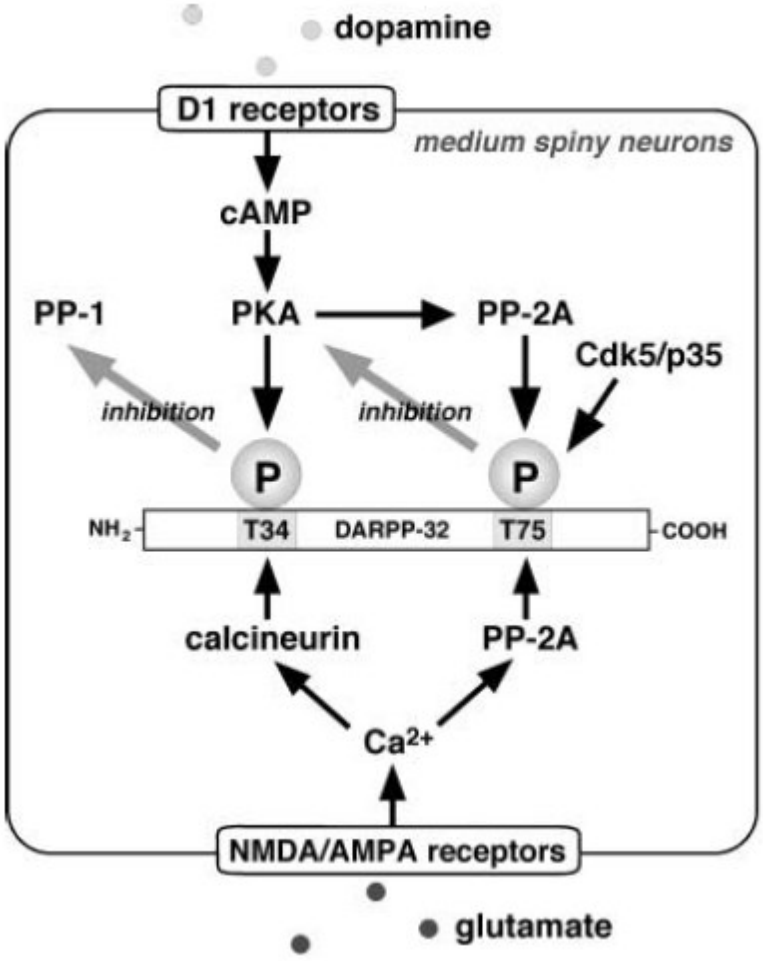


Figure 0.5 Regulation of DARPP-32 by both glutamate and dopamine in the striatum. From (Nishi et al., 2002).

DSI and DSE

CB_1Rs are predominantly expressed presynaptically and inhibit neurotransmitter release (Ishac et al., 1996; Kathmann et al., 1999; Nakazi et al., 2000; Shen et al., 1996; Szabo et al., 1999). Studies on the subcellular localization of CB_1Rs revealed that they are highly expressed on axon terminals and preterminal segments (Hajos et al., 2000; Katona et al., 2001). CB_1Rs play a role in refining neurotransmission by reducing presynaptic release of

neurotransmitters through the signaling systems described above. Unlike neurotransmitters, CB_1Rs are produced on demand and inhibit presynaptic neurotransmitter release through retrograde transmission primarily via release of 2-AG (Marsicano et al., 2003). In the hippocampus, excitation of CA1 pyramidal neurons leads to an influx of calcium, which promotes 2-AG synthesis in the neuron and 2-AG is released retrogradely and inhibits GABA release from nearby interneurons (Kano et al., 2009). This process is referred to as depolarization-induced depression of inhibition (DSI). Suppression of glutamate release on the projection neuron can also occur through the same process of retrograde signaling by 2-AG and is referred to as depolarization-induced depression of excitation (DSE). DSI and DSE were discovered to be mediated by CB_1Rs in both the cerebellum and hippocampus (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Ohno-Shosaku et al., 2012). The necessity of CB_1Rs in producing DSI and DSE is demonstrated by the loss of these processes in mice with genetic deletion of CB_1Rs. 2-AG is also necessary because these processes are lost in mice with genetic deletion of DAGL (Gao et al., 2010; Uchigashima et al., 2007). The duration of DSI/DSE is also dependent on the catabolism of 2-AG by DAGL (Hashimotodani et al., 2008) and breakdown of 2-AG by presynaptic MAGL (Hashimotodani et al., 2007).

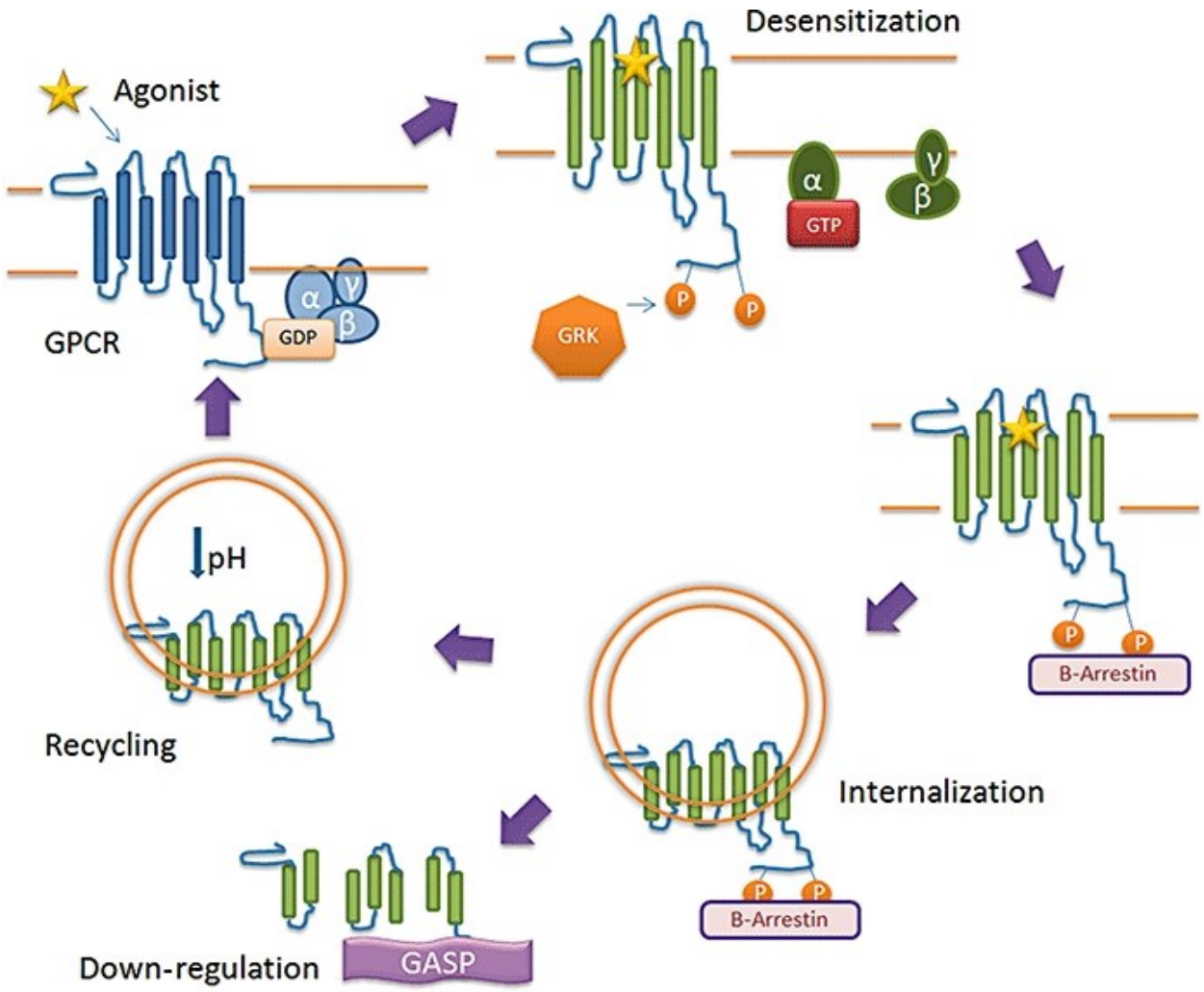


Figure 0.6 Schematic representation of CB_1R desensitization and downregulation following repeated cannabinoid administration. When an agonist binds, it causes dissociation of the G_α, subunit which leads to the phosphorylation of the receptor by GRK. β-arrestins bind to the phosphorylated receptor, which leads to internalization of the receptor. The receptor is either recycled back to the membrane or degraded in endosomes, which is mediated by GASP. From (Smith et al., 2010).

0.6 Tolerance, Desensitization and Downregulation following repeated THC administration

Tolerance develops to the *in vivo* effects of THC following repeated administration. Tolerance is a reduction in the effect of a drug following repeated administration of that drug. Repeated administration of THC, synthetic cannabinoid agonists and inhibition of 2-AG degradation in rodents produce tolerance to cannabinoid-mediated antinociception, hypothermia, catalepsy, and locomotor suppression (Carlini, 1968; Gonzalez et al., 2005; Pertwee et al., 1993; Schlosburg et al., 2010), and cross-tolerance develops among the different cannabinoid drugs (Fan et al., 1994). Surprisingly, tolerance does not develop to THC-mediated mouse killing (Miczek, 1979) and for some of the memory impairing effects of cannabinoids (Barna et al., 2007; Boucher et al., 2009; Ferraro and Grilly, 1974) but tolerance has been reported for the delayed match to sample performance test (Deadwyler, 1995). Studies in humans have demonstrated that tolerance develops to the cardiovascular (Benowitz and Jones, 1975) and memory/cognitive impairing (D'Souza et al., 2008) effects of cannabinoids whereas little tolerance develops to the motoric or "subjective high" effects (D'Souza et al., 2008; Haney et al., 1999a, b). Adaptation to chronic administration of cannabinoids is minimally represented by pharmacokinetic changes (Dewey et al., 1973; Martin et al., 1976), but relies more on pharmacodynamic changes, which include CB_1R desensitization and downregulation (Sim-Selley, 2003).

The mechanisms underlying G-protein coupled receptor (GPCR) desensitization and downregulation were initially determined using heterologously expressed β-adrenergic receptors (Gainetdinov et al., 2004; Inglese et al., 1993; Lefkowitz, 1998). Desensitization involves the phosphorylation of specific residues on the C-terminus of the receptor that causes a conformational change in the receptor (Lefkowitz, 1998). This process can occur through either

a heterologous or homologous pathway. Heterologous phosphorylation involves promiscuous protein kinases like PKA and PKC that phosphorylate either the active or inactive state of the receptor, typically through activation of other receptors (Chu et al., 2010). Homologous desensitization is more conservative and leads to phosphorylation of only activated receptors. This latter form of desensitization occurs in response to phosphorylation by G-protein receptor kinases (GRKs). The recruitment of GRKs to agonist-activated receptors occurs through Gβγ sequestration of GRKs to receptors (Daaka et al., 1997). Phosphorylation of specific residues on GPCRs facilitates the binding of arrestin molecules that reduce both G-protein coupling and initiate the internalization of receptors, and, as discussed previously, can lead to other signaling events. Specific residues of the CB_1R have been associated with receptor adaptions. The c-terminus of CB_1Rs is important for desensitization and requires mutation of four separate phosphorylation sites to suppress internalization (Daigle et al., 2008a), and residues between V459 and V464 are necessary for internalization (Hsieh et al., 1999). S425 and S429 are required for desensitization, but not endocytosis (Hsieh et al., 1999). L404F mutation can enhance agonist-induced trafficking (Anavi-Goffer et al., 2007). Truncation of the receptor at residue 417 attenuates desensitization (Jin et al., 1999). Mutation of CB_1R residues 425 and 429 does not alter β-arrestin recruitment or internalization, but attenuate ERK 1/2 phosphorylation (Daigle et al., 2008b) and GIRK channel activation (Jin et al., 1999). Class A GPCRs preferentially bind to β-arrestin 2 (Oakley et al., 2000); however, CB_1Rs can also interact with β-arrestin1 (Bakshi et al., 2007). In the brain, the major β-arrestin isoforms are β-arrestin1 and β-arrestin2, each of which is uniquely distributed in the CNS (Gurevich et al., 2002) (Figure 0.6).

CB_1R adaptation in response to repeated cannabinoid treatment has been investigated using both cell and animal models. There are seven known mammalian GRK isotypes whose

expression differs by brain region in the CNS. GRK2 and GRK3 are the most highly expressed in the CNS, although GRK4 is also expressed (Arriza et al., 1992). The role of GRK and β-arrestin in mediating CB_1R desensitization has been demonstrated in the *Xenopus* oocyte expression system (Jin et al., 1999). Repeated THC administration changes the expression of both GRKs and β-arrestins in a region-dependent manner (Rubino et al., 2006). However, it is unclear how these regional differences affect desensitization. The rate of internalization correlates with the relative efficacy of cannabinoid agonists to activate G-proteins. Lower efficacy agonists, like THC, produce a greater magnitude of internalization/desensitization (Wu et al., 2008). CB_1Rs are internalized through clathrin-coated pits into early endosomes (Hsieh et al., 1999). At the molecular level, repeated cannabinoid exposure results in the functional uncoupling of CB_1Rs from G-proteins (desensitization) (Sim et al., 1996) and agonist-promoted internalization (Jin et al., 1999); followed by either receptor degradation in lysosomes (downregulation) or recycling to the cell membrane (resensitization) (Tappe-Theodor et al., 2007). Downregulation involves targeting of CB_1Rs for degradation, which appears to require G-protein-associated sorting protein 1 (GASP1), a protein that has been shown to interact with CB_1Rs and was required for agonist-induced downregulation of CB_1Rs in spinal neurons (Tappe-Theodor et al., 2007). Genetic deletion of GASP1 abolishes CB_1R downregulation in the spinal cord and cerebellum of repeated WIN55,212-2-treated mice that is accompanied by a reduction in tolerance to cannabinoid-mediated antinociception, motor incoordination, and locomotor suppression (Martini et al., 2010; Tappe-Theodor et al., 2007).

Studies in rodents have determined that the development of desensitization and downregulation of CB_1Rs following repeated THC administration depends on both the dose and length of cannabinoid administration, while acute doses of CB_1R agonists do not produce

significant desensitization and downregulation *in vivo* ((Sim-Selley, 2003), Table 0.1). There are also brain regional differences in the development of desensitization and downregulation when cannabinoid dose and treatment time are constant ((Sim-Selley, 2003), Table 0.1). Specifically, regions of the basal ganglia (caudate-putamen, nucleus accumbens, globus pallidus and substantia nigra) show lower magnitude of desensitization and downregulation compared to areas like the hippocampus and periaqueductal gray (Sim-Selley, 2003), Table 0.1). Functionally, cannabinoid-mediated catalepsy and locomotor suppression, behaviors associated with the basal ganglia, exhibit less tolerance when compared to responses such as hypothermia (medial preoptic area) and antinociception (periaqueductal gray and spinal cord) (Bass and Martin, 2000; Whitlow et al., 2003). Both post-mortem studies and studies in live subjects using positron emission topography (PET) have found region-dependent differences in CB_1R levels in brains from marijuana users compared to non-users (Hirvonen et al., 2012; Villares, 2007). These findings correspond to studies showing that more tolerance develops to the memory impairing effects of THC, which is associated with hippocampal function compared to the motoric or "subjective high" effects of THC, which is associated with basal ganglia function (D'Souza et al., 2008). PET studies also showed differences in recovery of CB_1Rs after cessation of marijuana treatment (Hirvonen et al., 2012), which agreed with previous studies in rodents (Sim-Selley et al., 2006). In both studies, basal ganglia regions recovered faster than areas like the hippocampus.

TABLE 0.1 Summary of studies that have examined the effect of chronic cannabinoid treatment on several parameters of CB_1R function. Data from time course studies is not included because results vary based on duration of treatment. Adapted from (Sim-Selley, 2003)

Treatment	Tolerance	Receptor Binding	CB1 mRNA	$[^{35}S]GTP\gamma S$	cAMP/PKA
THC (10 mg/kg) 2X/day for 6.5 days Mice	SA	No change whole brain	No change whole brain		
THC (10 mg/kg) 2 weeks, rat CP (1,3,10 mg/kg) 2 weeks rat	Open field Open field	Dec: CPu Dec. str			
THC (6.4 mg/kg) 7 days, rat	SA	Dec str			
CP (0.4 mg/kg) 11 days, rat THC (10 mg/kg) 11 days rat	SA, analgesia		Dec Cpu No chg others Dec Cpu No chg others		
THC (3 mg/kg) 5 days, rat Anandamide (3mg/kg) 5 days, rat		Dec str Inc hip, cblm Inc cblm, hip No chg str			
CP (2 mg/kg) 2X/day for 6.5 days	Hypomot Hypotherm immobil	Dec cblm	Inc cblm		No chg in CB-inhibited in cblm
THC (10 mg/kg) 21 days, rat				Dec Cpu, GP, ctx, hip, cblm	
THC (10 mg/kg) 5 days, rat	SA	Dec Cpu ctx, hip, cblm No chg GP	Inc str No chg hip, cblm		
THC (10 mg/kg) 5 days, rat		Dec cblm, CPu, ctx No chg GP	Dec CPu No chg cblm, ctx, GP	No chg Cpu, GP	
R-methanandamide (10 mg/kg) 5 days, rats		Dec lCPu, cblm No chg hip, ctx, GP	Dec CPu, hip No chg hip, ctx	No chg lCPu, ctx, hip	
Anandamide (20 mg/kg) 15 days, rat	tetrad	No chg str, hip, ctx, cblm		Dec str, ctx, hip, cblm	No chg str, ctx, cblm
THC (15 mg/kg) 2X/day for 15 days, rat	analgesia	Dec str, hip, ctx, cblm			Basal cAMP/PKA Inc cblm, str, ctx
CP (0.4 mg/kg) 2X/day for 6.5.days rat		Dec str, ctx, hip, GP, cblm		Dec str, ctx, hip No chg cblm	No chg str, ctx, hip, cblm
THC (10-160 mg/kg) 15 days, mice	SA, hypotherm SA,	Dec Cpu, GP, ctx, hip, cblm Dec Cpu, GP, ctx,		Dec Cpu, GP, ctx, hip, cblm Dec Cpu, GP,	

WIN (3-48 mg/kg) 15 days, mice	hypotherm	hip, cblm		ctx, hip, cblm	

0.7 Signaling pathways known to modulate CB_1R desensitization and downregulation

The signaling mechanisms that underlie these brain-region dependent differences in desensitization and downregulation are not known, but studies have suggested a role for ERK and β-arrestin2. The role that ERK activation might play in CB_1R adaptation following repeated THC administration was further studied by Rubino et al. (2005). ERK activity was not increased following acute THC treatment in mice treated with SL327 (a MEK inhibitor) or Ras-GRF1 knockout mice (Rubino et al., 2005). Furthermore, tolerance to THC-mediated locomotor suppression was prevented in these mice after treatment with 10 mg/kg THC b.i.d for 4.5 days. In agreement with these findings, autoradiographic studies using [^{3}H]CP55,940 binding determined that CB_1Rs were not significantly decreased in the caudate-putamen or cerebellum, but were decreased in the hippocampus of mice that received SL327 treatment and in Ras-GRF1 knockout mice. Interestingly, Ras-GRF1 knockout mice had reduced CB_1R binding in the prefrontal cortex compared to wild type controls. This result was not seen with SL327 treated mice, suggesting that the loss of Ras-GRF1 affected CB_1R levels through a different mechanism than MEK inhibition alone. CP55,940-stimulated [^{35}S]GTPγS binding was not reduced in the caudate-putamen or hippocampus following repeated THC administration in Ras-GRF1 mice compared to controls, which agrees with the *in vivo* data. CP55,940-stimulated [^{35}S]GTPγS binding was reduced in Ras-GRF1 knockout compared to wild type mice in the prefrontal cortex and cerebellum. Inhibition of MEK using SL327 prevented CB_1R desensitization in the prefrontal cortex, caudate-putamen and cerebellum, but not in the hippocampus. Overall, these

data suggest that ERK plays a role in modulated CB_1R adaptations in a brain region-dependent way.

Our laboratory has reported that β-arrestin2 contributes to brain-region dependent differences in CB_1R desensitization and downregulation and in the development of tolerance to THC-mediated *in vivo* effects by using β-arrestin2 knockout mice (Nguyen et al., 2012). Vehicle-treated β-arrestin2 knockout mice had enhanced THC-mediated antinociception and hypothermia and increased [^{35}S]GTPγS binding in the piriform cortex, auditory and visual cortices and caudal hippocampus. After receiving twice-daily injections of 10 mg/kg THC for 6.5 days, β-arrestin2 knockout mice exhibited significantly greater tolerance to THC-mediated catalepsy and attenuated tolerance to antinociception. At the receptor level, β-arrestin2 knockout mice exhibited greater desensitization in the piriform cortex, auditory and visual cortex, somatosensory cortex, globus pallidus, hypothalamus and substantia nigra and attenuated desensitization and downregulation in the cerebellum, caudal periaqueductal gray and spinal cord (Nguyen et al., 2012). These results suggest that although ERK and β-arrestin2 might contribute to brain region-dependent differences in CB_1R adaptations, other factors must be involved.

0.8 Induction of transcription factors by cannabinoids

Transcription factors might also contribute to regional differences in CB_1R adaptions. Similar to the development of desensitization and downregulation, regional difference exist in the CNS regarding the induction of transcription factors by cannabinoid agonists. The regulation of gene expression by CB_1Rs is likely to begin with the activation of immediate early genes (IEGs), which are transcription factors that regulate the expression of downstream target genes. Immediate early genes can be constitutively expressed or induced by stimuli. For cannabinoids,

zif268 (or krox24), cAMP response element binding protein (CREB) and the Fos and Jun families of IEGS have been investigated most extensively. CREB is constitutively expressed and its binding to DNA is regulated by phosphorylation by upstream kinases. Inducible IEGs include zif268, the Fos (c-Fos, FosB, Fos-related antigen 1 (Fra-1), Fra-2 and ΔFosB) and Jun (c-Jun, JunB and JunD) families of transcription factors, which form AP-1 complexes that bind to AP-1 consensus sites on target genes. Inducible transcription factors are basally expressed in the brain and exhibit species-specific regional differences in basal expression (Herdegen and Leah, 1998). Transcriptional repressors also exist, such as cAMP response-element modulator (CREM), which reduces CREB transcription, and Fos-related antigen 1 (Fra1), which reduces the transcriptional ability of AP-1 complexes (Foulkes and Sassone-Corsi, 1992; Yoshioka et al., 1995). IEGs can also induce or repress the expression of other IEGs. For example, CREB can induce *c-fos* mRNA (Sheng et al., 1991), whereas ΔFosB, a truncated splice variant of FosB, can repress *c-fos* mRNA expression through epigenetic regulation by recruitment of histone deacetylase 1 (HDAC1) (Renthal et al., 2008). Co-regulation adds to the complexity of understanding interactions among IEGs and provides multiple points for interactions between these signaling pathways. Interpretation of results with cannabinoids is further complicated by differences in the particular drugs and doses administered, temporal paradigm and species examined. The role of specific IEGs in directly modulating the CB_1R gene, CNR1, has not been fully characterized, but a recent study in a mouse model of Huntington's disease suggests that the repressor element 1 silence transcription factor (REST) can regulate transcription of CB_1Rs (Blazquez et al., 2011).

Although there are numerous transcription factors found in mammalian cells, the majority of research has focused on the induction of zif268 (also known as Krox-24), CREB the Fos family of transcription factors (c-Fos, Fosb, ΔFosB, Fra-1 and Fra-2) and the Jun family of

transcription factors (c-Jun, JunB and junD) (Lazenka et al., 2013). Mailleux et al. (1994) first reported that *zif268* mRNA increased in the cingulate cortex, fronto-parietal cortex and caudate-putamen of rats 20 minutes after acute THC (5 mg/kg) injection. Separate studies in the caudate-putamen showed that zif268-immunoreactive (-ir) cells were restricted to striosomes when assessed 2 hours after injection of CP55,940 (2.5 mg/kg) (Glass and Dragunow, 1995). Studies in the hippocampus showed that acute THC (1 mg/kg) increased *zif268* mRNA in CA1 and CA3, but not dentate gyrus, in CD1 mice (Derkinderen et al., 2003). Zif268 is increased in the hippocampus of C57Bl/6J mice during learning tasks such as the Morris Water Maze task, but repeated administration of THC (1 mg/kg, 11 days) was shown to reduce zif268 in the hippocampus, suggesting zif268 could contribute to the memory impairing effects of THC (Boucher et al., 2009). This group also found a decrease in zif268 in caudate-putamen of these mice. The effects of THC have also been tested in zif268 knockout mice, but no genotype-specific differences were found for cannabinoid-induced analgesia or spontaneous withdrawal (Tzavara et al., 2001).

CREB has been proposed to be an important mediator of the effects of drugs of abuse (Nestler, 2004). Initial studies showed no changes in CREB bound to DNA in the caudate-putamen or cerebellum of rats that received THC (5-40 mg/kg b.i.d) for 5 days with brain collection 21 days after the last injection (Rubino et al., 2003). Subsequent studies using acute THC (15 mg/kg) administration found increased pCREB levels in the caudate-putamen, hippocampus and cerebellum, but not prefrontal cortex, of rats when measured 30 minutes following injection (Rubino et al., 2004). A different regional pattern emerged following repeated THC administration (15 mg/kg, b.i.d., 6.5 days), whereby pCREB was only increased in the prefrontal cortex of THC-treated rats. This finding could indicate that tolerance developed to

THC-induced activation of CREB in the other regions. A separate study examined CREB in the granule cell layer of the rat cerebellum. Results showed an increase in pCREB-ir cells in the granule cell layer following acute administration of 5 or 10 mg/kg THC, whereas repeated THC (10 mg/kg q.d., 4 weeks) administration produced a decrease in pCREB-ir that persisted for 3 weeks (Casu et al., 2005). This finding highlights the temporal nature of CREB activation, and suggests that alterations in CREB activity can persist after cessation of drug treatment. Measurement of CREB in the hippocampus following repeated THC administration has provided varying results. In one study, CREB and pCREB were decreased in the hippocampus in C57BL6 mice administered THC (10 mg/kg q.d.) for 7 days with levels assessed 24 hours after the last administration (Fan et al., 2010). Another group reported that repeated THC (10 mg/kg, b.i.d.) administration in rats for 4.5 days increased pCREB when tested 30 minutes after the final administration (Rubino et al., 2006). Differences in results could reflect methodological differences between the studies, most notably the survival time following final THC injection.

Fos (c-Fos, FosB, fos-related antigen 1 (Fra-1), Fra-2 and ΔFosB) and Jun (c-Jun, JunB and junD) families of transcription factors form AP-1 complexes that bind to AP-1 consensus sites on target genes. Mailleux et al. (1994) showed that c-Fos-ir and c-Jun-ir cells increased in the cingulate cortex when measured 20 minutes after THC (5 mg/kg) injection, whereas only c-Fos-ir cells increased in the fronto-parietal cortex and caudate-putamen. Subsequent studies showed an increase in c-Fos-ir cells in the caudate-putamen and nucleus accumbens of rats when measured 2 hours after THC injection (10 mg/kg) (Miyamoto et al., 1996). In this same study, pretreatment with a dopamine D_1 receptor (D_1R) antagonist (SCH-23390, 0.32 mg/kg), but not a D_2 receptor (D_2R) antagonist ((-)-sulpiride,100 mg/kg, i.p.), significantly attenuated c-Fos induction in these regions, suggesting that c-Fos induction was due to CB_1R-mediated dopamine

release and not through direct CB_1R signaling. The same group measured c-Fos-ir following repeated THC administration (10 mg/kg, q.d., 4 days) at 2 hours after final injection and compared the results to acute induction (Miyamoto et al., 1997). Repeated THC administration induced fewer c-Fos-ir cells as compared to acute administration, suggesting the development of tolerance. A similar study also suggested that tolerance developed to the induction of c-Fos in the prefrontal cortex and cerebellum following repeated, but not acute, THC (15 mg/kg) administration (Rubino et al., 2004).

Fewer studies have assessed FosB and its truncated isoforms (ΔFosB, Fra-1 and Fra-2) following cannabinoid treatment. Fos antigens are generally induced rapidly and transiently after acute drug administration (e.g. c-Fos). However, ΔFosB, a C-terminally truncated splice variant of FosB, is stable and accumulates with repeated induction over time (e.g. during repeated drug treatment), and can be detected in neurons for several weeks after cessation of drug treatment (Chen et al., 1997; Perrotti et al., 2005; Ulery et al., 2006). ΔFosB could therefore be important in regulating the long-term effects of repeated cannabinoid administration.

THC administration increased Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) and AP-1 DNA binding in the nucleus accumbens when measured one hour following administration of 10 or 15, but not 5, mg/kg of THC in rats (Porcella et al., 1998). AP-1 binding in the cingulate cortex and caudate-putamen was increased only after the highest dose of THC. In the cingulate cortex, this occurred in conjunction with increased c-Fos FosB, Fra-1 and Fra-2, whereas in the caudate-putamen, only c-Fos and FosB were significantly induced. ΔFosB was not significantly induced in any region examined, which is consistent with its low level of induction after a single drug injection. Induction of c-Fos, FosB, Fra-1 and Fra-2 was CB_1R-mediated because it was blocked by pretreatment with the antagonist SR141716A (Rimonabant) (Porcella et al., 1998).

Regional assessment of FosB following acute and repeated THC administration showed increased FosB in prefrontal cortex and hippocampus only after repeated THC administration (Rubino et al., 2004). The regional induction of ΔFosB following repeated THC administration has only been recently tested. Repeated THC administration significantly increased the number of FosB/ΔFosB-ir cells in the nucleus accumbens core with trends toward increases in the nucleus accumbens shell and caudate-putamen (Perrotti et al., 2008).

TABLE 0.2 Summary of brain region-dependent changes in immediate early gene (IEG) expression following acute or repeated THC administration. Adapted from (Lazenka et al., 2013)

Transcription factor	Treatment (time after last injection)	Increase in brain region	Decrease in brain region	Measure
Zif268				
Acute	5 mg/kg THC (20 min)	Cingulate cortex, fronto-parietal and caudate-putamen		mRNA immunohistochemistry
Acute	2.5 mg/kg CP55,940 (2 h)	Striosome of caudate-putamen		mRNA immunohistochemistry
Acute	1 mg/ml THC (60 min)	Hippocampus CA1 and CA3		mRNA immunohistochemistry
Repeated	1 mg/kg THC		Prefrontal cortex, caudate-	Protein
CREB				
Acute	15 mg/kg THC (30 min)	Caudate-putamen, hippocampus and cerebellum		pCREB protein bound to DNA ELISA
Acute	5 or 10 mg/kg THC (90 min)	Cerebellum		pCREB protein immunohistochemistry
Acute	1 µg, 5 µg or 10 µg THC microinjection (immediately after elevated plus maze)	Prefrontal cortex (10 µg) and ventral hippocampus (5 µg)	Basolateral amygdala (1 µg)	(pCREB) immunoblot

Repeated	15 mg/kg THC b.i.d. for 6.5 days (30 min)	Prefrontal cortex		pCREB protein bound to DNA ELISA
Repeated	10 mg/kg THC q.d. for 4 weeks (24 h or 3 weeks)		Cerebellum	pCREB protein immunohistochemistry
Repeated	10 mg/kg THC 4.5 days (30 min)	Hippocampus		pCREB bound to DNA
Repeated	10 mg/kg THC		Hippocampus	pCREB and total CREB
c-Fos				
Acute	5 mg/kg THC (20 min)	Cingulate cortex, fronto-parietal and caudate-putamen		mRNA immunohistochemistry
Acute	10 mg/kg THC (2 h)	Caudate-putamen and nucleus accumbens		Protein immunohistochemistry
Acute	25 mg/kg THC (1 h)	Lateral septum, paraventricular nucleus, caudate-putamen,	Prefrontal cortex and cerebellum	mRNA immunohistochemistry
Acute	5 mg/kg THC (1 h)	Prefrontal cortex, nucleus accumbens, caudate-putamen and hippocampus		mRNA RT-PCR
Repeated	15 mg/kg THC b.i.d. for 6.5 days (30 min)	Prefrontal cortex and cerebellum		c-Fos protein bound to DNA ELISA
FosB				
Acute	10 mg/kg and 15 mg/kg THC (1 h)	Nucleus accumbens		FosB, Fra-1 and Fra-2 protein immunoblot
Acute	15 mg/kg THC (1 h)	Caudate-putamen		FosB protein immunoblot
Acute	15 mg/kg THC (1 h)	Cingulate cortex		FosB, Fra-1 and Fra-2 protein immunoblot
Repeated	15 mg/kg THC b.i.d. for 6.5 days	Prefrontal cortex and hippocampus		FosB protein bound to DNA ELISA

	(30 min)			
ΔFosB				
Repeated	10–150 mg/kg q.d. for 14.5 days (24 h)	Nucleus accumbens core		Protein immunohistochemistry
Repeated	10 mg/kg q.d. for 13.5 days (24 h)	Prefrontal cortex, caudate-putamen, nucleus accumbens and cerebellum		Protein immunoblot

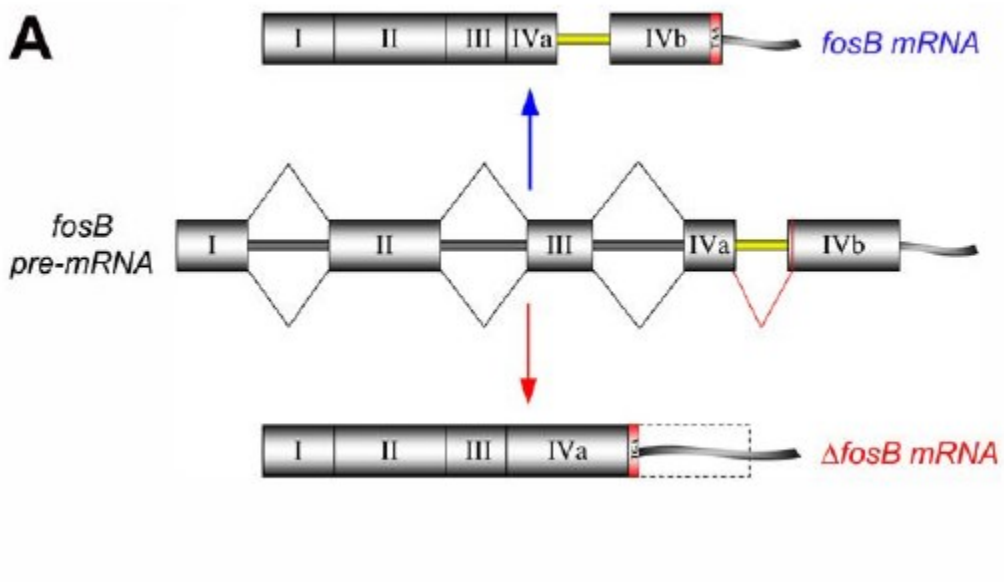


Figure 0.7. Representative figure of the FosB/ΔFosB mRNA transcript. ΔFosB is an isoform of FosB and the splicing out of region IVb reduces protesomal degradation of ΔFosB. Adapted from (Alibhai et al., 2007).

0.9 Transcriptional regulation by ΔFosB

ΔFosB is a member of the Fos family of transcription factors and is a truncated form of FosB (Figure 0.7). Early research into ΔFosB transcriptional regulation determined that it

repressed AP-1 activation when transiently transfected with various Fos and Jun family members (Nakabeppu and Nathans, 1991); however, another study determined that ΔFosB could activate transcription of an AP-1 reporter in a stably transfected cell line (Dobrazanski et al., 1991). In order to understand the overall pattern of ΔFosB-regulated gene expression *in* vivo, microarray studies were performed (McClung and Nestler, 2003). Gene expression changes in the nucleus accumbens were characterized following ΔFosB induction following repeated cocaine administration. These changes were compared to changes produced by overexpression of ΔFosB using bitransgenic mice and overexpression of ΔcJun, a dominant negative inhibitor of ΔFosB transcriptional regulation, in bitransgenic mice. These studies determined that initial overexpression/induction of ΔFosB produced similar effects as ΔcJun, meaning ΔFosB acted primarily as an AP-1 repressor. However, long-term overexpression/induction of ΔFosB had mostly opposing effects compared to ΔcJun, meaning ΔFosB acted as an AP-1 activator. At the behavioral level, differences also exist following short-term and long-term ΔFosB induction. Short term-ΔFosB induction and ΔcJun both reduce preference for cocaine, while long-term induction of ΔFosB increases preference for cocaine (McClung and Nestler, 2003).

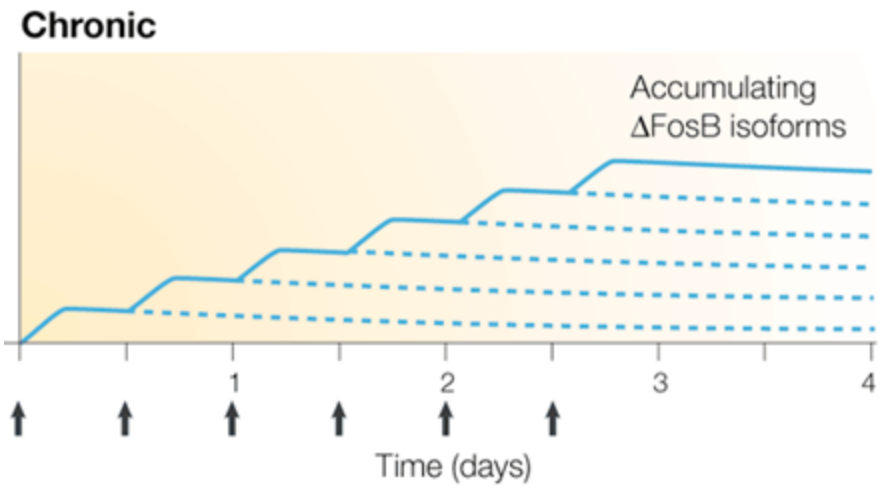


Figure 0.8 ΔFosB, due to its stability, accumulates following repeated drug administration. Adapted from (Nestler et al., 2001).

0.10 Genes targeted by ΔFosB

Unlike FosB, ΔFosB is minimally induced with acute drug administration but accumulates in cells due to its stability (Figure 0.8). ΔFosB regulates the N-methyl-D-aspartate (GluR2), as has been shown in the cerebral cortex following electroconvulsive seizures (Hiroi et al., 1998). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) glutamate receptor subunit 2 is also a ΔFosB target gene (Kelz et al., 1999). Overexpression of ΔFosB in bitransgenic mice increases GluR2 expression by over 50% in the nucleus accumbens, but no effect is seen on any other AMPA receptor subunit (Kelz et al., 1999). GluR2 is also up-regulated by cocaine, an effect ablated by overexpression of ΔcJun (Peakman et al., 2003) while ΔFosB binds the AP-1 consensus sequence at the GluR2 promoter region. Cyclin-dependent kinase 5 (CDK5) and its activating cofactor, p35, were identified as a ΔFosB target gene in the hippocampus and striatum by use of DNA microarrays (Bibb et al., 2001a; Chen et al., 2000b). CDK5 mRNA, protein, and activity are up-regulated in response to ΔFosB overexpression or

chronic cocaine treatment (Bibb et al., 2001a; Chen et al., 2000a) and this effect is blocked by overexpression of ΔcJun (Peakman et al., 2003). In addition, chromatin immunoprecipitation assays demonstrated that ΔFosB is selectively associated with the *CDK5* promoter following chronic, but not acute, cocaine administration (Kumar et al., 2005). CDK5 is involved in the regulation of cocaine-induced changes in dendritic spine density (Norrholm et al., 2003). CDK5 also increases the phosphorylation of DARPP-32 at threonine 75, which inhibits PKA activity (Bibb et al., 1999). Dynorphin appears to be another target for ΔFosB (Andersson et al., 2003), and is an example of a gene repressed by the transcription factor (Zachariou et al., 2006a). Finally, ΔFosB can recruit histone deacetylases (HDAC) to gene promoters, perhaps regulating gene expression through epigenetic mechanisms (Renthal et al., 2008). ΔFosB is known to repress cFos expression following repeated amphetamine administration through recruitment of HDAC1, which deacetylates histones at the promoter site, causes DNA to condense, and represses transcription.

Rationale and Hypothesis

CB_1Rs belong to the superfamily of GPCRs and are one of the most abundantly expressed GPCRs in the mammalian central nervous system. These receptors mediate the psychoactive and therapeutic effects of THC, the main psychoactive constituent of marijuana. Repeated administration of THC is known to produce brain region-dependent differences in CB_1R desensitization and downregulation and induction of transcription factors, suggesting a role for transcription factors in modulating these CB_1R adaptations. One transcription factor, ΔFosB, is induced primarily in striatal regions following repeated THC administration, and these regions are also known to be more resistant to CB_1R adaptations. As an overall hypothesis for this thesis, studies were performed to determine if THC-mediated induction of ΔFosB is regulated through interactions between cannabinoid and dopamine systems and that brain region-dependent differences in ΔFosB transcriptional regulation could explain some aspects of long-term CB_1R signaling and CB_1R adaptations.

In Chapter 1, studies were performed to determine the brain regional relationship between the THC-mediated induction of ΔFosB and CB_1R desensitization and downregulation. I hypothesize that regions with induction of ΔFosB will have less CB_1R desensitization than regions where ΔFosB is not induced. Further, I predict that CB_1Rs are expressed in those cells where ΔFosB is induced, and that THC-mediated induction of ΔFosB is CB_1R-dependent. If ΔFosB regulates CB_1R signaling, then it would require that ΔFosB and CB_1Rs are co-expressed.

Chapter 2 addresses the overall hypothesis by determining if ΔFosB can modulate CB_1R desensitization. To test this, bitransgenic mice with overexpression of ΔFosB or ΔcJun (a dominant negative inhibitor of ΔFosB transcription) in specific neuronal populations will be used to determine the effect of overexpression of these transcription factors on CB_1R desensitization.

This approach allowed direct testing of whether expression of ΔFosB would affect CB_1R adaptation in distinct brain regions. ΔFosB is overexpressed in the D_1R/dynorphin MSN population of the caudate-putamen and nucleus accumbens, as well as in the hippocampus and parietal cortex. ΔcJun is overexpressed in both the D_1R/dynorphin and D_2R/enkephalin MSN population, as well as in the hippocampus and parietal cortex. The specific overexpression of ΔFosB in the D_1R/dynorphin MSN population is functionally relevant since several drugs of abuse specifically induce ΔFosB in this neuronal population. THC-medited in vivo effects were also tested in these mice to determine if any ΔFosB-mediated changes in CB_1R desensitization were associated with altered tolerance following repeated THC administration. To address this possibility, tolerance to THC-mediated antinociception, hypothermia, locomotor suppression and catalepsy was assessed. It is hypothesized that ΔFosB overexpression will reduce CB_1R desensitization in the caudate-putamen, substantia nigra and nucleus accumbens, but have no effect in the hippocampus. Overexpression of ΔcJun is predicted to enhance desensitization in the caudate-putamen, nucleus accumbens, globus pallidus and substantia nigra by blocking ΔFosB-mediated transcription, but have no effect in the hippocampus. Further, less tolerance is expected to develop to the locomotor suppressing effects of THC in mice overexpressing ΔFosB while enhancing tolerance is expected in the ΔcJun overexpressing mice, because these brain contribute to THC-mediated locomotor suppression.

CB_1Rs are found primarily on axon terminals, suggesting that THC-mediated ΔFosB induction could be mediated indirectly by trans-synaptic events involving other receptors as opposed to directly by CB_1Rs in a cell autonomous manner. It is hypothesized that CB_1R-mediated ΔFosB induction can be indirectly mediated by CB_1R-mediated release of dopamine and the activation of D_1Rs. Other studies with drugs of abuse that produce dopamine release in

striatal regions have found that antagonism of D_1Rs can abolish the induction of ΔFosB in these regions. In chapter 3, this question was addressed pharmacologically by administering D_1R antagonists (SCH23390 and SCH39166) to determine if antagonism of D_1Rs blocks THC-mediated induction of ΔFosB. If THC-mediated induction of ΔFosB is dependent on D_1Rs, it is hypothesized that ΔFosB will be specifically induced in D_1R/dynorphin MSNs. To address this possibility, dual staining studies were conducted using antibodies directed against dynorphin and FosB/ΔFosB. This question is important since cocaine and natural rewards are known to increase ΔFosB expression specifically in this neuronal population. Activation of D_1Rs is known to alter the activity of DARPP-32 via phosphorylation at threonine-34 and genetic deletion of both DARPP-32 and mutation of the threonine-34 site attenuates cocaine-mediated induction of ΔFosB in striatal regions. Therefore, it is hypothesized that genetic deletion of DARPP-32 will attenuate THC-mediated induction of ΔFosB. If striatal ΔFosB induction is attenuated in DARPP-32 knockout mice, it is hypothesized that greater tolerance will develop to THC-mediated locomotor suppression, as predicted after inhibition of ΔFosB.

Finally, if ΔFosB regulates CB_1R desensitization, it is likely occurring through regulation of transcription and changes in the expression of known targets. It is hypothesized that expression of CDK5 and p35, two known transcriptional targets of ΔFosB, will be increased in regions where ΔFosB is induced following repeated THC administration. I also predict that repeated THC administration will increase the phosphorylation state of DARPP-32 at threonine 75 since CDK5, when dimerized with p35, phosphorylates DARPP-32 at this site. Understanding the signaling mechanisms that underlie CB_1R adaptation will provide insights into the development of possible therapeutic targets that can then selectively enhance or reduce CB_1R adaptation.

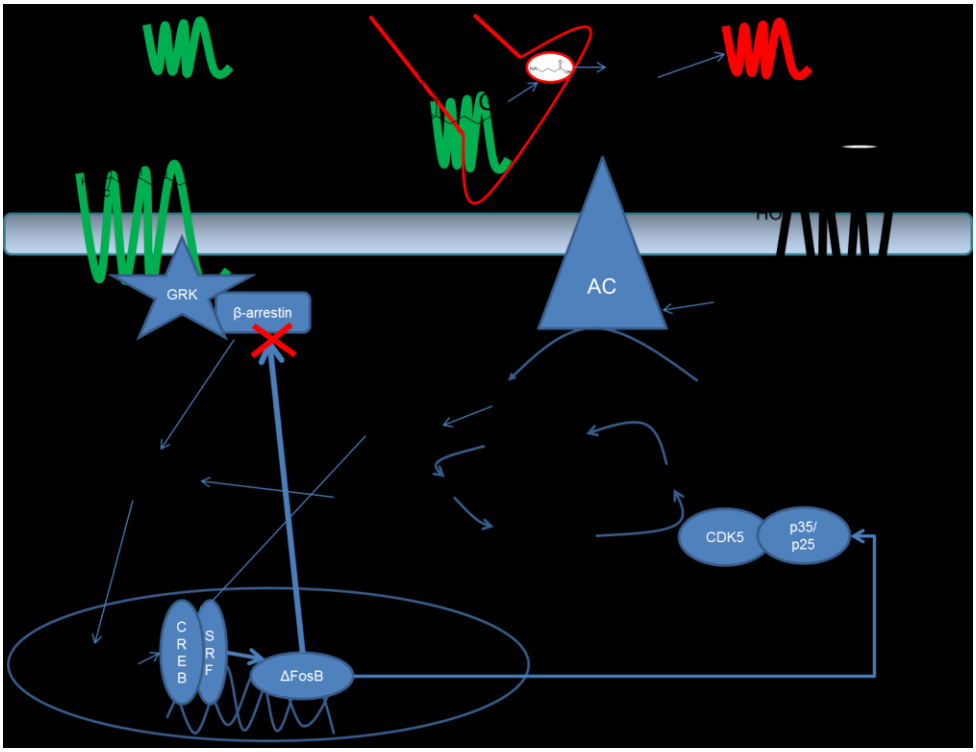


Figure 0.9 Hypothesized mechanism of THC-mediated ΔFosB induction in striatum. THC promotes release of dopamine through inhibition of GABA release from GABAergic MSN terminals. Dopamine activates D_1Rs on D_1R/dynorphin MSNs, which activates AC, increases cAMP and subsequently activates PKA. PKA then phosphorylates DARPP-32 at threonine 34, which indirectly increases phosphorylation of ERK through increased phosphorylation of striatal-enriched protein tyrosine phosphatase (STEP, not shown), which inactivates ERK. PKA and ERK (through ETS domain-containing protein 1, ELK-1) phosphorylate CREB, which regulates expression of ΔFosB when dimerized with serum response factor (SRF). ΔFosB can increase CDK5 and p35/p25 expression, which would feed back on the DARPP-32 pathway, providing one mechanism through which ΔFosB could regulate CB_1R desensitization. CB_1Rs could also increase ERK phosphorylation through the recruitment of βarrestin.

Chapter 1: ΔFosB induction correlates inversely with CB_1 receptor desensitization in a brain region- dependent manner following repeated Δ^9-THC administration

1.1 Introduction

Marijuana is the most widely used illicit drug in the United States and its repeated use leads to the development of both tolerance and withdrawal symptoms, which are included in the DSM-IV criteria for cannabis use disorder (American Psychiatric Association, 2000; SAMHSA, 2010). THC is the main psychoactive constituent of marijuana and produces its behavioral effects via CB_1Rs, which are G-protein-coupled receptors that are widely distributed in the brain (Howlett et al., 2002). Cannabinoid-mediated effects in rodents include antinociception, hypothermia, catalepsy, hypolocomotion and memory impairment (Howlett et al., 2002; Varvel and Lichtman, 2002). Repeated THC administration produces tolerance to these effects and withdrawal occurs upon cessation of treatment or antagonist administration (Lichtman and Martin, 2005). Studies have revealed alterations in CB_1R signaling following repeated cannabinoid treatment, but the relationship between these molecular adaptations and tolerance and dependence are not well understood. Repeated THC administration decreases both CB_1R levels (downregulation) and CB_1R-mediated G-protein and effector activity (desensitization) in rodent brain (Sim-Selley, 2003). Several studies have demonstrated that there are differences among brain regions in the magnitude and temporal properties of CB_1R desensitization and downregulation. Specifically, CB_1R desensitization and downregulation occur at lower agonist doses and develop more rapidly in the hippocampus than in the striatum (caudate-putamen and nucleus accumbens) (Breivogel et al., 1999; McKinney et al., 2008). These findings appear to translate to human cannabis users. CB_1R levels were lower in the brains of marijuana users compared to non-users, and the magnitude of apparent downregulation exhibited a similar

regional pattern as seen in rodents (Villares, 2007). Region-specific reductions in CB_1R binding have also been reported using in vivo imaging in subjects that were marijuana users (Hirvonen et al., 2012). The recovery of CB_1R levels and activity after cessation of cannabinoid treatment was slower in the hippocampus than striatum in rodents (Sim-Selley et al., 2006). Similarly, reduced CB_1R binding in human brain persisted in the hippocampus after ~4 weeks of abstinence from marijuana, whereas binding in other regions appeared similar to pre-drug levels at this time point (Hirvonen et al., 2012). These observations are important because the hippocampus is associated with cognitive and memory impairing effects of cannabinoids, whereas the striatum mediates motivational and motor effects of these drugs(Breivogel and Sim-Selley, 2009). In fact, studies in human marijuana users suggest that greater tolerance develops to memory impairment compared to motor or subjective measures such as "high" (D'Souza et al., 2008; Haney et al., 2004; Ramaekers et al., 2009)

The mechanisms underlying regional differences in CB_1R adaptations are not known, but differences in the expression of signaling and regulatory proteins among brain regions, and changes in their expression following repeated THC administration, could contribute to these findings. ΔFosB, a truncated splice variant of the transcription factor FosB, is modestly induced following a single drug injection, but accumulates upon repeated drug administration and is stable for weeks after cessation of treatment (Chen et al., 1997). Treatment with several drugs of abuse, including opiates and cocaine, induces ΔFosB in the striatum (Nestler et al., 2001). We showed that THC significantly increased the number of FosB/ΔFosB-immunoreactive (-ir) cells in the nucleus accumbens core (Perrotti et al., 2008). Semi-quantitative analysis also showed that THC-induced FosB/ΔFosB-ir cells in other forebrain regions, but protein levels could not be quantified with this technique.

Bitransgenic mice that overexpress ΔFosB in dopamine D_1R/dynorphin containing striatal MSNs exhibit increased rewarding effects of several drugs of abuse and natural rewards (Nestler, 2008). These same mice also had increased G-protein signaling and AC inhibition for mu- and kappa-opioid receptors, respectively, in the nucleus accumbens, suggesting that ΔFosB modulates signaling at the receptor/effector level (Sim-Selley et al., 2011). The striatum and its projection regions appear resistant to CB_1R desensitization and downregulation (Sim-Selley, 2003) and the striatum is involved in the rewarding effects of drugs of abuse (Koob, 1999; Koob and Volkow, 2010). Taken together, these findings suggest that ΔFosB might modulate CB_1Rs after repeated drug administration, but the regional expression pattern of ΔFosB and CB_1R desensitization and downregulation has not been directly compared in brains from animals that received the same THC administration paradigm. Therefore, this study investigated the brain regional relationship between ΔFosB induction and CB_1R desensitization and downregulation after repeated THC treatment. Studies were also conducted to determine the neuroanatomical relationship between CB_1Rs and ΔFosB positive cells in the striatum. Finally, the role of CB_1Rs in THC-mediated ΔFosB induction was assessed in CB_1R knockout mice. Results showed an inverse regional relationship between CB_1R desensitization and ΔFosB induction and neuroanatomical results support the possibility of both cell-autonomous and trans-synaptic interactions.

1.2 Materials and Methods

Materials

THC and [(-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol] (CP55,940) were provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). [^{35}S]GTPγS (1250 Ci/mmol) was purchased

from PerkinElmer Life Sciences (Boston, MA). Bovine serum albumin (BSA) and guanosine diphosphate (GDP) were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit anti-FosB antibodies (sc-7203 and sc-48) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-CB_1R and guinea-pig anti-CB_1R antibodies (against residues 401-473 of the CB_1R) (Pickel et al., 2006) were generously provided by Dr. Ken Mackie (Indiana University, Bloomington, IN). Secondary antibodies were purchased from either LI-COR (Lincoln, NE) or Invitrogen (Grand Island, NY). ProLong® Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen. All other reagent grade chemicals were obtained from Sigma Chemical Co. or Fisher Scientific.

Subjects

Male ICR mice (Harlan Laboratories, Indianapolis, IN) weighing 25-30 grams (n=8 per group) were used to assess CB_1R adaptations and ΔFosB induction. THC (10 mg/kg) was dissolved in a 1:1:18 solution of ethanol, emulphor and saline (vehicle). Mice were injected subcutaneously with either vehicle or THC at 07:00 and 16:00 h for 13 days. On day 14, mice received a morning injection only, and 24 hours later mice were sacrificed by decapitation and brains were extracted. Brains were then hemisected, with one half dissected for immunoblot analysis and the other half frozen in isopentane at -30°C for autoradiography and immunohistochemistry to measure [^{35}S]GTPγS binding and CB_1R levels, respectively. Based on initial results, a second group of ICR mice was treated as described above, and the lateral and basomedial nuclei of the amygdala were dissected to determine ΔFosB expression.

For immunohistochemical studies to determine whether CB_1Rs and ΔFosB are co-localized in striatal neurons, male ICR mice (n=4) were treated with vehicle or a ramping dose of THC (10-20-30 mg/kg) twice daily for 6.5 days. We have previously determined that this

treatment paradigm induces a high level of ΔFosB in the striatum (unpublished data). Brains were collected 24 hours after final drug administration to maximize the detection of ΔFosB, which is more stable than FosB.

The role of CB_1Rs in ΔFosB induction was determined using CB_1R knockout mice on a C57Bl/6J background and littermate controls (Zimmer et al., 1999) (n = 7-8 per group). CB_1R knockout and wild type (WT) mice were treated with THC (10mg/kg) or vehicle for 13.5 days as described above, and the caudate-putamen and nucleus accumbens were dissected 24 hours after final treatment. A separate group of C57Bl/6J mice (Jackson Laboratories, Bar Harbor, Maine) were treated with increasing doses of THC to determine whether results in CB_1R knockout mice were due to an inability of this dose of THC (10 mg/kg) to further induce ΔFosB above levels in vehicle-treated mice. Mice received vehicle, 10 mg/kg THC or 30 mg/kg THC for 13.5 days as described above and the caudate-putamen was dissected 24 hours after the final injection.

Mice were housed four to six per cage and maintained on a 12-hr light/dark cycle in a temperature controlled environment (20-22°C) with food and water available ad libitum. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health guide for the care and use of Laboratory animals 7th edition.

Dissections

Brain regions of interest were dissected from hemisected or whole fresh brains. The prefrontal cortex was dissected by making a cut at the posterior extent of the anterior olfactory nucleus after which the olfactory nuclei were removed. This sample included frontal association, primary and secondary motor, anterior cingulate, prelimbic and orbital frontal cortices. The next

cut was made anterior to the optic chiasm to produce a thick coronal section. The nucleus accumbens was dissected by removing the cortex ventrally and the septum and nucleus of the horizontal limb of the diagonal band medially and then collecting the tissue surrounding the anterior commissure. The caudate-putamen was dissected by removing the cortex and then collecting the caudate-putamen that remained after removal of the nucleus accumbens. The hippocampus was exposed by removing the cortex from the remaining brain, then dissecting the whole hippocampus from the surface of the brain. In a separate experiment, the lateral amygdala (including the ventrolateral, dorsolateral, and anterior and posterior basolateral nuclei) and basomedial amygdala were dissected. These dissections were made by first cutting caudal to the optic chiasm, and then making a second cut directly caudal to the median eminence. The basomedial amygdala was isolated by removing the surrounding ventral amygdaloid regions and separating dorsally at the ventral extent of the bifurcated corpus callosum. The lateral amygdala was isolated by removing the tissue found within the bifurcated corpus callosum.

Agonist stimulated [^{35}S]GTPγS autoradiography

Assays were conducted as previously published from our laboratory (Nguyen et al., 2010; Sim et al., 1995). Briefly, coronal sections (20 μm) were cut on a cryostat maintained at -20°C, thaw-mounted onto gelatin-coated slides and stored desiccated at 4°C overnight. Sections were collected at 3 levels to include 1) prefrontal cortex, 2) nucleus accumbens and caudate-putamen, and 3) hippocampus, lateral amygdala and basomedial amygdala. Slides were stored desiccated at -80°C until use. For assays, slides were brought to room temperature, and then rinsed in 50 mM Tris-HCl buffer (pH 7.4) with 3 mM $MgCl_2$, 0.2 mM ethylene glycol tetraacetic acid (EGTA) and 100 mM NaCl (Assay Buffer) for 10 min at 25°C. Next, slides were transferred to Assay Buffer + 0.5% BSA, with 2 mM GDP and 10 mU/ml adenosine deaminase for 15 min at

25°C. Slides were then incubated in Assay Buffer + 0.5% BSA containing 0.04 nM [^{35}S]GTPγS with 3 μM CP55,940 or vehicle (basal) for 2 hours at 25°C. CP55,940 was used because we have previously shown that it does not stimulate [^{35}S]GTPγS binding in autoradiography of CB_1R knockout mouse brains (Nguyen et al., 2010). The maximally effective concentration of CP55,940 was previously determined in cerebellar sections and homogenates (Nguyen et al., 2010). After final incubation, slides were rinsed twice in 50 mM Tris buffer (pH 7.4) at 4°C, and then in deionized water. Slides were then dried and exposed to Kodak Biomax MR film with [^{14}C] microscales for 18 hrs. Films were digitized at 8-bits per pixel with a Sony XC-77 video camera. Brain regions of interest (ROIs) were determined using The Mouse Brain Atlas (Franklin and Paxinos, 2008). Images were analyzed using NIH Image J software as described previously and resulting values are expressed as nanocuries of [^{35}S] per gram of tissue (nCi/g). Net agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal (without agonist) binding from agonist-stimulated binding. Values were obtained in quadruplicate sections collected from eight hemisected brains per group and averaged for statistical analysis.

Immunohistochemistry

CB_1R immunofluorescence was used to assess receptor levels in hemisected brains. Slide-mounted sections were washed in 0.1 M phosphate buffer (pH 7.4) with 0.9% NaCl (PBS) for 5 minutes and fixed with 4% paraformaldehyde dissolved in 0.05 M phosphate buffer (pH 7.4), 0.9% NaCl, 1% Triton-X100 (PBST) for 30 minutes. Slides were rinsed 3 X 5 minutes in 0.1 M Tris buffer (pH 7.4), with 0.9% NaCl and 0.1% Triton-X100 (TBST), and then blocked in TBST containing 5% normal donkey serum. Slides were incubated overnight at 4°C in TBST containing 2.5% normal donkey serum and goat-anti CB_1R (1:2000). Slides were then washed 3 X 10 minutes in TBS containing 0.05% Tween-20 and incubated in Alexa 800 donkey anti-goat

IgG (1:5000) for 2 hours. After incubation, slides were washed 2 X 10 minutes in TBS containing 0.05% Tween-20 and 1 X 5 minutes in TBS. Fluorescent immunoreactivity was detected with the LI-COR Odyssey scanner (42 μm resolution, 1 mm offset with highest quality, channel sensitivity set at 4.0) and LI-COR software v 2.1 was used to measure the average intensity of ROIs (Franklin and Paxinos, 2008) with the free form shape tool. Average intensity values were used to account for differences in the size of ROIs between slices because this is not corrected using integrated intensity.

CB_1R and ΔFosB/FosB dual staining was assessed in coronal sections of the striatum to determine the anatomical relationship between these two proteins. Slide-mounted sections (20 μm) were washed in 0.1 M phosphate buffer (pH 7.4) with 0.9% NaCl (PBS) for 5 minutes and fixed with 4% paraformaldehyde (30 minutes) dissolved in 0.05 M PBS. Slides were washed 3 X 5 minutes in PBS and incubated in PBS containing 1% Triton-X100 for 15 minutes. Slides were then washed 3 X 5 minutes in PBS and incubated in PBS containing 5% normal goat serum for 1 hour. Slides were incubated overnight at 4°C in PBS containing 2.5% normal donkey serum and antibodies against CB_1R (1:1000; guinea-pig) and FosB (1:500; sc-48/rabbit). Slides were then washed 3 X 5 minutes in PBS containing Alexa Fluor® 488 goat anti-guinea pig IgG (1:500) and Alexa Fluor® 594 goat anti-rabbit IgG for 2 hours. After incubation, slides were washed 3 X 10 minutes in PBS and once for 5 minutes in double-distilled water. Slides were coverslipped using ProLong® Gold anti-fade reagent with DAPI. Images were captured on a Zeiss 700 laser scanning confocal microscope utilizing the ZEN 2011 software. Pinhole diameter was set to 1 Airy unit for the 488 wavelength to which the optical slice thicknesses were matched for the 405 and 594 detectors. Scan resolution was optimized to meet Nyquist sampling criteria in the X and Y dimensions. Signal crosstalk was eliminated by separating each wavelength into

individual tracks and scanning sequentially. Scanning line-by-line, averaging four passes in a single direction, then yielded an image at a 16 bit depth. All images were taken under a Zeiss Plan-Apochromat 40x/1.3 Oil objective.

Immunoblots

Immunoblotting was performed as previously described (Sim-Selley et al., 2006; Zachariou et al., 2003). Tissue was homogenized in 20 mM HEPES buffer (pH 7.8) with 0.4 M NaCl, 20.0% glycerol, 5.0 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 0.1 mM EGTA and 1% NP-40 (EMSA buffer) containing 0.5 mM phenylmethanesulfonylfluoride, 10 µg/ml leupepsin, 100 µg/ml benazamide, 2 µg/ml aprotinin, 500 µM dithiothreitol and Halt™ protease inhibitor cocktail. Samples (50 µg protein) were loaded in 10% Tris-HCl gels and separated by electrophoresis. Gels were transferred onto nitrocellulose paper, blocked in 0.1 M TBS with 5% Carnation™ instant nonfat dry milk for 1 hour, incubated in antibodies against α-tubulin (1:1000) and FosB (1:500) in 0.1 M TBS containing 0.1% Tween-20 (TBST) with 5% nonfat dry milk. Blots were washed 3 X 10 minutes in TBST and incubated with Alexa 680 goat anti-rabbit IgG (1:12000) and Alexa 800 goat anti-mouse IgG (1:12000) in TBST for 45 minutes. Fluorescent intensity was visualized using the Odyssey LI-COR infrared scanner. LI-COR software v 2.1 was used to measure integrated intensity between treatments for the band of interest, with subtraction of the background (average of intensities 3 border widths above and below the band). In order to verify that bands for the α-tubulin loading control were not saturated and ensure the accuracy of results, an experiment was conducted in which varying concentrations of protein (25-100 µg) were loaded onto the gel and intensity was measured using the LI-COR system. Linear regression analysis showed that these data fitted at $r^2 = 0.9978$, thereby confirming that the signal was not saturated at 50 µg, the amount of protein used in these

studies.

Data Analysis

For all experiments, data were analyzed with Prism® version X (GraphPad Software, San Diego, CA). For desensitization and downregulation studies and immunoblots comparing only vehicle and 10 mg/kg THC, student t-tests were used to compare means of repeated THC and vehicle groups based on planned comparisons by region. For studies in CB_1R knockout and wild type mice, data were analyzed by two-way ANOVA and Bonferroni post-hoc test and one-way ANOVA with Dunnett's post-hoc test in instances where an interaction was found. For all other studies, one-way ANOVAs were performed with Bonferroni post-hoc test. To determine whether ΔFosB induction correlated with CB_1R desensitization, linear regression analysis was performed and the significance of correlations was determined with F-tests to determine whether the slope of the line was significantly non-zero. Significance was determined with $p < 0.05$.

1.3 Results

Repeated THC administration reduces CP55,940-stimulated [^{35}S]GTPγS binding in a region-specific manner

CP55,940-stimulated [^{35}S]GTPγS binding was conducted to determine whether 13.5 day treatment with 10 mg/kg THC (b.i.d.) produced CB_1R desensitization in the forebrain. No differences in basal [^{35}S]GTPγS binding were found between THC- and vehicle-treated mice in any region examined (data not shown). Densitometric analysis revealed a region-dependent reduction in CP55,940-stimulated [^{35}S]GTPγS binding in brains from THC- compared to vehicle-treated mice. THC treatment produced a significant reduction in CP55,940-stimulated [^{35}S]GTPγS binding in the prefrontal cortex (29% decrease, $df=14$, $p < 0.05$) and hippocampus

(50% decrease, df=14, $p < 0.01$) compared to vehicle-treated mice (Figure 1.1, Table 1.1). THC treatment significantly reduced CP55,940-stimulated [^{35}S]GTPγS binding by 27% (df=14, $p < 0.05$) in both the lateral amygdala (including the lateral and basolateral nuclei) and basomedial amygdala of THC-compared to vehicle-treated mice. In contrast, there was no significant difference in CP55,940-stimulated [^{35}S]GTPγS binding in the caudate-putamen or nucleus accumbens of THC- versus vehicle-treated mice (Figure 1.1, Table 1.1). Therefore, the regional profile of relative CB_1R desensitization was hippocampus >> prefrontal cortex ≥ basomedial amygdala = lateral amygdala > > caudate-putamen = nucleus accumbens.

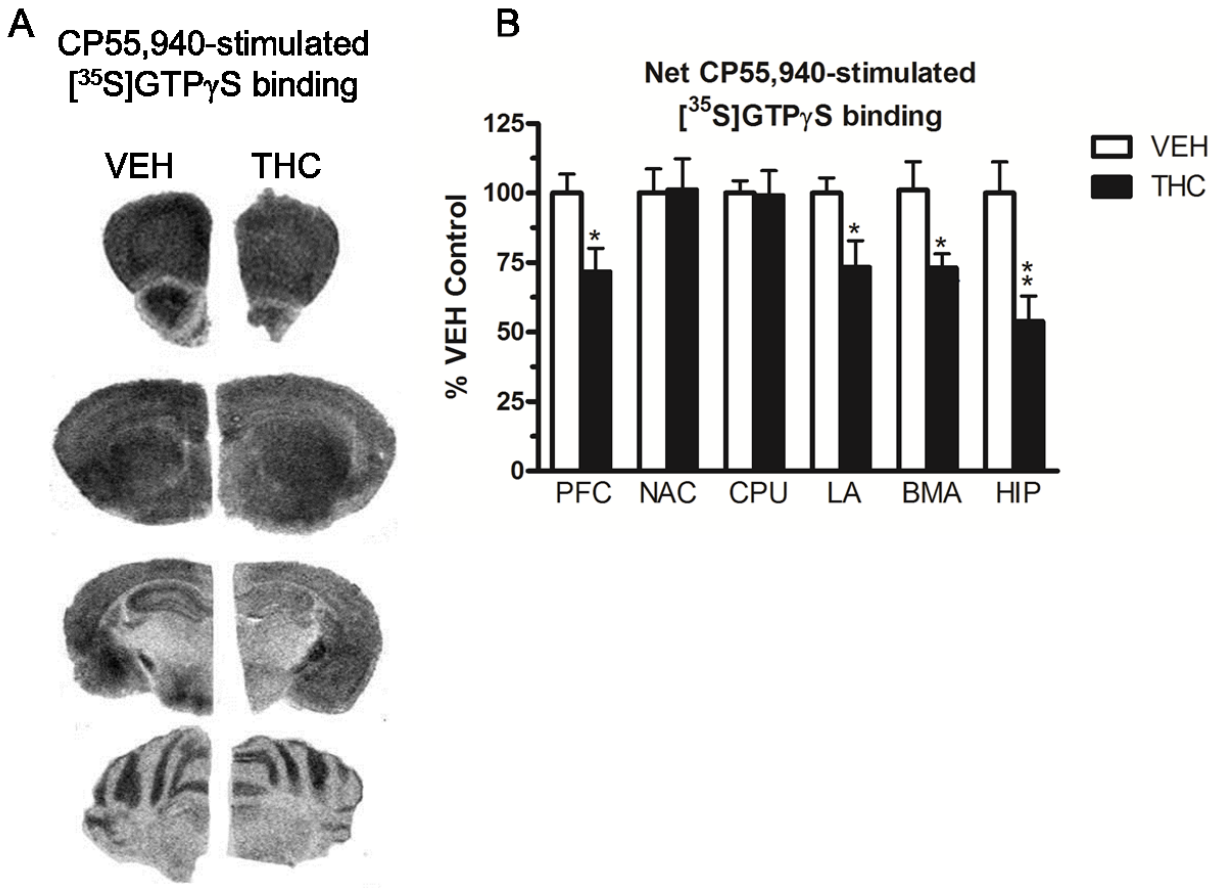


Figure 1.1 (A) Representative autoradiograms showing CP55,940-stimulated [^{35}S]GTPγS binding in brains from vehicle and THC-treated mice. Prefrontal cortex is shown in row 1, nucleus accumbens and caudate-putamen in row 2 and hippocampus, lateral amygdala and basomedial amygdala in row 3. (B) Graph representing differences in net-stimulated [^{35}S]GTPγS binding expressed as a percent of net-stimulated binding in vehicle-treated mice. Data are means ± SEM with * $p < 0.05$ and ** $p < 0.01$ versus vehicle controls, un-paired, two-tailed Student t-test, n = 8 mice per group.

CB_1R-ir is reduced by repeated THC treatment in a subset of brain regions

CB_1R-ir was measured using immunohistochemistry in brain sections that were near-adjacent to those used for [^{35}S]GTPγS autoradiography. CB_1R-ir in brain sections was analyzed using the Odyssey LI-COR system, which can scan images with a resolution up to 24 μm, allowing accurate measurements of differences in fluorescent intensity (Brunet et al., 2009; Kearn, 2004). CB_1R-ir was measured in the same regions as described above for agonist-stimulated [^{35}S]GTPγS binding. Decreased CB_1R-ir, indicative of downregulation, was found in many of the same regions as CB_1R desensitization, although the magnitude of the decrease was generally greater for desensitization. CB_1R-ir was significantly reduced in the prefrontal cortex (19% decrease, df=14, $p < 0.01$), lateral amygdala (15% decrease, $p < 0.05$) and hippocampus (22% decrease, df=14, $p < 0.05$) of THC- compared to vehicle-treated mice (Figure 1.2, Table 1.1). CB_1R-ir did not significantly differ between THC- and vehicle-treated mice in the nucleus accumbens, caudate-putamen or basomedial amygdala. These results demonstrate a similar regional pattern for CB_1R desensitization and downregulation.

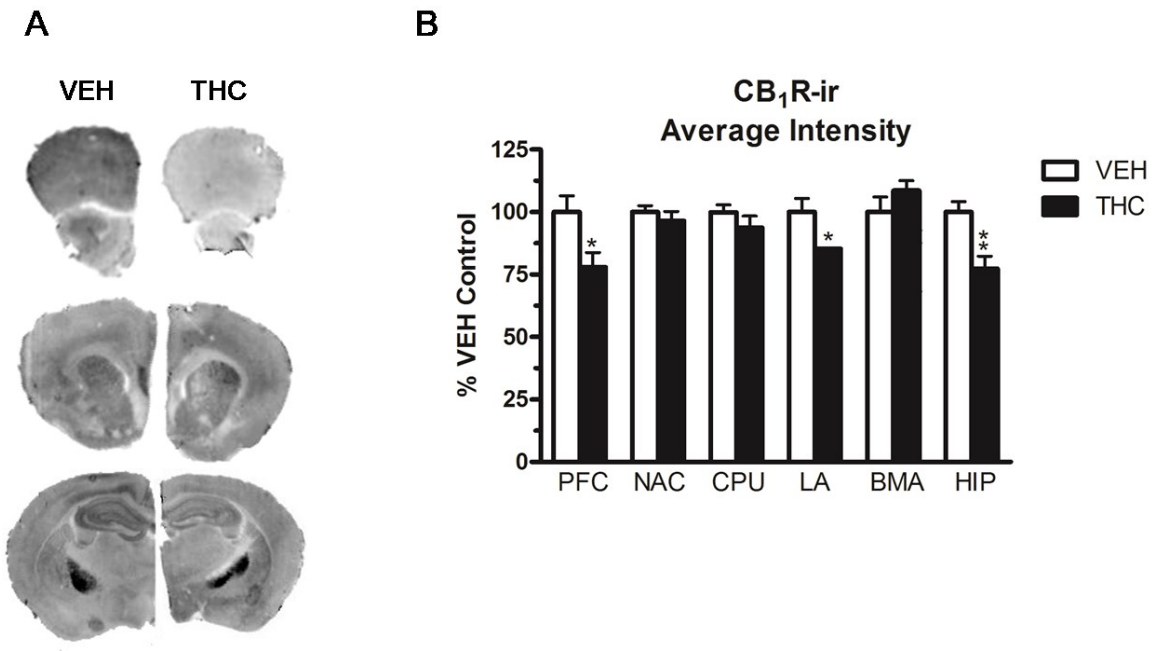


Figure 1.2 (A) Representative images of LI-COR scans for CB_1R-ir. Prefrontal cortex is shown in row 1, nucleus accumbens and caudate-putamen in row 2, and hippocampus, lateral amygdala and basomedial amygdala in row 3. (B) Graph showing differences in average intensity for CB_1R-ir as a percent of vehicle. Data are means ± SEM with * $p < 0.05$ versus vehicle controls, un-paired, two-tailed Student t-test, n = 8 mice per group.

TABLE 1.1

Net CP55,940-stimulated [^{35}S]GTPγS binding and CB_1R-ir measured in brain sections from vehicle- and THC- treated mice

	Net [^{35}S]GTPγS binding		CB_1R-ir Average intensity	
Region	VEHICLE	THC	VEHICLE	THC
Prefrontal cortex	541 ± 35	388 ± 42*	1173 ± 66	946 ± 20*
Nucleus accumbens	286 ± 25	290 ± 32	432 ± 12	417 ± 16
Caudate-putamen	319 ± 14	316 ± 29	1160 ± 34	1134 ± 33
Lateral amygdala	397 ± 21	290 ± 36*	461 ± 18	393 ± 2*
Basomedial amygdala	349 ± 35	253 ± 16*	351 ± 33	365 ± 14
Hippocampus	380 ± 25	188 ± 32**	498 ± 7	392 ± 12**

Brain sections were incubated in 0.04 nM [^{35}S]GTPγS, 3 μM CP55,940 and 2 mM GDP for autoradiography and results are expressed as net CP55,940-stimulated [^{35}S]GTPγS binding (nCi/g) ± SEM. Near-adjacent sections were processed with an antibody to CB_1R for immunohistochemistry and results are expressed as CB_1R-ir average intensity in units of counts/pixels ± SEM. *$p < 0.05$ **$p < 0.01$ different from vehicle by Student's t-test, n=8 mice per group.

ΔFosB is induced by THC treatment in specific forebrain regions

Immunoblots were performed to determine the relative expression levels of ΔFosB between vehicle- and THC-treated mice. Immunoblot results showed region-specific induction of ΔFosB expression by THC. Repeated THC treatment produced significant increases in ΔFosB-ir in the prefrontal cortex (43% increase, df=14, $p < 0.05$), caudate-putamen (62% increase, df=14, $p < 0.001$), nucleus accumbens (87% increase, df=14, $p < 0.001$) and lateral amygdala (38% increase, df=14, $p < 0.05$) of THC- compared to vehicle-treated mice (Figure 1.3, Table 1.2). In contrast, ΔFosB-ir in the basomedial amygdala and hippocampus did not significantly differ between treatment groups. Therefore, the regional profile of THC-mediated ΔFosB induction was nucleus accumbens > caudate-putamen > prefrontal cortex > lateral amygdala >> basomedial amygdala = hippocampus.

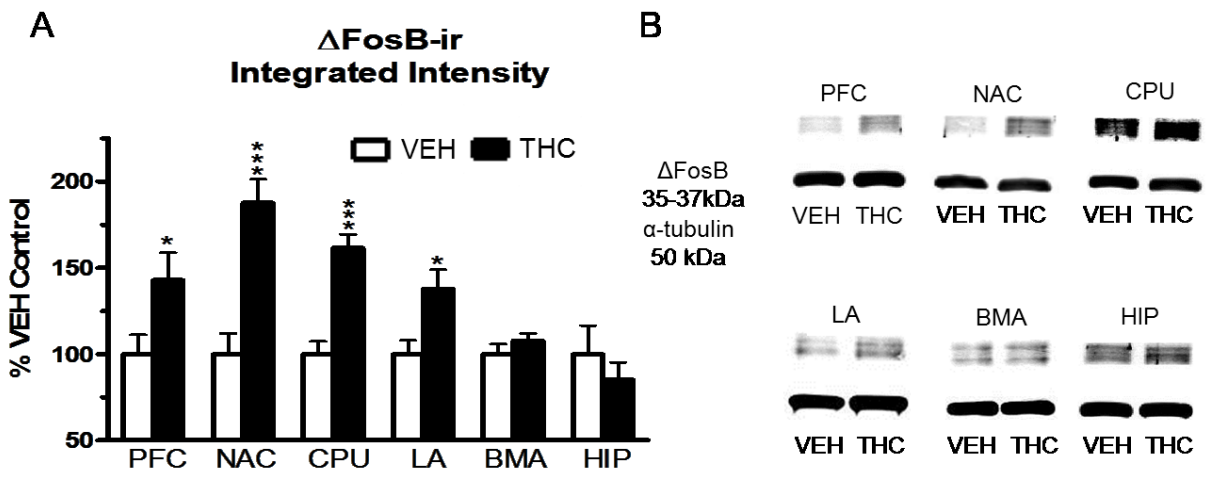


Figure 1.3 Immunoblot results for ΔFosB expression in the prefrontal cortex, nucleus accumbens, caudate-putamen, lateral amygdala, basomedial amygdala and hippocampus of mice that received repeated vehicle or THC administration. Blots were probed with antibodies directed against ΔFosB and α-tubulin (loading control). (A) Graph showing densitometric analysis of brain regions from vehicle- and THC-treated mice expressed as percent vehicle control. Data are means ± SEM with * $p < 0.05$ and *** $p < 0.001$ versus vehicle controls, un-paired, two-tailed student t-test, n = 8 per group. (B) Representative blots showing ΔFosB-ir and α-tubulin-ir in vehicle- and THC-treated brains for each region examined.

TABLE 1.2

ΔFosB expression measured by immunoblot in brains from vehicle- and THC-treated mice

Region	ΔFosB-ir Integrated intensity VEHICLE	THC
Prefrontal cortex	2.94 ± 0.33	4.20 ± 0.47*
Nucleus accumbens	1.81 ± 0.22	3.39 ± 0.25***
Caudate-putamen	1.56 ± 0.11	2.52 ± 0.12***
Lateral amygdala	2.94 ± 0.11	4.05 ± 0.08*
Basomedial amygdala	1.98 ± 0.11	2.13 ± 0.08
Hippocampus	1.40 ± 0.23	1.20 ± 0.14

ΔFosB-ir was measured in homogenates prepared from brain regions of interest using an antibody against FosB that recognizes all FosB isoforms, as described in Methods. The 35-37 kDa band, defined as ΔFosB, was measured for analysis. Results are expressed as integrated intensity in units of counts-mm^2 ± SEM. *$p < 0.05$ ***$p < 0.001$ different from vehicle by Student's t-test, n=8 mice per group

CB_1R *desensitization and ΔFosB expression are inversely correlated*

Reductions in CB_1R-ir and CB_1R-mediated G-protein activity exhibited a similar regional pattern, whereas THC-mediated ΔFosB induction was most robust in regions with less CB_1R desensitization. In order to determine whether these observations represented significant correlations, the mean percent changes in [^{35}S]GTPγS binding, CB_1R-ir and ΔFosB-ir of vehicle- versus THC-treated mice were plotted for each region. Desensitization ([^{35}S]GTPγS autoradiography, y-axis) and downregulation (CB_1R-ir, y-axis) were each compared to ΔFosB expression (immunoblots, x-axis). For the comparison between ΔFosB-ir and downregulation, the slope of the linear regression line was not determined to be significantly non-zero $r(4) = 0.20$, $p = 0.67$. For the comparison between ΔFosB-ir and desensitization, the slope of the linear regression line was determined to be significantly non-zero $r(4) = 0.94$, ($p < 0.01$) (Figure 1.4). These analyses confirmed initial observations and showed a significant inverse regional correlation between CB_1R desensitization and ΔFosB expression.

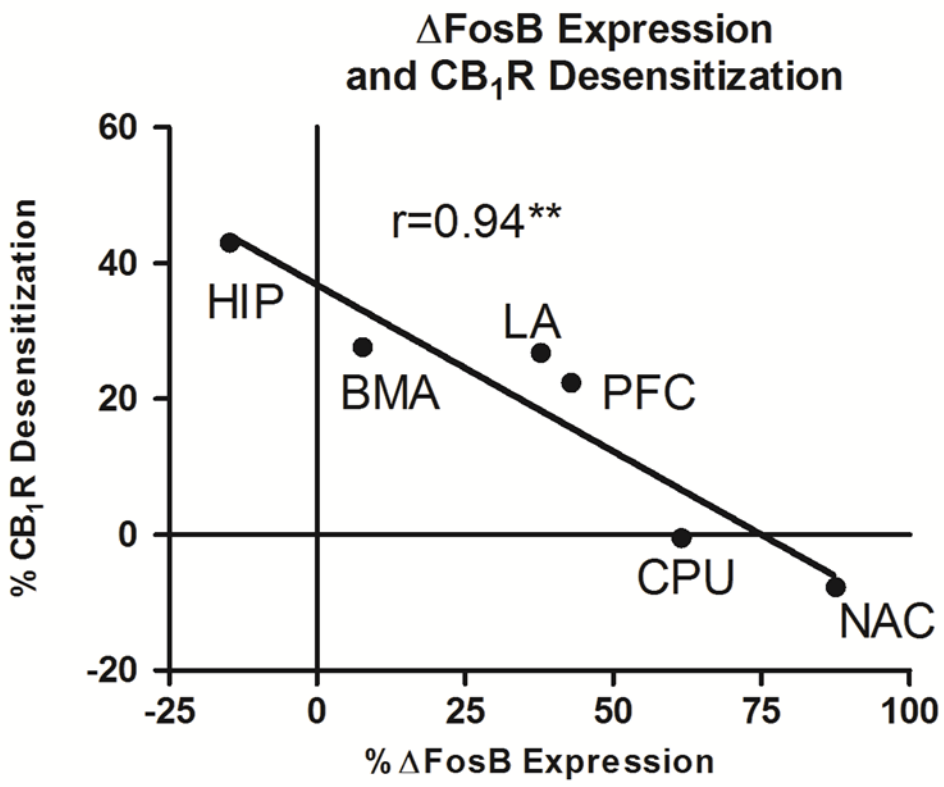


Figure 1.4 Correlation of percent change in measured parameters for THC-compared to vehicle-treated mice for the brain regions examined between desensitization (y-axis) and ΔFosB expression (x-axis). Correlation is presented as percent change from vehicle with corresponding r-squared values. Data are means ± SEM with ** $p < 0.01$, F-test, n=8 per group.

CB_1Rs co-localize with and contact ΔFosB/FosB-ir neurons

Immunohistochemistry was performed in order to determine whether the interaction between CB_1Rs and ΔFosB occurs within the same cell or is a trans-synaptic effect. Mice were treated with a ramping dose of THC (10-20-30mg/kg) that strongly induces ΔFosB expression in the striatum. The antibody used to assess ΔFosB recognizes FosB/ΔFosB, but the 24-hour post-treatment survival time used in this experiment favors detection of ΔFosB (Perrotti et al., 2004). CB_1R-ir was visualized in green and FosB/ΔFosB-ir was visualized in red (Figure 1.5). DAPI (blue) was used to identify cell nuclei. The distribution of CB_1R-ir in the caudate-putamen and nucleus accumbens of both vehicle- and THC-treated mice was similar to that previously described by (Tsou et al., 1998) (Figure 1.5 A, D). CB_1R-ir in both the caudate-putamen and nucleus accumbens appeared as bright puncta that were distributed in the neuropil and surrounding cell bodies, as indicated by nuclear markers (Figure 1.5 C, F, G-I). More diffuse staining was also observed in the caudate-putamen that appeared to represent fiber bundles. Although most of the CB_1R-ir appeared to be on fibers, green fluorescent CB_1R-ir cell bodies were also observed (Figure 1.5 A, C, G and H). FosB/ΔFosB-ir nuclei were seen in the caudate-putamen and nucleus accumbens of both vehicle- and THC-treated mice (Figure 1.5 B, E), but fewer FosB/ΔFosB-ir nuclei were observed in brain sections from vehicle- compared to THC-treated mice (not shown). Dual staining for DAPI showed that FosB/ΔFosB-ir was localized in cell nuclei (Figure 1.5 C, F, G-I), as previously reported (Perrotti et al., 2008). DAPI stained nuclei that were immunonegative for FosB/ΔFosB were also observed in brains from both groups of mice (Figure 1.5 C, F, G-I). Examination of dual staining in brains from THC-treated mice revealed that in many cases CB_1R-ir puncta appeared to be surrounding cells that contained FosB/ΔFosB-ir nuclei (Figure 1.5 G-I). Cells were also observed in the caudate-putamen with

green fluorescence that surrounded DAPI/ΔFosB positive nuclei (Figure 1.5 G and H). There were no instances where CB_1R-ir and FosB/ΔFosB-ir were dual stained in the nucleus (Figure 1.5 C, F, G-I). Therefore, it appeared that CB_1R-ir was both co-localized with FosB/ΔFosB-ir in cells and also in puncta that contacted cells with FosB/ΔFosB-ir nuclei.

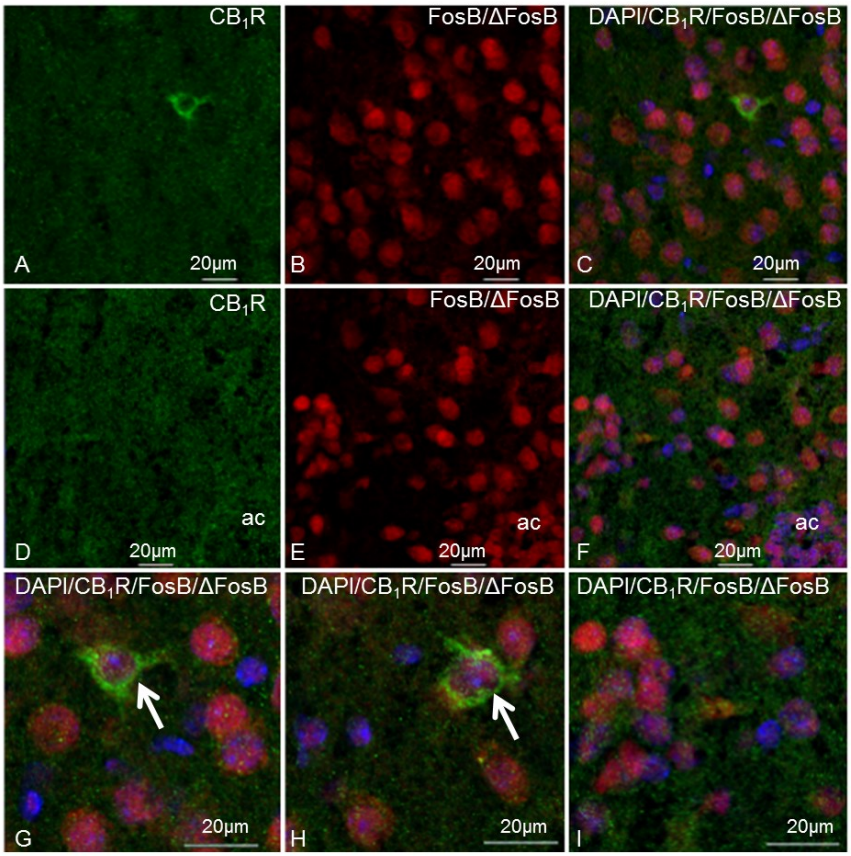


Figure 1.5 Representative images showing CB_1R-ir (green), FosB/ΔFosB-ir (red) and DAPI (blue) in the caudate-putamen and nucleus accumbens of mice that received repeated THC treatment. CB_1R-ir fibers and puncta were seen in the caudate-putamen (A) and nucleus accumbens (B) and CB_1R-ir cells were occasionally found in the caudate-putamen (A). FosB/ΔFosB-ir was localized to nuclei of cells in the caudate-putamen (B, C) and nucleus accumbens (E, F). FosB/ΔFosB-ir and DAPI were seen in a subset of cell nuclei that were surrounded by CB_1R-ir puncta in the caudate-putamen (C, G, H) and nucleus accumbens (F, I). CB_1R-ir was also seen in cells that contained FosB/ΔFosB-ir nuclei in the caudate-putamen (indicated by arrows in G, H). ac: anterior commissure

THC-mediated ΔFosB induction is abolished in CB_1R *knockout mice*

The role of CB_1Rs in THC-mediated ΔFosB induction was determined in the nucleus accumbens and caudate-putamen, regions that showed the highest magnitude of ΔFosB induction. CB_1R knockout and littermate wild type mice were treated with 10 mg/kg THC or vehicle for 13.5 days (b.i.d.) as described above. ΔFosB expression was significantly increased in THC- versus vehicle-treated wild-type mice in both the caudate-putamen (39% increase, $F_{1,25}$, $p < 0.05$) and nucleus accumbens (45% increase, $F_{1,25}$, $p < 0.05$) (Figure 6). There was no significant difference in ΔFosB-ir between vehicle- and THC-treated CB_1R knockout mice in either the caudate-putamen or nucleus accumbens. In the caudate-putamen, two-way ANOVA determined a significant interaction between the factors of genotype × treatment $F_{1, 25} = 4.86$, $p< 0.05$. One-way ANOVA, followed by Dunnett's post-hoc test, determined that both vehicle- and THC-treated CB_1R-knockout mice exhibited significantly greater ΔFosB expression ($F_{3,25}$, $p < 0.01$) compared to wild type vehicle-treated mice (Figure 6). Because ΔFosB-ir was elevated in the caudate-putamen of vehicle-treated CB_1R knockout compared to wild type mice, it is possible that further increases in ΔFosB-ir might not be detected in this region after this THC treatment paradigm, essentially producing a ceiling effect. Therefore, C57Bl/6J mice were repeatedly administered vehicle, 10 mg/kg and a higher dose (30 mg/kg) of THC twice daily for 13.5 days. Results showed that this 30 mg/kg THC administration paradigm produced a significantly greater increase in ΔFosB-ir than the 10 mg/kg THC administration paradigm ($F_{2,21}$, $p < 0.05$, Figure 1.7), indicating that the 10 mg/kg paradigm did not induce maximal ΔFosB expression in this brain region. This result shows that THC-mediated ΔFosB induction is dose-dependent and that ΔFosB induction does not occur in CB_1R knockout mice.

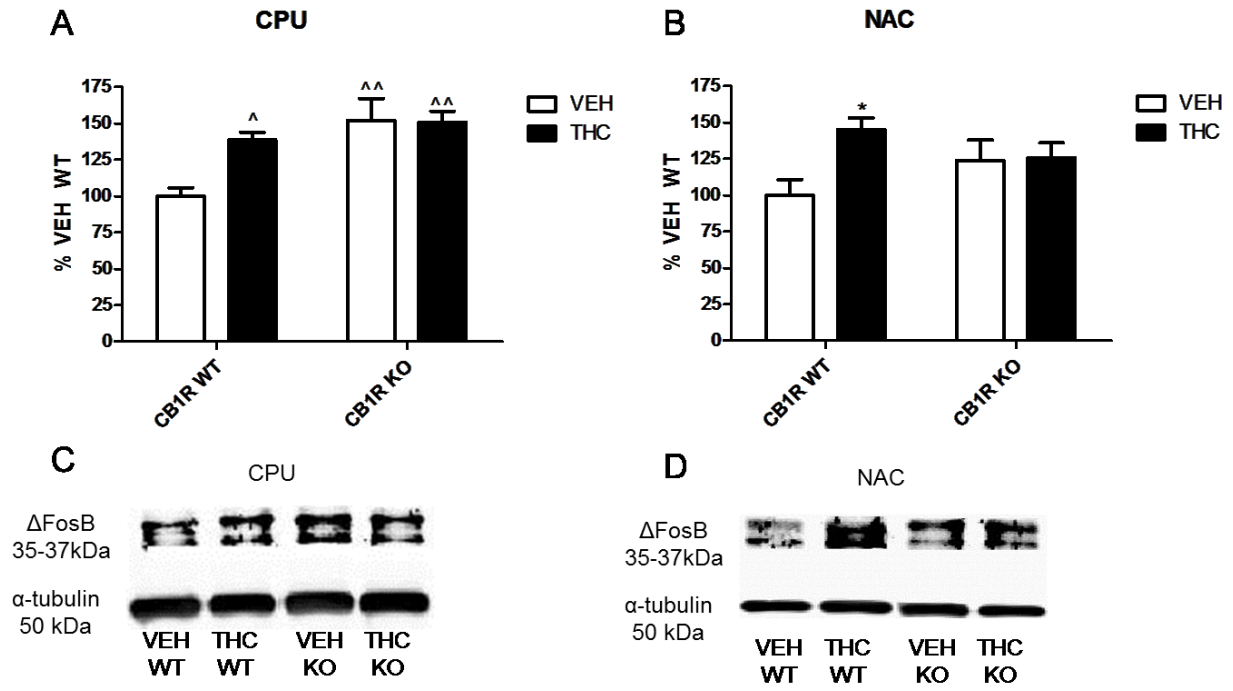


Figure 1.6 Immunoblot results for ΔFosB expression in the caudate-putamen and nucleus accumbens following repeated vehicle or THC administration in wild type and CB_1R knockout mice. Blots were probed with antibodies directed against ΔFosB and α-tubulin (loading control). (A and B) Graphs showing densitometric analysis of brain regions from vehicle- and THC-treated mice expressed as percent vehicle control. For CPU, data are means ± SEM with ^ $p < 0.05$ and ^^ $p < 0.01$ versus wild type vehicle controls, Dunnett's post-hoc test following a one-way ANOVA, n = 7-8 mice per group. For NAC, data are means ± SEM with * $p < 0.05$ versus wild type vehicle controls, Bonferroni post-hoc test following a two-way ANOVA, n = 7-8 mice per group. (C and D) Representative blots showing ΔFosB-ir and α-tubulin-ir in vehicle- and THC-treated brains of wild type and CB_1R knockout mice for each region examined.

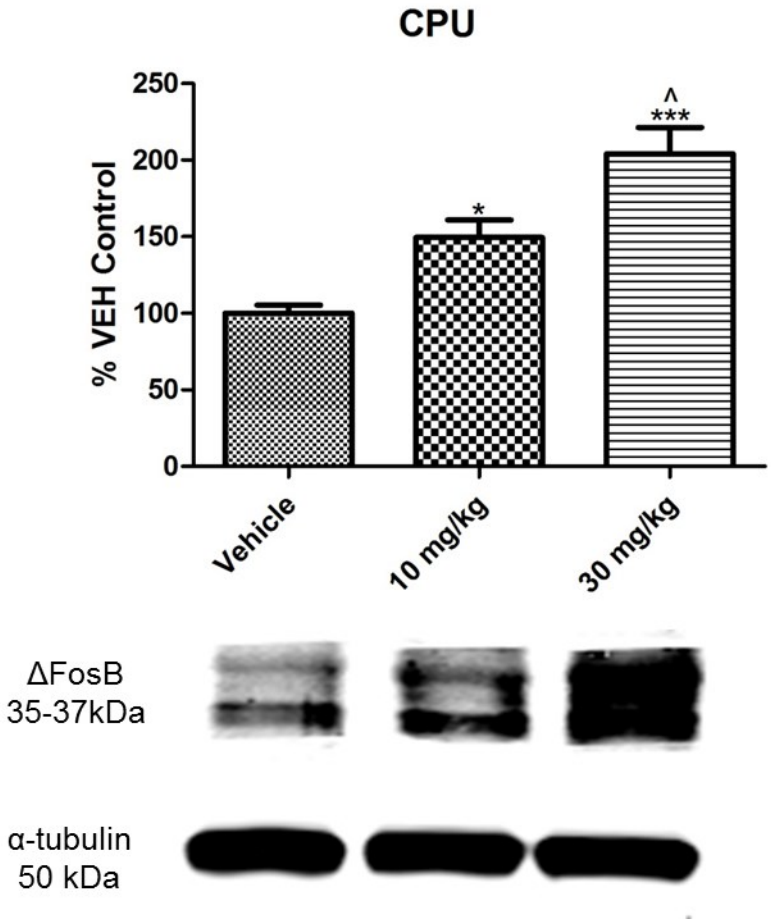


Figure 1.7 Immunoblots showing ΔFosB-ir in the caudate-putamen of mice that received vehicle, 10 mg/kg THC or 30 mg/kg THC administration twice daily for 13.5 days. ΔFosB expression was significantly increased by THC treatment ($F_{2,21}$ =17.78, $p < 0.0001$). ΔFosB levels were 50% ± 11% ($p < 0.05$) and 104% ± 17% ($p < 0.001$) above levels in vehicle control mice following 10 mg/kg and 30 mg/kg THC administration, respectively. ΔFosB-ir was also significantly greater in mice that received 30 mg/kg THC administration compared to mice treated with 10 mg/kg THC ($p < 0.05$). Results are presented as % vehicle control ± SEM with significance determined following one-way ANOVA with Bonferroni post-hoc test, n = 8 mice per group.

1.4 Discussion

This study demonstrated an inverse regional correlation between THC-mediated induction of ΔFosB and CB_1R desensitization in the forebrain. Repeated THC treatment induced ΔFosB in the caudate-putamen and nucleus accumbens, regions that did not exhibit THC-induced CB_1R desensitization and downregulation. In contrast, THC treatment did not induce ΔFosB in the hippocampus, which exhibited the highest magnitude of CB_1R desensitization and downregulation. Areas with intermediate levels of CB_1R desensitization and downregulation, such as prefrontal cortex, lateral amygdala and basomedial amygdala, demonstrated either no change or an intermediate level of ΔFosB induction. Immunohistochemical results showed that CB_1R-ir puncta surrounded cells with FosB/ΔFosB-ir nuclei and also that CB_1R and FosB/ΔFosB were co-localized in some cells. Previous studies have shown that CB_1R are expressed primarily in GABAergic MSN of the striatum (Hohmann and Herkenham, 2000). Thus, these results support the idea that ΔFosB could regulate CB_1Rs and/or that CB_1R signaling could modulate ΔFosB expression via both direct and trans-synaptic mechanisms. The role of CB_1Rs in THC-mediated ΔFosB induction has not previously been assessed. Studies in CB_1R knockout and wild type mice revealed that ΔFosB induction was CB_1R-dependent in the caudate-putamen and nucleus accumbens, showing that CB_1Rs are required for THC-mediated ΔFosB induction.

Studies in rodents have established that there are brain region-dependent differences in the magnitude, rate of development and rate of recovery of CB_1R desensitization and downregulation (McKinney et al., 2008; Sim-Selley, 2003; Sim-Selley et al., 2006). Similar regional relationships have been found in brains from human marijuana users, where greater apparent downregulation and slower recovery of ligand binding were found in the hippocampus

compared to other brain regions (Hirvonen et al., 2012; Villares, 2007). The similar regional relationship in CB_1R adaptations between rodents and humans suggests that this is a fundamental property of adaptation of brain CB_1Rs to repeated THC exposure. The present study has extended our previous findings by showing that brain regional specificity also exists for induction of the stable transcription factor ΔFosB in rodents.

We have previously assessed THC-mediated desensitization and downregulation and induction of ΔFosB in separate studies using a 15-day ramping-dose THC paradigm (Perrotti et al., 2008; Sim-Selley and Martin, 2002). This treatment paradigm produced significant CB_1R desensitization and downregulation in almost all regions examined, but the relative magnitude varied across regions. The hippocampus exhibited a higher magnitude of desensitization and the caudate-putamen and its projection regions of substantia nigra and globus pallidus exhibited a lower magnitude of desensitization (Sim-Selley and Martin, 2002). FosB/ΔFosB induction was examined in a separate study by treating mice with this THC ramping dose paradigm and counting the number of FosB/ΔFosB-ir cells (Perrotti et al., 2008). Results showed significant THC-induced increases in FosB/ΔFosB-ir cells in the nucleus accumbens core, with trends toward increases in the nucleus accumbens shell and caudate-putamen. Semi-quantitative analysis showed greater numbers of FosB/ΔFosB-ir neurons throughout the forebrains of THC- compared to vehicle-treated mice (Perrotti et al., 2008). The current study extends those findings by using immunoblot analysis, which provides a quantitative measure that distinguishes between ΔFosB and full length FosB and measures total protein expression. Results showed significant THC-mediated ΔFosB induction in the nucleus accumbens, as well as prefrontal cortex, caudate-putamen and lateral amygdala. The finding that THC-mediated ΔFosB induction occurs in these forebrain regions could have important implications for understanding the mechanisms that

contribute to the motivational effects of THC. The distribution of THC-induced ΔFosB expression in the prefrontal cortex, caudate-putamen, nucleus accumbens and lateral amygdala corresponds to previous findings reported after treatment with other drugs of abuse or exposure to chronic stress (Perrotti et al., 2004; Perrotti et al., 2008). Neuroplasticity of these brain regions is critical in the transition from acute to compulsive drug use and has been suggested to be a neural substrate of addiction (Koob and Volkow, 2010). ΔFosB-mediated regulation of target genes in these regions could therefore affect behaviors that contribute to the motivational effects of THC as well as other drugs of abuse. In fact, overexpression of ΔFosB in D1/dynorphin-containing striatal MSN enhanced the rewarding effects of morphine and cocaine (Colby et al., 2003; Zachariou et al., 2006a). Moreover, if ΔFosB or its target genes regulate CB_1R desensitization and/or downregulation in these regions, these molecular changes could also modulate the motivational effects of THC. For example, if ΔFosB or its targets could inhibit CB_1R desensitization, then less tolerance might develop to behaviors mediated by the striatum versus hippocampus in which ΔFosB is not induced by THC. In fact, studies in humans suggest that tolerance develops to the memory-impairing effects of THC, whereas subjective criterion, such as THC-induced "high", are less susceptible to development of tolerance (D'Souza et al., 2008; Haney et al., 1997; Haney et al., 2004).

A significant inverse correlation was found between desensitization and ΔFosB induction, whereas ΔFosB induction did not correlate with CB_1R downregulation. One explanation for this difference is that this THC paradigm did not produce sufficient downregulation to allow a direct comparison with ΔFosB induction. It is also possible that ΔFosB might directly or indirectly regulate genes involved in CB_1R desensitization, but not downregulation. For example, desensitization involves phosphorylation of G-protein coupled receptors (GPCRs) by G-protein

receptor kinases (GRKs), and subsequent recruitment of β-arrestins to the receptor that can produce desensitization by interfering with receptor-G-protein coupling and initiating endocytosis (Claing et al., 2002; Jin et al., 1999). β-arrestin-mediated GPCR endocytosis promotes trafficking to endosomes, which leads to either recycling of the receptor to the plasma membrane (resensitization) or degradation (downregulation). Trafficking of CB_1Rs to lysosomes for degradation is regulated by G protein-coupled receptor associated sorting protein 1 (GASP1) (Martini et al., 2007). Thus, a number of regulatory proteins could contribute to the molecular changes shown in the present study. ΔFosB has not yet been linked to pathways involved in GPCR trafficking, but this possibility has not been addressed directly.

The gene targets of ΔFosB that could regulate desensitization are not fully known, but previous studies have identified candidate proteins that regulate CB_1R adaptions. Our laboratory showed that genetic deletion of β-arrestin-2 in mice attenuated CB_1R desensitization in the periaqueductal gray, cerebellum and spinal cord, and enhanced desensitization in the projection areas of the caudate-putamen (substantia nigra and globus pallidus) following repeated THC administration (Nguyen et al., 2012). Inhibition of the ERK pathway has also been shown to modulate CB_1R receptor desensitization and downregulation, suggesting that inhibition of proteins in this pathway could reduce desensitization. Alternative interpretations are also suggested by the current findings. It is possible that CB_1R desensitization in regions such as the hippocampus inhibits induction of ΔFosB, thus regions in which CB_1R desensitization occurs would show less ΔFosB induction. This mechanism could also explain the inverse regional relationship identified between CB_1R desensitization and ΔFosB induction.

Although the current results support the idea that CB_1R desensitization and ΔFosB induction after repeated THC exposure might be related, it also is possible that the two events

could be coincident and not linked. For example, signaling pathways upstream of CB_1Rs and ΔFosB might regulate both processes. Studies using rat sarcoma(Ras)-specific guanine nucleotide exchange factor 1 (GRF1) knockout mice, which blunts ERK activation through this signaling pathway, showed that the Ras/ERK pathway was necessary for CB_1R desensitization and downregulation in the striatum (Rubino et al., 2005) and was also involved in cocaine-mediated ΔFosB induction in the striatal neurons (Fasano et al., 2009). These findings provide a mechanism upstream of ΔFosB induction that could also regulate CB_1R desensitization. However, if ERK was solely responsible for both events, one would predict a positive correlation between desensitization and ΔFosB induction, whereas results showed a negative correlation in this study. Thus, it will be important in future studies to determine whether there is indeed a direct relationship between ΔFosB induction and CB_1R desensitization and identify the signaling processes that regulate these events.

The finding that ΔFosB expression was significantly higher in the caudate-putamen of CB_1R knockout compared to wild type mice suggests that CB_1Rs modulate basal ΔFosB expression in this region. A recent study showed that reduction of CB_1R expression in striatal cells using RNA interference-directed knockdown decreased the levels of D_2R mRNA and protein, as well as D_2R-stimulated G-protein activity (Blume et al., 2013). Moreover, administration of the D_2R antagonist, haloperidol, is known to induce ΔFosB expression (Atkins et al., 1999). Taken together, these findings suggest that loss of striatal CB_1Rs in knockout mice could reduce D_2R signaling, which, like haloperidol, would enhance dopamine release. A potential interaction between CB_1Rs and dopamine receptors in dopamine-mediated regulation of ΔFosB could have important implications in understanding the cellular consequences of drugs of abuse.

THC has previously been reported to induce ΔFosB, a property common to drugs of abuse (Perrotti et al., 2008), but we believe that this is the first study to directly assess the relationship between THC-mediated ΔFosB induction and THC-mediated desensitization and downregulation in CB_1Rs. CB_1Rs and ΔFosB were co-localized in a subset of striatal neurons, demonstrating that adaptations in these pathways following THC exposure could be cell autonomous. The anatomical proximity of CB_1R-ir puncta with cells that express ΔFosB indicates that CB_1Rs might also trans-synaptically regulate ΔFosB. Results suggest several possible functional interactions between CB_1R signaling and ΔFosB in the striatum. The inverse regional relationship between CB_1R desensitization and ΔFosB induction suggests that ΔFosB induction and subsequent changes in the expression of gene targets might inhibit CB_1R desensitization. A non-mutually-exclusive possibility is that CB_1R desensitization impairs a signaling pathway that normally induces ΔFosB expression, so that CB_1R desensitization would attenuate ΔFosB induction. These possibilities will need to be directly assessed in future studies to determine the mechanism(s) underlying functional interactions between CB_1Rs and ΔFosB and potential consequences after repeated THC administration.

These results suggest that THC-mediated ΔFosB induction could inhibit CB_1R desensitization or modulate resensitization, and/or that CB_1R desensitization could attenuate THC-mediated ΔFosB induction. Future studies will be required to distinguish among these mechanisms. The demonstration that CB_1Rs are both co-localized with ΔFosB and in puncta that contact ΔFosB expressing cells indicates that both direct interactions and trans-synaptic effects could occur. These studies also demonstrate the requirement for CB_1Rs in THC-mediated ΔFosB induction and that induction of ΔFosB is THC dose-dependent. The finding that THC treatment induces ΔFosB in several regions important for functions related to reward highlights the role

this transcription factor might play in human marijuana use.

Chapter 2: ΔFosB modulation of CB_1R desensitization and tolerance to cannabinoid-mediated effects

2.1 Introduction

THC, the main psychoactive constituent of marijuana (Gaoni, 1964), produces its behavioral effects by activating CB_1Rs in the CNS (Rinaldi-Carmona et al., 1994; Zimmer et al., 1999). Repeated THC administration produces tolerance to THC-mediated *in vivo* effects, including cognitive impairment, locomotor suppression, catalepsy, hypothermia and antinociception (Lichtman and Martin, 2005). Tolerance occurs concomitantly with CB_1R desensitization (Sim-Selley, 2003), but the mechanism(s) underlying these adaptations are not well understood. CB_1R desensitization varies in magnitude by brain region depending on the dose and duration of repeated cannabinoid administration and the regional profile of these adaptations correspond with the development of tolerance to specific cannabinoid-mediated responses (Sim-Selley, 2003). For example, tolerance to THC-mediated hypothermia develops more rapidly and at lower doses than tolerance to locomotor suppression and catalepsy (McKinney et al., 2008; Whitlow et al., 2003) consistent with the lower level of desensitization observed in structures of the basal ganglia and nucleus accumbens compared to other regions (Sim-Selley, 2003). In human marijuana users, greater tolerance develops to the memory impairing effects of THC, which involve hippocampal function, compared to motor impairment and subjective "high", which involve striatal circuits (D'Souza et al., 2008; Haney et al., 1999a, b). Studies in human brain using post-mortem autoradiography or *in vivo* imaging have revealed a greater decrease in CB_1R levels in the hippocampus compared to the caudate-putamen of marijuana users compared to non-users (Hirvonen et al., 2012; Villares, 2007). These data agree with findings in rodent studies and suggest the potential functional relevance of regional

differences in CB_1R adaptation, but the regulatory mechanisms that underlie these regional differences are not known.

We have proposed that regional differences in the interaction of CB_1Rs with specific signaling and regulatory proteins might contribute to region-specific differences in CB_1R adaptation (Nguyen et al., 2012; Sim-Selley, 2003), and recently suggested that induction of transcription factors following repeated THC administration might modulate CB_1R desensitization (Lazenka et al., 2013). This idea was based, in part, on the demonstration that an inverse regional correlation exists between THC-mediated CB_1R desensitization and induction of ΔFosB (Chapter 1). ΔFosB belongs to the Fos family of transcription factors that dimerize with Jun proteins to produce an AP-1 complex that regulates the transcription of target genes (Chen et al., 1997; Herdegen and Leah, 1998). ΔFosB, a truncated splice variant of FosB, is a stable transcription factor that accumulates with repeated drug administration (Nestler et al., 2001). Transgenic overexpression of ΔFosB in dopamine type 1 receptor (D_1R) positive striatal MSNs enhanced the rewarding effects of drugs of abuse and natural rewards (Nestler, 2008; Werme et al., 2002). Expression of ΔcJun, which functionally inhibits ΔFosB, reduced cocaine- (Peakman et al., 2003) and morphine- (Zachariou et al., 2006a) induced condition place preference. Microarray studies have determined that ΔFosB regulates expression of certain receptors (e.g., adenosine A_{2A} receptor) and signaling proteins (G-protein $G\alpha_o$, protein kinase C and calcium/calmodulin-dependent protein kinase II) (McClung and Nestler, 2003). Inducible transgenic overexpression of ΔFosB enhanced mu opioid, but not CB_1, receptor-mediated G-protein activity in the nucleus accumbens (Sim-Selley et al., 2011), supporting the idea that ΔFosB can regulate GPCR signaling. However, a possible role for ΔFosB in regulating CB_1R desensitization has not been investigated. The current study addressed this question by

administering repeated THC to transgenic mice that have inducible overexpress ΔFosB or ΔcJun in the forebrain and assessing CB_1R-mediated G-protein activity and THC-mediated *in vivo* responses.

2.2 Materials and Methods

Materials

Materials are provided in Chapter 1

Mice and Drug Treatments

Subjects were male, bitransgenic *NSE-tTA* x *TetOp-ΔFosB* mice (on an FVB/C57BL/6J background) and *NSE-tTA* ×*TetOp-FLAG-Δc-Jun* mice (on an FVB background) with brain-region specific, tetracycline-regulated inducible expression of either ΔFosB or ΔcJun, respectively (Chen et al., 1998; Peakman et al., 2003). ΔFosB or ΔcJun expression is controlled by adding doxycycline to the drinking water, which prevents ΔFosB/ΔcJun expression. Omission of doxycycline from the drinking water allows ΔFosB/ΔcJun to be expressed. In mice that overexpress ΔFosB (ΔFosB-ON), ΔFosB is expressed in D_1R MSNs in the caudate-putamen and nucleus accumbens, deep layers of cerebral cortex and hippocampus (Chen et al., 1998). In mice that overexpress ΔcJun (ΔcJun-ON), expression occurs in both D_1R and dopamine type 2 receptor (D_2R) positive MSNs of the caudate-putamen and nucleus accumbens, parietal cortex and hippocampus (Peakman et al., 2003). ΔcJun is a dominant negative functional inhibitor of Fos-mediated transcription, thus this model provides a strategy to block the effects of ΔFosB expression. Mice were housed four to six per cage and maintained on a 12-hr light/dark cycle in a temperature-controlled environment (20-22°C) with food and water available ad libitum. Mice were maintained on drinking water that contained doxycycline (100 μg/ml) throughout gestation

and were either taken off doxycycline for 8 weeks prior to experiments to induce expression of ΔFosB or ΔcJun or maintained on doxycycline (control). After 8 weeks with/without doxycycline, mice were treated twice daily (08:00 and 16:00) with vehicle (1:1:18 solution of ethanol, emulphor and saline) or a ramping dose of THC (10-30-60 mg/kg, subcutaneous injection) for 6 days, with doses increasing every 2 days (McKinney et al., 2008). On day 7, mice received only the morning THC injection, and 24 hours later separate groups of mice were either tested for THC-induced *in vivo* responses or were sacrificed and brains were collected for CP55,940-stimulated [^{35}S]GTPγS binding. This THC treatment regimen was employed because it produces CB_1R desensitization throughout the brain, including in the striatum, therefore should reveal whether ΔFosB expression alters CB_1R desensitization. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health guide for the care and use of Laboratory animals 7th edition.

Agonist-stimulated [^{35}S]GTPγS Autoradiography

Assays were conducted as previously described in Chapter 1. For this study, sections were collected to include 1) prefrontal cortex, 2) nucleus accumbens, 3) caudate-putamen, 4) globus pallidus, 5) hippocampus and amygdala (including central, basolateral and basomedial nuclei), 6) VTA, 7) substantia nigra and 8) cerebellum.

In vivo Assessment

Mice were evaluated 24 hours after the last THC injection to determine whether overexpression of ΔFosB or ΔcJun affected THC-induced *in vivo* responses after either repeated vehicle or THC treatment. ΔFosB-ON and ΔFosB-OFF vehicle-treated mice (n = 8 mice per group) were initially evaluated for THC-induced hypothermia, antinociception and catalepsy

using a cumulative dosing procedure to determine whether expression of ΔFosB affected THC-mediated responses. Dose-response data were also used to determine the appropriate challenge dose of THC to administer in subsequent experiments for both ΔFosB and ΔcJun bitransgenic mice. Baseline measures were first assessed in the absence of THC, and then mice received intraperitoneal (i.p.) injections of increasing doses (3, 7, 20 and 70 mg/kg) of THC and were assessed again after each injection. Subjects were evaluated for all measures beginning at 30 minutes after injection of each dose of THC, and the entire dose-response assessment was completed in less than 3 hours (Falenski et al., 2010; Schlosburg et al., 2010). Catalepsy was determined in the bar test, antinociception was evaluated in the warm water tail immersion test at 52.0 °C, and body temperature was measured by inserting a thermocouple probe 2.0 cm into the rectum (Falenski et al., 2010; Long et al., 2009). For locomotor activity, each mouse was placed in a clear Plexiglas box (42.7 x 21.0 x 20.4 cm) for a 5 min assessment period and Anymaze software (Stoelting, Wood Dale, Illinois) was used to determine the amount of time spent immobile (Long et al., 2009). Mice were tested in separate chambers for baseline and THC trials to avoid habituation. Thigmotaxis was also measured during locomotor activity trials by using Anymaze to draw a zone in the center of the activity chamber that subtracted the width of a mouse (~4 cm) from each side of the chamber, thereby by creating two separate zones. The outside zone represented time spent exhibiting thigmotaxis and the inside zone represented time spent within the center of the chamber (Simon et al., 1994). Data are presented as: (time spent in the outside zone/ time spent in the inside zone) x 100. To circumvent the possibility that mice might acclimate to activity chambers with repeated testing, all remaining experiments tested a single dose (i.p.) of 100 mg/kg THC for both ΔFosB and ΔcJun bitransgenic mice (n = 8 mice per group) using the testing procedures described above. Baseline measures were taken, and

then mice were injected with THC and tested 20 minutes later for locomotor activity. Catalepsy, antinociception and hypothermia were tested 3 hours after THC injection because initial studies determined that maximal effects were produced at this time point (data not shown). Because neither control nor ΔFosB-ON mice that received repeated THC injection exhibited catalepsy at the 100 mg/kg dose, a separate group of mice was tested at a dose of 200 mg/kg THC.

Analysis

Data were analyzed with Prism® version X (GraphPad Software, San Diego, CA) for all experiments. For *in vivo* studies, repeated measures ANOVA were performed with Bonferroni post-hoc test (cumulative dosing) or two-way ANOVA with Bonferroni post-hoc test (single injection). For [^{35}S]GTPγS autoradiography, net-stimulated [^{35}S]GTPγS binding was determined by (CP55,940-stimulated [^{35}S]GTPγS binding – basal [^{35}S]GTPγS binding). Two-way ANOVA with Bonferroni post-hoc test was used to determine significant differences. Desensitization was calculated as (net-stimulated [^{35}S]GTPγS binding in THC-treated mice / net-stimulated [^{35}S]GTPγS binding in vehicle-treated mice), and Student's t-tests were used based on planned comparisons by region. Significance was determined with $p < 0.05$ and all results are presented as mean ± SEM.

2.3 Results

CB_1R *desensitization is attenuated in the ventral midbrain and amygdala of mice that overexpress ΔFosB*

CP55,940-stimulated [^{35}S]GTPγS binding was measured in repeated vehicle- and THC-treated ΔFosB-ON and ΔFosB-OFF mice to assess CB_1R-mediated G-protein activity and desensitization. Basal levels of [^{35}S]GTPγS binding did not differ between any group of ΔFosB-

ON and ΔFosB-OFF mice in any region examined (data not shown). Net CP55,940-stimulated [^{35}S]GTPγS binding was first compared in vehicle-treated ΔFosB-ON and ΔFosB-OFF mice to determine whether ΔFosB expression altered cannabinoid-mediated G-protein activity in drug-naïve mice. [^{35}S]GTPγS binding in the amygdala was significantly lower ($p < 0.01$) in ΔFosB-ON mice (339 ± 16 nCi/g, when compared to ΔFosB-OFF mice (393 ± 16 nCi/g) (Figure 2.1, Table 2.1). No differences in CP55,940-stimulated [^{35}S]GTPγS binding were found between vehicle-treated ΔFosB-ON and ΔFosB-OFF mice in any other region examined. The effect of repeated THC administration on CP55,940-stimulated [^{35}S]GTPγS binding was then compared between ΔFosB-ON and ΔFosB-OFF mice. CP55,940-stimulated [^{35}S]GTPγS binding was significantly lower in repeated THC- compared to vehicle-treated brains from both ΔFosB-OFF and ΔFosB-ON mice in almost all regions examined (Figure 2.1, Table 2.1). The exception was the VTA, where there was no significant difference in CP55,940-stimulated [^{35}S]GTPγS binding between vehicle- and THC-treated ΔFosB-ON mice (118 ± 15 nCi/g for vehicle- versus 82 ± 5 nCi/g for THC-treated), but ΔFosB-OFF mice exhibited a significant reduction in CP55,940-stimulated [^{35}S]GTPγS binding after THC treatment (118 ± 14 nCi/g for vehicle versus 76 ± 7 nCi/g for THC-treated, $p < 0.05$).

CB_1R desensitization was then calculated as previously reported (Sim-Selley and Martin, 2002) to compare results between ΔFosB-ON and ΔFosB-OFF mice. Significant differences in CB_1R desensitization between ΔFosB-OFF and ΔFosB-ON mice were found in the substantia nigra and amygdala. In the substantia nigra, significantly less desensitization was found in ΔFosB-ON mice (78% ± 4% of ΔFosB-ON vehicle-treated mice) compared to ΔFosB-OFF mice (56% ± 3% of ΔFosB-OFF vehicle-treated mice) ($p < 0.001$, Figure 2.5 A). Similarly, significantly less desensitization was found in the amygdala of ΔFosB-ON mice (45% ± 2% of

ΔFosB-ON vehicle-treated mice) compared to ΔFosB-OFF mice (35% ± 3% of ΔFosB-OFF vehicle-treated mice) ($p < 0.05$, Figure 2.5 A). CB_1R desensitization following repeated THC administration was not significantly different between ΔFosB-ON and ΔFosB-OFF mice in the prefrontal cortex, nucleus accumbens, caudate-putamen, globus pallidus, hippocampus or cerebellum.

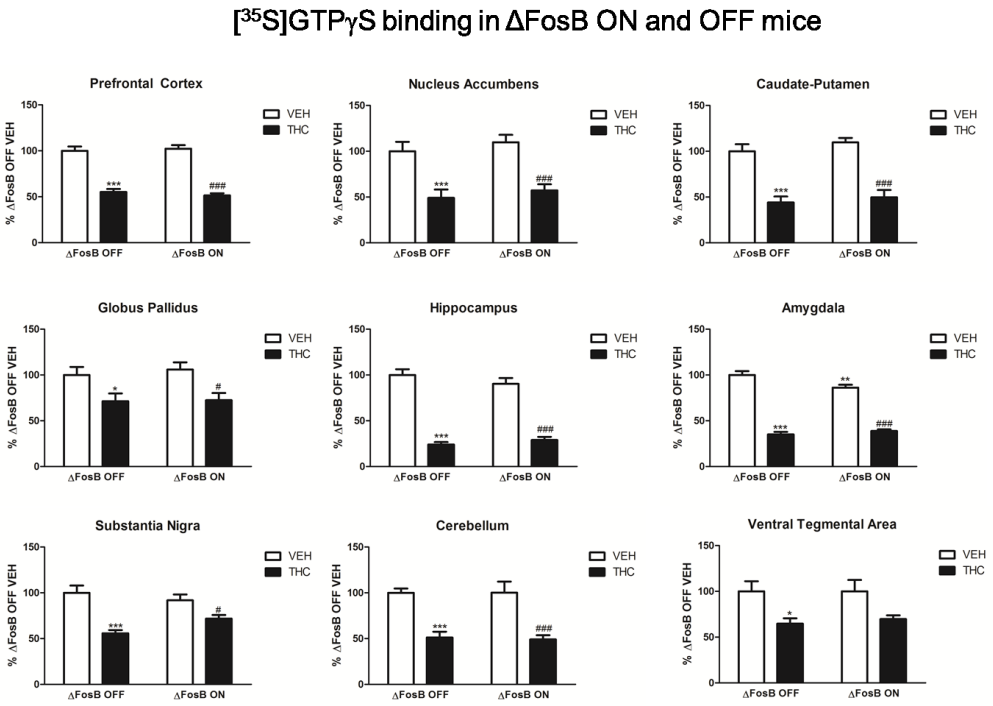


Figure 2.1 Net-stimulated [^{35}S]GTPγS binding in brain regions of vehicle- and THC-treated ΔFosB overexpressing (ΔFosB-ON) and control (ΔFosB-OFF) mice expressed as percent of net-stimulated binding in control vehicle-treated mice. Vehicle-treated ΔFosB-ON mice exhibited significantly less net-stimulated [^{35}S]GTPγS binding in the amygdala compared to ΔFosB-OFF mice ($p < 0.01$, Bonferroni post-hoc test). Net-stimulated [^{35}S]GTPγS binding was significantly decreased in all brain regions of ΔFosB-ON and ΔFosB-OFF mice, with the exception of ventral tegmental area of ΔFosB-ON mice. There were no differences in net-stimulated [^{35}S]GTPγS binding between ΔFosB-ON and ΔFosB-OFF mice following repeated THC-administration. Data are normalized to percent vehicle-treated control mice and presented as means ± SEM (n = 8-10 mice per group) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-treated control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ as compared to vehicle-treated ΔFosB-ON mice following two-way ANOVA and Bonferroni post-hoc test.

TABLE 2.1 Net CP55,940-stimulated [^{35}S]GTPγS binding in brain sections from ΔFosB-ON and ΔFosB-OFF mice following repeated vehicle or THC treatment.

	Net CP55,940-stimulated [^{35}S]GTPγS binding (nCi/g) ± SEM			
Brain Region	ΔFosB-OFF Vehicle	ΔFosB-OFF THC	ΔFosB-ON Vehicle	ΔFosB-ON THC
Prefrontal Cortex	446 ± 21	246 ± 15***	456 ± 17	229 ± 11$^{###}$
Nucleus Accumbens	403 ± 41	198 ± 37***	443 ± 33	230 ± 27$^{###}$
Caudate-Putamen	205 ± 16	90 ± 13***	225 ± 10	102 ± 16$^{###}$
Globus Pallidus	613 ± 54	437 ± 53*	649 ± 49	444 ± 48$^{#}$
Hippocampus	273 ± 17	65 ± 7***	247 ± 17	66 ± 11$^{###}$
Amygdala	393 ± 16	138 ± 11***	339 ± 12**	153 ± 7 $^{###}$
VTA	118 ± 13	76 ± 7*	118 ± 15	82 ± 5
Substantia Nigra	608 ± 48	339 ± 21***	558 ± 40	436 ± 24$^{#}$
Cerebellum	293 ± 14	150 ± 19***	293 ± 36	143 ± 14$^{###}$

Brain sections were incubated in 0.04 nM [^{35}S]GTPγS, 3 μM CP55,940 and 2 mM GDP and autoradiograms were analyzed using densitometry. Results are expressed as net CP55,940-stimulated [^{35}S]GTPγS binding (nCi/g) ± SEM, one-way ANOVA, Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, $p < 0.001$ vs. ΔFosB-OFF vehicle. # $p < 0.05$, ### $p < 0.001$ vs. ΔFosB-ON vehicle. (n = 8-10 per group)

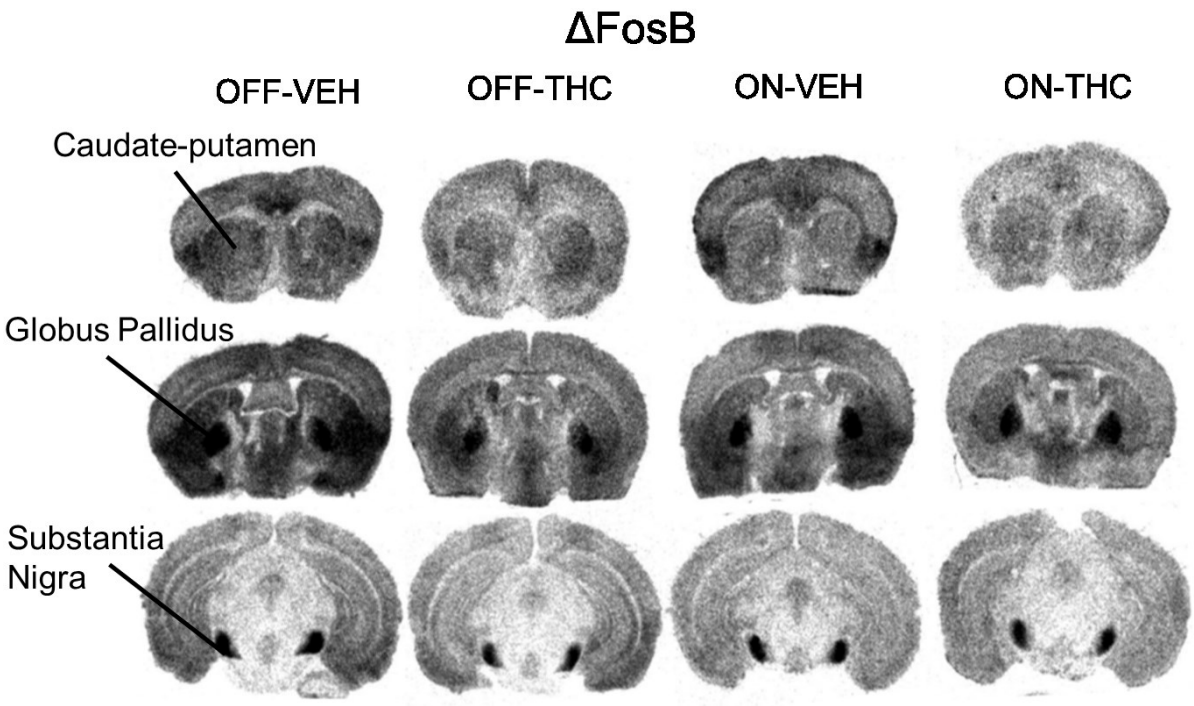


Figure 2.2 Representative autoradiograms showing CP55,940-stimulated [^{35}S]GTPγS binding in ΔFosB-OFF and ΔFosB-ON mice following repeated vehicle or THC (10-30-60 mg/kg, b.i.d., 6.5 days) treatment in regions of the basal ganglia.

CB_1R desensitization is enhanced in the caudate-putamen and reduced in the hippocampus and ventral midbrain of ΔcJun-ON mice

Studies were conducted to determine whether the expression of ΔcJun, a dominant negative inhibitor of FosB-mediated transcription, would alter CB_1R-mediated G-protein activity or desensitization. ΔcJun-ON and ΔcJun-OFF mice received the same repeated THC treatment as the ΔFosB overexpressing mice, and CP55,940-stimulated [^{35}S]GTPγS binding was assessed in the same regions described above. Basal levels of [^{35}S]GTPγS binding did not differ between any group of ΔcJun-ON and ΔcJun-OFF mice in any region examined (data not shown). Analysis of brains from vehicle-treated mice revealed that CP55,940-stimulated [^{35}S]GTPγS binding in the amygdala was significantly higher in ΔcJun-ON (282 ± 15) compared to ΔcJun-OFF (218 ± 16) mice ($p < 0.01$, Figure 2.3, Table 2.2). No significant differences were found in CP55,940-stimulated [^{35}S]GTPγS binding between ΔcJun-ON and ΔcJun-OFF mice in any other region examined. Repeated THC treatment significantly reduced CP55,940-stimulated [^{35}S]GTPγS binding compared to vehicle-treatment in ΔcJun-ON and ΔcJun-OFF mice in all regions examined, except for the caudate-putamen in ΔcJun-OFF mice. CP55,940-stimulated [^{35}S]GTPγS binding did not differ in the caudate-putamen of repeated THC- compared to vehicle-treated ΔcJun-OFF mice (114 ± 16 nCi/g for vehicle- versus 70 ± 17 nCi/g for THC-treated, Figure 2.3, Table 2.2). In contrast, a significant decrease was found in CP55,940-stimulated [^{35}S]GTPγS binding in the caudate-putamen of ΔcJun-ON mice following repeated THC compared to vehicle treatment (153 ± 16 nCi/g for vehicle- versus 68 ± 16 nCi/g for THC-treated, $p < 0.01$, Figure 2.3, Table 2.2).

CB_1R desensitization was then calculated and compared between ΔcJun-ON and ΔcJun-OFF mice. Significantly greater desensitization was found in the caudate-putamen of THC-

treated ΔcJun-ON (39% ± 6% of vehicle-treated ΔcJun-ON mice) compared to ΔcJun-OFF (62% ± 13% of ΔcJun-OFF vehicle-treated mice) mice (Figure 2.5 B). Significantly less CB_1R desensitization was found in the hippocampus of THC-treated ΔcJun-ON compared to ΔcJun-OFF mice (37% ± 6% of ΔcJun-ON vehicle-treated mice vs. 18% ± 4% of ΔcJun-OFF vehicle-treated mice, $p < 0.05$, Figure 2.5 B). CB_1R desensitization was also less in the VTA of ΔcJun-ON compared to ΔcJun–OFF mice (36% ± 4% of ΔcJun-ON vehicle-treated mice vs. 24% ± 3% of ΔcJun-OFF vehicle-treated mice, $p < 0.05$, Figure 2.5 B). No significant differences in desensitization between THC-treated ΔcJun-ON and ΔcJun-OFF mice were found in any of the other regions examined.

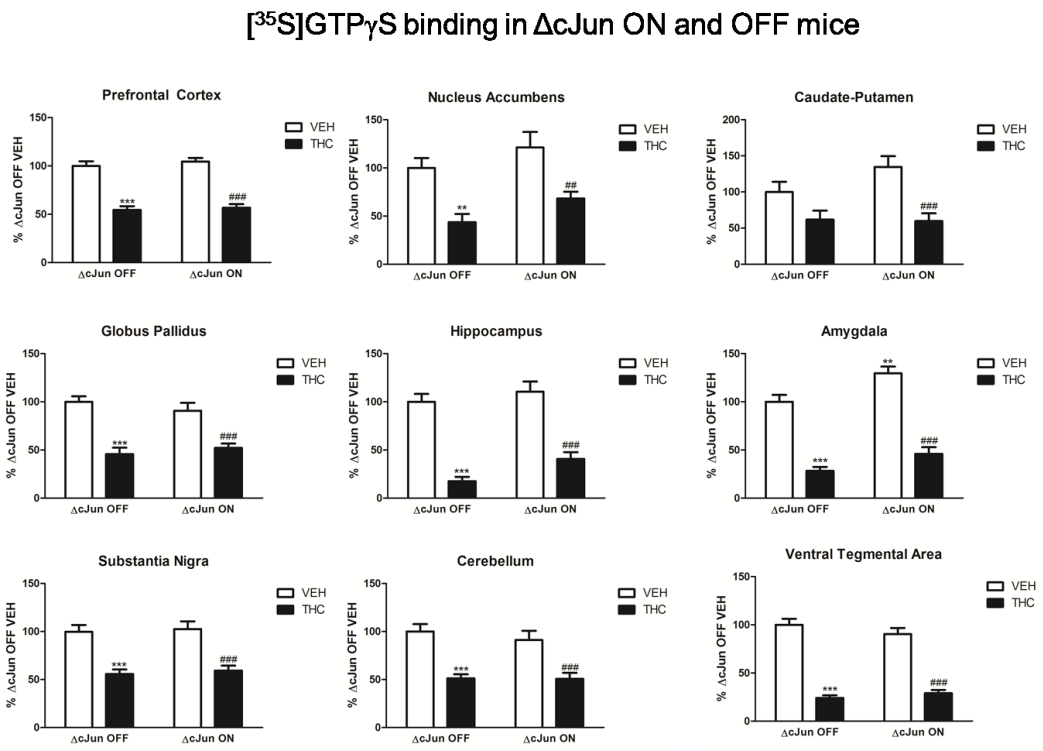


Figure 2.3 Net-stimulated [^{35}S]GTPγS binding in brain regions of vehicle- and THC-treated ΔcJun overexpressing (ΔcJun-ON) and control (ΔcJun-OFF) mice expressed as percent of net-stimulated binding in control vehicle-treated mice. Vehicle-treated ΔcJun-ON mice exhibited significantly greater net-stimulated [^{35}S]GTPγS binding in the amygdala compared to ΔcJun-OFF mice ($p < 0.01$, Bonferroni post-hoc test). Net-stimulated [^{35}S]GTPγS binding was significantly decreased in all brain regions of THC- versus vehicle-treated ΔcJun-ON and ΔcJun-OFF mice, with the exception of caudate-putamen of ΔcJun-OFF mice. There were no differences in net-stimulated [^{35}S]GTPγS binding between ΔcJun-ON and ΔcJun-OFF mice following repeated THC-administration. Data are normalized to percent vehicle-treated control mice and presented as means ± SEM (n = 8-10 mice per group) ** $p < 0.01$ and *** $p < 0.001$ as compared to vehicle-treated control. ## $p < 0.01$ and ### $p < 0.001$ as compared to vehicle-treated ΔcJun-ON mice following two-way ANOVA and Bonferroni post-hoc test.

TABLE 2.2

Net CP55,940-stimulated [^{35}S]GTPγS binding in brain sections from ΔcJun-OFF and ΔcJun-ON mice following repeated vehicle or THC treatment.

	Net CP55,940-stimulated [^{35}S]GTPγS binding (nCi/g) ± SEM			
Brain Region	ΔcJun-OFF Vehicle	ΔcJun-OFF THC	ΔcJun-ON Vehicle	ΔcJun-ON THC
Prefrontal Cortex	340 ± 16	185 ± 13***	355 ± 13	193 ± 12$^{###}$
Nucleus Accumbens	227 ± 23	99 ± 19**	275 ± 36	155 ± 16$^{##}$
Caudate-Putamen	114 ± 16	70 ± 17	153 ± 16	68 ± 16$^{##}$
Globus Pallidus	535 ± 30	244 ± 36***	486 ± 44	279 ± 24$^{###}$
Hippocampus	107 ± 9	19 ± 13***	118 ± 36	44 ± 23$^{###}$
Amygdala	218 ± 16	62 ± 9***	282 ± 15**	100 ± 15$^{###}$
VTA	274 ± 17	66 ± 7***	247 ± 18	79 ± 10$^{###}$
Substantia Nigra	467 ± 29	260 ± 28***	479 ± 29	277 ± 29$^{###}$
Cerebellum	250 ± 19	128 ± 11***	227 ± 24	126 ± 16$^{###}$

Brain sections were incubated in 0.04 nM [^{35}S]GTPγS, 3 μM CP55,940 and 2 mM GDP and autoradiograms were analyzed using densitometry. Results are expressed as net CP55,940-stimulated [^{35}S]GTPγS binding (nCi/g) ± SEM, one-way ANOVA, Bonferroni post-hoc test. ** $p < 0.01$, ***$p < 0.001$ vs. ΔcJun-OFF vehicle. ## $p < 0.01$, ### $p < 0.001$ vs. cJun-ON vehicle. (n = 8-10 per group)

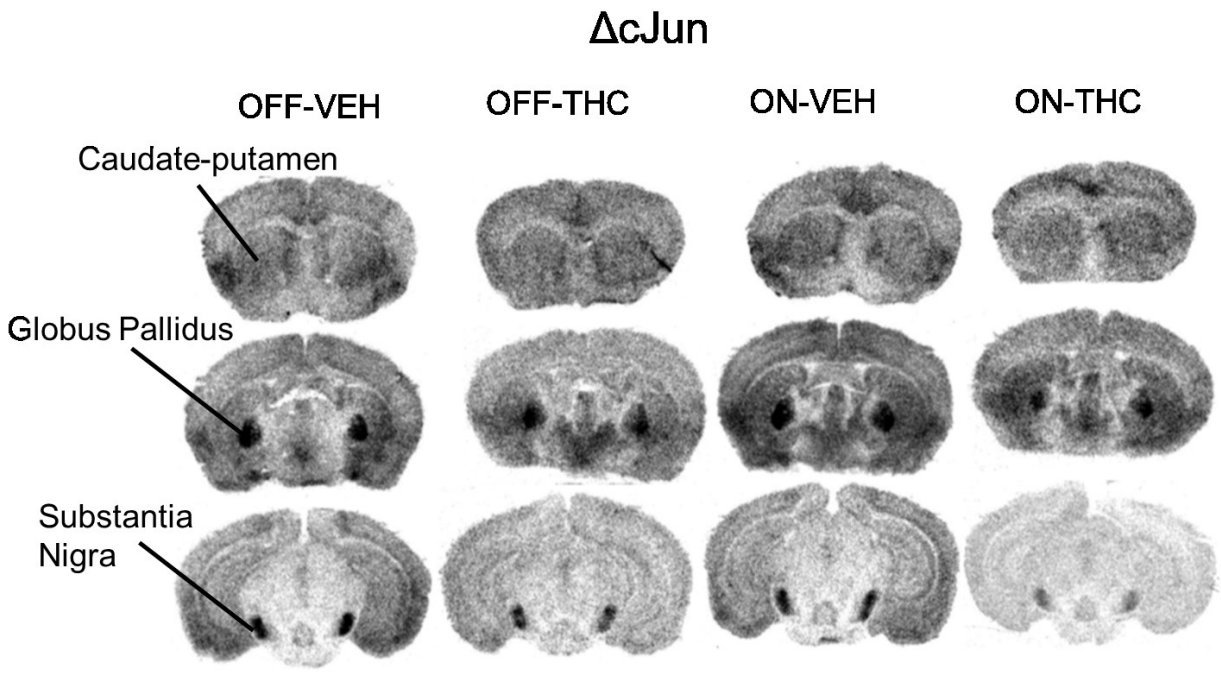


Figure 2.4 Representative autoradiograms showing CP55,940-stimulated [^{35}S]GTPγS binding in ΔcJun-OFF and -ON mice following repeated vehicle or THC (10-30-60 mg/kg, b.i.d., 6.5 days) treatment in regions of the basal ganglia.

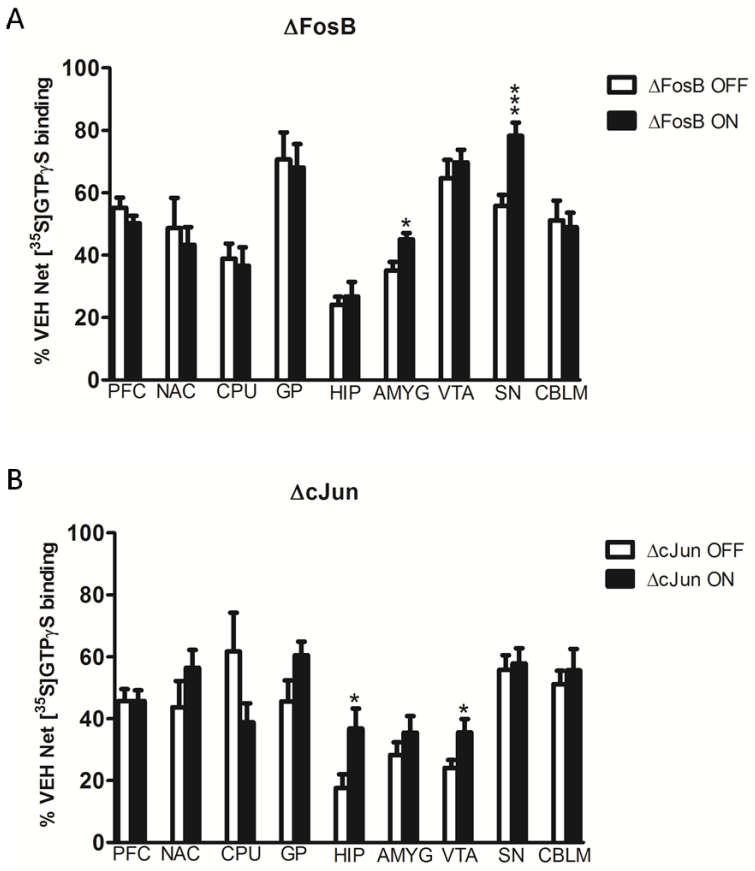


Figure 2.5 Net-stimulated [^{35}S]GTPγS binding in ΔFosB (A) and ΔcJun (B) overexpressing mice expressed as a percent of net-stimulated [^{35}S]GTPγS binding in the respective vehicle-treated mice. As a percentage of their respective vehicles, ΔFosB-ON mice had less desensitization following repeated THC administration in substantia nigra (**$p < 0.001$) and amygdala (*$p < 0.05$). ΔcJun-ON mice had less desensitization in hippocampus (*$p < 0.05$) and ventral tegmental area (*$p < 0.05$). Data are normalized to values in respective vehicle-treated mice and represented as mean ± SEM, Student's t-tests based on planned comparisons by region. PFC, prefrontal cortex; NAC, nucleus accumbens; CPU, caudate-putamen; GP, globus pallidus; HIP, hippocampus; AMYG, amygdala; VTA, ventral tegmental area; SN, substantia nigra; CBLM, cerebellum.

Tolerance to THC-induced locomotor suppression is enhanced in ΔFosB-ON mice following repeated THC treatment

THC-mediated *in vivo* effects were assessed to determine the effect of ΔFosB overexpression on THC-mediated responses and the development of tolerance after repeated THC administration. The dose-effect response for THC in ΔFosB-OFF and ΔFosB-ON mice was first determined for THC-mediated hypothermia, antinociception and catalepsy (data not shown). Based on these results, mice were tested with 100 mg/kg THC in subsequent studies. Responses were then compared in repeated vehicle- versus THC-treated mice of each genotype. No significant differences in baseline measures of body temperature, nociception or locomotor activity were found between vehicle and THC-treated ΔFosB-ON or ΔFosB-OFF mice (data not shown). Neither ΔFosB-ON nor ΔFosB-OFF mice exhibited baseline catalepsy. Acute THC (100 mg/kg) injection produced hypothermia, antinociception and catalepsy in vehicle-treated ΔFosB-ON and ΔFosB-OFF mice, with no significant effect of genotype on THC-mediated responses. Comparison of THC-mediated hypothermia in repeated vehicle- versus THC-treated ΔFosB-ON and ΔFosB-OFF mice showed a main effect of repeated treatment ($F_{1,28} = 84.01$, $p < 0.001$, Figure 2.6 A). There was no significant difference in hypothermia between ΔFosB-ON and ΔFosB-OFF mice that received repeated THC. An effect of repeated THC treatment was also found for antinociception ($F_{1,28} = 69.66$, $p < 0.001$, Figure 2.1 B), but there were no significant differences between ΔFosB-OFF and ΔFosB-ON mice that received repeated THC. A main effect of repeated THC treatment was also found for catalepsy ($F_{1,28} = 94.54$, $p < 0.001$, Figure 2.6 C). Because catalepsy was not produced by100 mg/kg THC for either ΔFosB-OFF or ΔFosB-ON mice that received repeated THC, a separate group of mice was tested at 200 mg/kg. At this dose, these mice exhibited catalepsy, but no significant difference was found between

groups (data not shown). There was a significant main effect of THC treatment for locomotor activity ($F_{1,27} = 39.00$, $p < 0.001$, Figure 2.6 D). Bonferroni post-hoc test determined that THC-treated ΔFosB-ON mice exhibited significantly less THC-mediated locomotor suppression compared to vehicle-treated ΔFosB-ON mice ($p < 0.001$, Figure 2.6 D), whereas THC-mediated locomotor suppression was similar between vehicle- and THC-treated ΔFosB-OFF mice. A significant interaction $F(_{1,27} = 9.986$, $p < 0.01$, Figure 2.6 D) was also found and Bonferroni post-hoc test determined that ΔFosB-ON mice that received repeated THC administration exhibited less locomotor suppression compared to ΔFosB-OFF mice that received repeated THC administration ($p < 0.01$, Figure 2.6 D). Overall, these results show that THC-treated ΔFosB-ON and ΔFosB-OFF mice developed tolerance to THC-mediated hypothermia, antinociception and catalepsy that was similar between genotypes. A genotype-specific difference in the effect of repeated THC was found for locomotor activity, where tolerance appeared to develop in the ΔFosB-ON, but not ΔFosB-OFF, mice.

Activity data were analyzed to assess thigmotaxis, which is defined as hugging the wall and can be considered a measure of anxiogenic-like behavior (Simon et al., 1994). There were no significant differences in baseline measures of thigmotaxis between any groups. ΔFosB-OFF and ΔFosB-ON mice, with mice spending equal time near the wall and center of the chamber (Figure 2.8 A). Following acute THC administration, there was a significant main effect of genotype ($F_{1,27} = 11.71$, $p < 0.05$) and an interaction ($F_{1,27} = 11.43$, $p < 0.05$), suggesting differences in the expression of thigmotaxis between ΔFosB-ON and ΔFosB-OFF mice. Bonferroni post-hoc test determined that repeated THC-treated ΔFosB-OFF mice exhibited a significant increase in time spent on the outside zone of the chamber (333% ± 109%, $p < 0.05$, Figure 2.8 C) compared to ΔFosB-OFF mice that received repeated vehicle (102% ± 33%) and ΔFosB-ON mice that

received repeated THC (85% ± 23%). These findings suggest that repeated THC administration can unmask thigmotaxis in mice that receive 100 mg/kg THC.

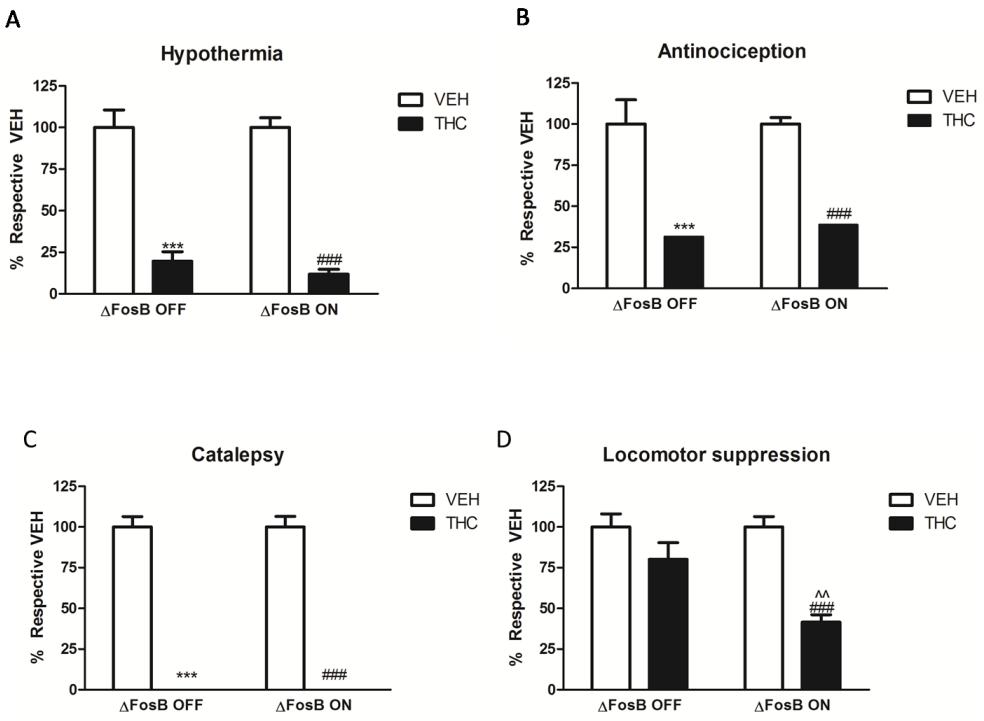


Figure 2.6 THC-mediated hypothermia (A), antinociception (B), catalepsy (C) and locomotor suppression (D) in ΔFosB overexpressing (ΔFosB-ON) and control (ΔFosB-OFF) mice following repeated vehicle or THC treatment. ΔFosB-ON and ΔFosB-OFF mice treated with repeated vehicle exhibited THC-mediated hypothermia, antinociception, catalepsy and locomotor suppression and repeated THC-administration reduced these effects. ΔFosB-ON mice exhibited significantly less THC-mediated locomotor suppression following repeated THC administration compared to ΔFosB-OFF mice that received repeated THC administration ($p < 0.001$ THC-treated ΔFosB-ON vs. THC-treated ΔFosB-OFF mice). Data are presented as mean percent of respective vehicle ± SEM (n = 8 mice per group). * $p < 0.05$, *** $p < 0.001$ as compared to vehicle-treated control. # $p < 0.05$ ###, $p < 0.001$ compared to vehicle-treated ΔFosB-ON mice. ^^, $p < 0.01$ compared to THC-treated control mice. Two-way ANOVA following Bonferroni post-hoc test.

Tolerance to THC-mediated catalepsy is reduced, whereas tolerance to locomotor suppression is enhanced, in ΔcJun-ON mice following repeated THC administration

ΔcJun-ON and ΔcJun-OFF mice were assessed to determine whether blocking ΔFosB-mediated transcription would affect THC-mediated *in vivo* effects or tolerance. No significant differences were found in baseline measures of body temperature, nociception, or locomotor activity between any groups (data not shown). Acute THC administration in repeated vehicle-treated ΔcJun-OFF and ΔcJun-ON mice, produced hypothermia, antinociception, catalepsy and locomotor suppression. THC-mediated responses were then compared between THC- and vehicle-treated mice of each genotype. There was a significant main effect of repeated treatment on hypothermia ($F_{1, 32} = 80.98$, $p < 0.001$, Figure 2.7 A), but not a significant interaction between treatment and genotype. Bonferroni post-hoc test determined no significant difference between ΔcJun-ON and ΔcJun-OFF mice that received either repeated vehicle or THC treatments. Similarly, there was a significant main effect of repeated treatment for antinociception ($F_{1, 32} = 84.15$, $p < 0.001$, Figure 2.7 B) in both ΔcJun-ON mice and ΔcJun-OFF mice. Bonferroni post-hoc test determined no differences between genotypes for this measure. For catalepsy, there was a significant main effect of treatment ($F_{1, 30} = 58.66$, $p < 0.001$, Figure 2.7 C), as well as a significant main effect of genotype ($F_{1, 30} = 6.36$, $p < 0.05$) and an interaction ($F_{1, 30} = 6.36$, $p < 0.05$). Bonferroni post-hoc test determined that ΔcJun-ON mice exhibited significantly more THC-induced catalepsy compared to ΔcJun-OFF mice ($p < 0.001$, Figure 2.7 C). Likewise, there was a significant main effect of treatment for locomotor suppression in ΔcJun-ON and ΔcJun-OFF mice ($F_{1, 30} = 59.57$, $p < 0.001$, Figure 2.7 D), as well as a significant main effect of genotype ($F_{1, 30} = 6.36$, $p < 0.05$) and an interaction ($F_{1, 30} = 6.36$, $p < 0.05$). Bonferroni post-hoc

test determined that ΔcJun-ON mice that received repeated THC administration exhibited less locomotor suppression compared to control mice that received repeated THC administration ($p < 0.01$, Figure 2.7 D). These results indicate that less tolerance to THC-mediated catalepsy and more tolerance to THC-mediated locomotor suppression developed in ΔcJun-OFF compared to ΔcJun-ON mice, whereas tolerance to hypothermia and antinociception did not differ between genotypes. Thigmotaxis was also measured in ΔcJun-ON and ΔcJun-OFF mice. There was no significant difference in baseline or THC-induced thigmotaxis between ΔcJun-OFF and ΔcJun-ON mice following either repeated vehicle or THC administration, (Figure 2.8 B and D).

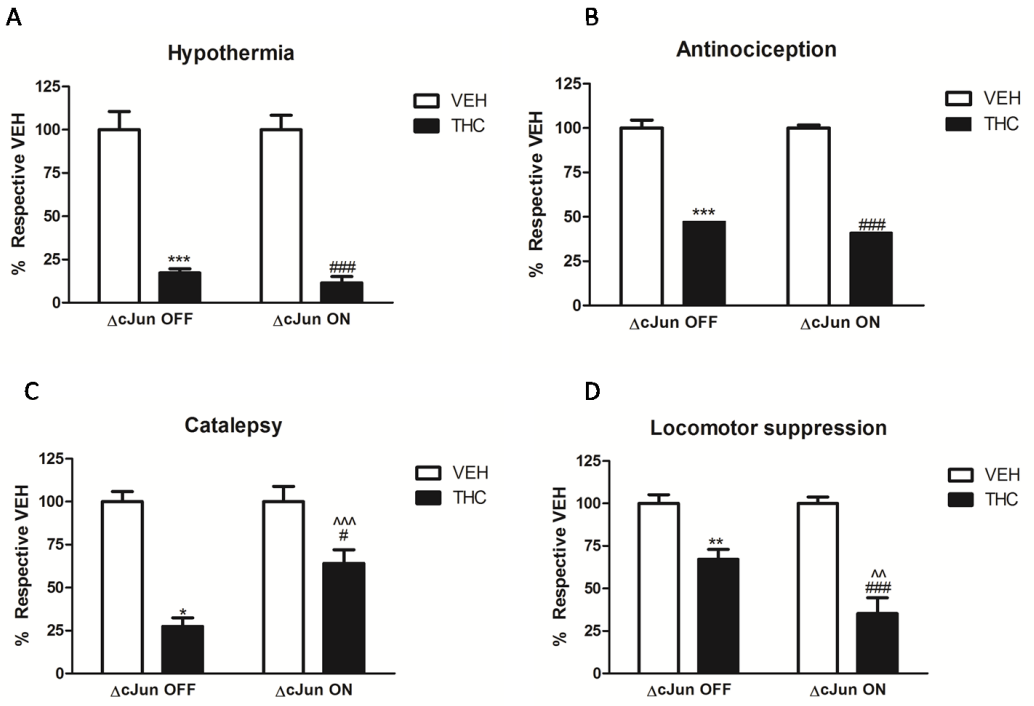


Figure 2.7 THC-mediated hypothermia (A), antinociception (B), catalepsy (C) and locomotor suppression (D) in ΔcJun overexpressing (ΔcJun-ON) and control (ΔcJun-OFF) mice following repeated vehicle or THC treatment. ΔcJun-OFF and ΔcJun-ON mice treated with repeated vehicle exhibited THC-mediated hypothermia, antinociception, catalepsy and locomotor suppression and repeated THC-administration reduced these effects. ΔcJun-ON mice that received repeated THC administration exhibited significantly less catalepsy compared to control mice ($p < 0.001$). ΔcJun-ON mice that received repeated THC administration also exhibited signficantly less locomotor suppression compared to control mice ($p < 0.01$). Data are presented as percent of respective vehicle with mean ± SEM (n = 8-10 mice per group). * $p < 0.05$, *** $p < 0.001$ as compared to vehicle-treated control. # $p < 0.05$ ###, $p < 0.001$ compared to vehicle-treated ΔcJun-ON. ^^^, $p < 0.001$ compared to THC-treated control mice. Two-way ANOVA following Bonferroni post-hoc test.

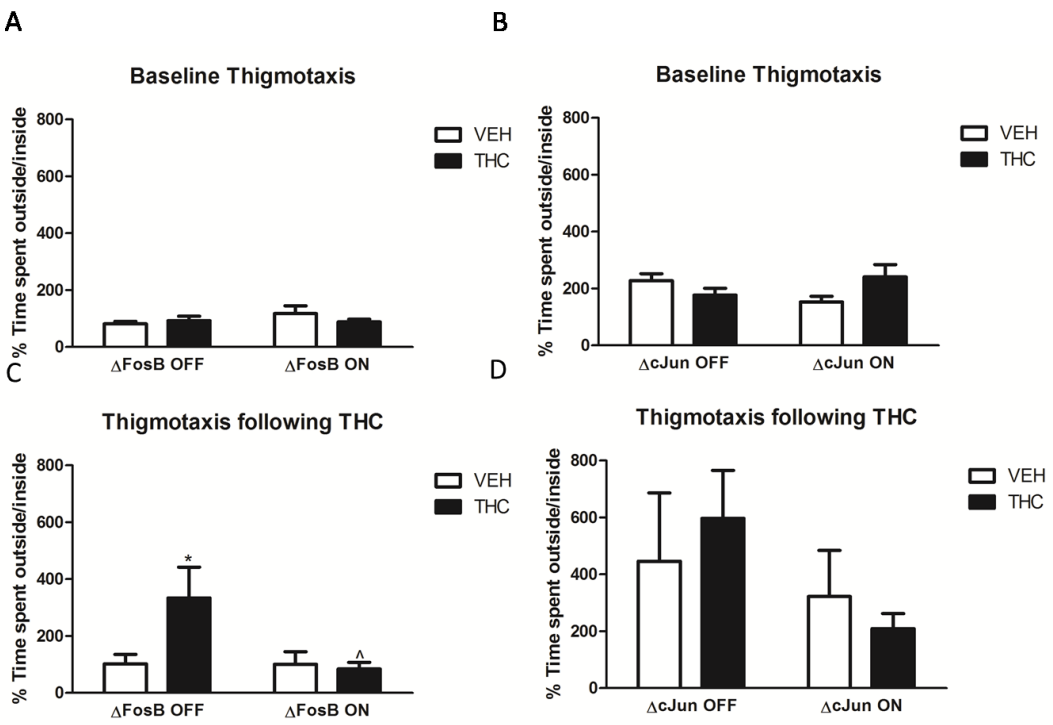


Figure 2.8 Baseline thigmotaxis in (A) ΔFosB-OFF and ΔFosB-ON mice and (B) ΔcJun-OFF and ΔcJun-ON mice. Baseline thigmotaxis did not differ for either ΔFosB-ON/OFF or ΔcJun-ON/OFF mice following either repeated vehicle or THC. (C) ΔFosB-OFF mice that received repeated THC administration exhibited significantly greater THC-mediated thigmotaxis compared to both control mice that received repeated vehicle ($p < 0.05$) and ΔFosB-ON mice that received repeated THC ($p < 0.05$). (D) THC-mediated thigmotaxis was similar in ΔcJun-ON/OFF mice that received either repeated vehicle or THC. Data are presented as percent of time spent in the outside zone/time spent in the inside zone x 100 ± SEM (n = 8-10 mice per group). * $p < 0.05$ compared to vehicle-treated control mice and ^ $p < 0.05$ compared to THC-treated control mice following two-way ANOVA following Bonferroni post-hoc test.

2.4 Discussion

The present study was conducted to determine whether expression of ΔFosB regulates CB_1R-mediated G-protein signaling after acute or repeated activation by cannabinoids. Regional analyses in brains from vehicle-treated mice showed that overexpression of ΔFosB attenuated CP55,940-stimulated [^{35}S]GTPγS binding in the amygdala, whereas functional inactivation of ΔFosB by expression of ΔcJun enhanced cannabinoid-stimulated activity. Expression of ΔFosB also attenuated CB_1R desensitization in the amygdala, further supporting a role for this transcription factor in CB_1R signaling in the amygdala. ΔFosB expression did not affect CB_1R-mediated G-protein activity in the striatum of vehicle-treated mice, consistent with our previous findings (Sim-Selley et al., 2011) and suggesting that the effect of ΔFosB on CB_1R signaling is region-dependent. We proposed that ΔFosB might inhibit CB_1R desensitization following repeated THC treatment based in part on the finding that THC-induced CB_1R desensitization and ΔFosB induction exhibited an inverse regional relationship (Chapter 1). Overexpression of ΔFosB in D_1R-positive MSNs attenuated CB_1R desensitization in the substantia nigra and VTA, targets of MSNs of the direct striatal pathway. Expression of ΔcJun in D_1R and D_2R positive MSNs enhanced CB_1R desensitization in the caudate-putamen and enhanced tolerance to THC-mediated locomotor suppression. However, ΔcJun expression also reduced tolerance to THC-mediated catalepsy and ΔFosB expression enhanced tolerance to locomotor suppression. Despite these unexpected results, the effects of ΔFosB and ΔcJun expression on CB_1R desensitization in striatal circuits and of ΔcJun on tolerance to locomotor suppression support our hypothesis that ΔFosB can inhibit CB_1R desensitization.

Bitransgenic mice overexpress ΔFosB or ΔcJun in the caudate-putamen, nucleus accumbens, cerebral cortex and hippocampus (Chen et al., 1998; Peakman et al., 2003).

Tolerance to THC-mediated hypothermia and antinociception did not differ between mice overexpressing ΔFosB or ΔcJun and their controls, which agree with the restricted anatomical overexpression of ΔFosB/ΔcJun in these mice. Cannabinoid-induced hypothermia is associated with CB_1R activity in the preoptic area (Rawls et al., 2002) and antinociception involves CB_1Rs in the PAG and spinal cord (Lichtman and Martin, 1991). Both mouse lines overexpress the appropriate transcription factor in D_1R/dynorphin MSNs of the caudate-putamen and nucleus accumbens, whereas ΔcJun is also overexpressed in D_2R/enkephalin MSNs. Overexpression of ΔFosB or ΔcJun did not affect CB_1R signaling the caudate-putamen or nucleus accumbens of drug naïve mice, as we previously reported in homogenates prepared from the nucleus accumbens (Sim-Selley et al., 2011). Mice overexpressing ΔFosB that were treated with repeated THC did not exhibit differences in CB_1R desensitization in the caudate-putamen or nucleus accumbens when compared to control mice. Mice overexpressing ΔcJun showed enhanced CB_1R desensitization in the caudate-putamen, but no difference in the nucleus accumbens. The finding that functional inhibition of ΔFosB by ΔcJun expression enhanced CB_1R desensitization supports our hypothesis, but ΔFosB overexpression did not enhance desensitization as we would predict. It is possible that the result in the ΔFosB overexpressing mice is due to the restricted overexpression to only D_1R-positive MSNs. CB_1Rs in the caudate-putamen and nucleus accumbens are expressed by both D_1R and D_2R MSN populations, as well as on glutamatergic, but not dopaminergic, afferent projections (Hohmann and Herkenham, 2000; Pickel et al., 2004). It is also possible that the dose of THC administered in this study was sufficient to overcome the effects of ΔFosB in reducing CB_1R desensitization.

CB_1R desensitization was measured in the substantia nigra and VTA, the projection regions of the caudate-putamen and nucleus accumbens, respectively. D_1R/dynorphin MSNs

comprise the direct pathway that projects from the striatum to the substantia nigra, which has a very high density of CB_1Rs, and VTA (Fitzgerald et al., 2012). Mice overexpressing ΔFosB in D_1R-positive MSNs exhibited less CB_1R desensitization in both the substantia nigra and VTA, suggesting that ΔFosB inhibited CB_1R desensitization in these terminal field regions. The direct pathway is associated with activation of locomotor activity (Kravitz et al., 2010), but it is not clear if the locomotor suppressing effect of THC is mediated through activation of CB_1Rs in this pathway (Monory et al., 2007). THC-treated ΔcJun mice showed enhanced tolerance to THC-mediated locomotor inhibition, which corresponds to the enhanced CB_1R desensitization measured in the caudate-putamen. Surprisingly, THC-treated mice that overexpressed ΔFosB exhibited greater tolerance to locomotor suppression. However, studies have reported that unilateral intra-nigral injections of THC alone (Sanudo-Pena et al., 1996) or in combination with muscimol (Wickens and Pertwee, 1995) produced contralateral circling, an indicator of hyperactivity (Amalric and Koob, 1989). Furthermore, systemic THC administration in mice that have a unilateral lesion of the substantia nigra produced ipsilateral circling, similar to amphetamine (Sakurai et al., 1985). Therefore, it is possible that mice overexpressing ΔFosB exhibited less desensitization in the substantia nigra, but similar desensitization occurred in other basal ganglia regions, therefore they exhibited greater locomotor activity. This would agree with studies indicating that inhibition of glutamatergic neurotransmission contributes to cannabinoid-mediated locomotor suppression (Monory et al., 2007).

The finding that control mice exhibited significantly more thigmotaxis when compared to mice overexpressing ΔFosB might also be relevant to the interpretation of these data. Thigmotaxis is considered an anxiogenic-like phenotype (Simon et al., 1994) and mice exhibiting this behavior also tend to exhibit locomotor suppression (Hoy et al., 1999). It is

possible that ΔFosB overexpressing mice displayed more exploratory behavior because they did not exhibit an anxiogenic-like phenotype.

Mice overexpressing ΔcJun exhibited significantly more catalepsy following repeated THC administration compared to controls, suggesting that less tolerance developed. Early research suggested that the globus pallidus was involved in modulating cannabinoid-mediated catalepsy (Wickens and Pertwee, 1993); however more recent research has also implicated the nucleus accumbens (Sano et al., 2008). Our finding that mice overexpressing ΔcJun exhibited less desensitization in the VTA would support this more recent finding because D_1R/dynorphin MSNs in the nucleus accumbens project to the VTA. One caveat is that there was also less desensitization in the VTA of mice overexpressing ΔFosB, but no difference in catalepsy was found between ΔFosB-ON and ΔFosB-OFF mice. ΔFosB bitransgenic mice are a cross between FVB and C57BL/6J mouse strains (Chen et al., 1998), whereas ΔcJun mice are on a pure FVB background (Peakman et al., 2003). There are ~49% more dopaminergic neurons in the VTA of FVB mice compared to C57BL/6J mice (Nelson et al., 1996), suggesting a possible strain-dependent difference that could affect the results.

Overexpression of ΔcJun in both the D_1R/dynorphin and D_2R/enkephalin MSN populations did not enhance desensitization in either the substantia nigra or globus pallidus, but did reduce desensitization in the VTA. This finding suggests that dominant negative inhibition of ΔFosB can also reduce CB_1R desensitization; however, ΔcJun also inhibits the transcriptional regulation of other Fos family members (Peakman et al., 2003), making it difficult to determine if this effect is due to inhibition of ΔFosB alone. Moreover, this same effect was found in the hippocampus of ΔcJun overexpressing mice, a region in which ΔFosB is not induced by repeated THC administration (Chapter 1). This finding in the hippocampus is the first to demonstrate a

possible mechanism through which CB_1R desensitization could be inhibited in this region. It is not clear which Fos family member(s) might be involved, but c-Fos and FosB are likely candidates because they are also induced by THC administration in both nucleus accumbens and hippocampus (Marie-Claire et al., 2003; Porcella et al., 1998; Rubino et al., 2006). ΔFosB is known to regulate c-Fos induction (Renthal et al., 2008) so it is possible that overexpression of both ΔFosB and ΔcJun could inhibit c-Fos transcription and reduce CB_1R desensitization.

Overexpression of ΔFosB or ΔcJun produced opposing effects on CB_1R G-protein signaling in the amygdala of drug-naive mice. Basal levels of ΔFosB are normally low in the amygdala, but administration of drugs of abuse, including opioids, cocaine, ethanol and THC, induce ΔFosB expression (Perrotti et al., 2008). This suggests that CB_1R signaling in the amygdala could be altered after use of these drugs. Systemic administration of cannabinoids typically produces a biphasic effect in anxiety-like behaviors, where lower doses produce anxiolytic-like effects and higher doses produce anxiogenic-like effects (Viveros et al., 2005), and these anxiogenic effects are mediated by the basolateral amygdala (Rubino et al., 2008). It is important to note that Rubino et al. 2008 found that these anxiogenic effects were evident at lower doses of THC (1 µg/microinjection) but not at higher doses. The amygdala is also involved in drug reinstatement as research suggests its involvement in consolidation of drug-paired cues (Luo et al., 2013). Specifically, excitotoxic lesion of the basolateral amygdala abolishes cocaine conditioned place preference (Fuchs et al., 2002) and heroin-induced reinstatement (Fuchs and See, 2002).

Following repeated THC administration, there was a significant difference between ΔFosB-ON and ΔFosB-OFF mice for both CB_1R-desensitization in amygdala and THC-mediated thigmotaxis. Mice overexpressing ΔFosB had significantly less CB_1R desensitization

in the amygdala and also exhibited a similar amount of thigmotaxis as mice that received repeated vehicle. It appears that significant desensitization in amygdala can unmask an anxiogenic-like phenotype in mice given 100mg/kg THC. The differences in CB_1R G-protein signaling in the amygdala are surprising because neither ΔFosB nor ΔcJun overexpression is found in the amygdala of these transgenic mice (Chen et al., 1998; Peakman et al., 2003), which suggests that these effects result from afferent projections to amygdala from another brain region. Immunohistochemical and electron microscopic data suggest that CB_1Rs are found primarily on cholecystokinin (CCK)-positive GABAergic interneurons and on symmetrical (glutamatergic) synapses in the amygdala (Katona et al., 2001; Marsicano and Lutz, 1999; Tsou et al., 1998).

Overexpression of either ΔFosB or ΔcJun reduced CB_1R desensitization in a brain region-dependent manner. These results suggest that transcriptional regulation of CB_1Rs by Fos family members regulates desensitization in different brain regions. Inhibition of ERK phosphorylation modulated CB_1R desensitization in the caudate-putamen and cerebellum, but not in the prefrontal cortex and hippocampus (Rubino et al., 2005). However, it is not known whether ΔFosB or ΔcJun modulates ERK activity. Another possible mechanism could be the repression of c-Fos expression by ΔFosB (Renthal et al., 2008). THC-mediated c-Fos induction is attenuated following repeated THC administration in the striatum (Miyamoto et al., 1997) and prefrontal cortex (Rubino et al., 2004). Therefore, inhibition of c-Fos by either ΔFosB or ΔcJun could explain reduced CB_1R desensitization in some regions.

Overall, these studies suggest a role for the Fos family of transcription factors in modulating CB_1R desensitization; specifically, ΔFosB can reduce desensitization and dominant negative inhibition of ΔFosB can enhance CB_1R desensitization in certain forebrain regions.

Results in mice with overexpression of ΔcJun also suggest a possible role for Fos family members in reducing CB_1R desensitization, especially in hippocampus. This result may provide a mechanism through which the memory impairing effects of THC could be mitigated. Reductions in CB_1R desensitization led to reductions in the development of tolerance to certain cannabinoid-mediate behaviors, whereas enhanced CB_1R desensitization led to enhanced tolerance. These findings further support the hypothesis that CB_1R desensitization contributes to the development of tolerance to cannabinoid-mediated effects and provide new insights into the role transcription factors play in mediating both desensitization and tolerance.

Chapter 3: Role of dopamine type 1 receptors and DARPP-32 in THC-mediated induction of ΔFosB in forebrain regions

3.1 Introduction

Cannabinoids including THC, the primary psychoactive constituent of marijuana, produce rewarding and motor effects by activating CB_1Rs in the mesolimbic and nigrostriatal systems (Haring et al., 2011; Shi et al., 2005; Steiner et al., 1999; Tanda and Goldberg, 2003). Anatomical and functional studies have shown that cannabinoid-mediated reward and motor effects are produced by interactions of CB_1Rs with dopamine systems in these circuits (Fitzgerald et al., 2012; Glass and Felder, 1997; Julian et al., 2003; Seif et al., 2011). For example, CB_1Rs enhance dopamine release in the striatum directly and by regulating the activity of midbrain dopaminergic neurons (Cheer et al., 2003; Gardner, 2005a; Wu and French, 2000). CB_1Rs in the caudate-putamen and nucleus accumbens are located on both axonal projections from other regions, including glutamatergic projections from the cortex, and expressed by GABAergic MSNs of the direct and indirect pathways, which predominantly express D_1Rs and dynorphin or D_2Rs and enkephalin, respectively (Hohmann and Herkenham, 2000; Pickel et al., 2004). THC produces some of the same cellular effects as other drugs of abuse, including an increase in phosphorylation of the dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32) at threonine 34 (Bateup et al., 2008; Borgkvist et al., 2008) and induction of ΔFosB in the striatum (McClung et al., 2004; Perrotti et al., 2008)(Chapter 1). Dopamine D_1Rs and DARPP-32 increase neuronal activity in D_1R/dynorphin MSNs, and this activity is thought to contribute to the rewarding effects of drugs of abuse (Le Foll et al., 2009). Currently, the role of the D_1R system in THC-mediated ΔFosB induction in the striatum is not clearly defined.

ΔFosB, a stable transcription factor, accumulates in striatal neurons during repeated

treatment with drugs of abuse (Perrotti et al., 2008). Transgenic overexpression of ΔFosB enhanced the rewarding effects of cocaine (Colby et al., 2003) and morphine (Zachariou et al., 2006a), whereas expression of a dominant negative form of its binding partner, ΔcJun, reduced conditioned place preference at lower doses of cocaine and at higher doses of morphine (Peakman et al., 2003; Zachariou et al., 2006a). We reported that repeated THC-mediated ΔFosB induction in the striatum was abolished in mice lacking CB_1Rs (Chapter 1). Anatomical studies showed that CB_1Rs were both co-localized with ΔFosB in striatal neurons and also expressed in puncta surrounding FosB/ΔFosB positive neurons (Chapter 1). The latter observation suggests that THC might trans-synaptically induce ΔFosB in striatal neurons. CB_1Rs enhance dopamine release in the striatum (Oleson and Cheer, 2012), which would activate D_1Rs and provides a potential trans-synaptic mechanism for ΔFosB induction. Consistent with this hypothesis, ΔFosB expression is primarily restricted to the D_1R/dynorphin containing MSNs in the striatum following repeated cocaine administration (Moratalla et al., 1996; Nye et al., 1995). Moreover, previous studies showed that the D_1R antagonist, SCH23390, blocked induction of ΔFosB by cocaine (Nye et al., 1995) and morphine (Muller and Unterwald, 2005). Thus, by analogy with other abused drugs, THC might also induce ΔFosB via D_1R activation.

The role of D_1Rs in the central nervous system has been demonstrated for several drugs of abuse, but the signaling pathways that mediate these effects are under investigation. D_1R agonists and psychomotorstimulants increase phosphorylation of DARPP-32 at threonine 34 in D_1R/dynorphin MSNs (Bateup et al., 2008). When DARPP-32 is phosphorylated at this site, it becomes an inhibitor of protein phosphatase-1, which results in the enhancement in the phosphorylation of substrates downstream of protein kinase A (PKA) (Desdouits et al., 1995; Hemmings et al., 1984a; Kwon et al., 1997). ΔFosB induced by repeated cocaine administration

was attenuated in mice with genetic deletion of DARPP-32 or mutation of the threonine 34 site to prevent protein kinase A (PKA)-mediated phosphorylation (Hiroi et al., 1999; Zachariou et al., 2006b). The CB_1R agonist CP55,940 increased phosphorylation of DARPP-32 at threonine 34 in the striatum, which was blocked by adenosine $_{2A}$ (A_{2A}) or D_2R antagonists and in mice with genetic deletion of these receptors (Andersson et al., 2005). Administration of THC also increased phosphorylation of DARPP-32 at threonine 34 in the striatum, and this effect was blocked by antagonism of A_{2A} or D_1 receptors (Borgkvist et al., 2008). DARPP-32 also contributes to cannabinoid-mediated *in vivo* effects. Genetic deletion of DARPP-32 or mutation of the PKA site at threonine 34 reduced CP55,940-induced catalepsy (Andersson et al., 2005). Phosphorylation of DARPP-32 at threonine 34 is known to increase PKA activity (Blank et al., 1997), which could also interfere with the development of tolerance to this cannabinoid-mediated effect because inhibition of PKA has been shown to reduce tolerance to the locomotor suppressing effects of THC (Bass et al., 2004).

While it is clear that D_1Rs can modulate the induction of ΔFosB produced by psychomotorstimulants and opioids, the role of D_1Rs in THC-mediated ΔFosB induction is not known. The current study was conducted to determine whether THC-mediated induction of ΔFosB is D_1R-dependent and whether THC-induced ΔFosB is localized to D_1R-positive MSNs of the caudate-putamen and nucleus accumbens. The role of DARPP-32 in THC-mediated ΔFosB induction was also investigated because this protein is downstream of dopamine receptors and also modulates ΔFosB induction. The contribution of dopamine-mediated signaling to THC-mediated in vivo responses was determined by testing naïve and THC-treated DARPP-32 knockout mice. Results showed that D_1Rs and DARPP-32-mediated signaling are involved in THC-mediated ΔFosB induction and that genetic deletion of DARPP-32 enhances both acute

THC-mediated locomotor suppression and tolerance to this response.

3.2 Materials and Methods

Materials

Sources of THC and antibodies are provided in Chapter 1. Goat anti-preprodynorphin antibody was purchased from Millipore (Billerica, MA). (*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390) and (6a*S*-*trans*)-11-Chloro-6,6a,7,8,9,13b-hexahydro-7-methyl-5*H*-benzo[*d*]naphth[2,1-*b*]azepin-12-ol hydrobromide (SCH39166) were purchased from Tocris Bioscience (Minneapolis, MN). Refer to Chapter 1 for secondary antibodies and mounting media. All other reagent grade chemicals were obtained from Sigma Chemical Co. or Fisher Scientific.

Subjects and Drug treatments

Male ICR mice (n=8 per group) (Harlan Laboratories, Indianapolis, IN) weighing 25-30 grams were used to assess the effect of D_1R antagonists on THC-mediated ΔFosB induction. All mice were housed four to six per cage and maintained on a 12-hr light/dark cycle in a temperature controlled environment (20-22°C) with food and water available ad libitum. THC was dissolved in a 1:1:18 solution of ethanol, emulphor and saline (vehicle). SCH23390 and SCH39166 were dissolved in saline. SCH23390 is a high affinity D_1R antagonist with agonist properties at $5HT_{1/2c}$ receptors and SCH39166 is a high affinity D_1R antagonist with lower affinity for D_2R, 5-HT and A_{2A} receptors. Mice were pretreated with an intraperitoneal (i.p.) injection of either saline or 1 mg/kg SCH23390 or SCH39166 and 30 minutes later were injected subcutaneously (s.c.) with either THC (ramping doses of 10-20-30 mg/kg increased every 2 days) or vehicle at 08:00 and 16:00 h for 6 days. On day 7, mice received morning injections

only, and 24 hours later mice were sacrificed by decapitation and brains were extracted. A separate group of male ICR mice (n = 4) was treated with THC or vehicle using the same treatment protocol for co-localization studies.

DARPP-32 knockout mice on a C57BL/6J background and littermate controls (Hiroi et al., 1999)(n = 8 per group) were used to determine the role of DARPP-32 in THC-mediated ΔFosB induction and THC-mediated *in vivo* effects. Mice were treated using a protocol that we have shown produces ΔFosB induction in C57BL/6J mice (Chapter 1). Mice were injected (s.c.) with 10 mg/kg THC or vehicle at 08:00 and 16:00 h for 13 days. On day 14, mice received only a single injection (08:00), and 24 hours later mice were assessed for THC-induced antinociception, hypothermia, catalepsy and locomotor suppression. 24 hours after in vivo assessment, mice were sacrificed by decapitation and brains were extracted. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health guide for the care and use of Laboratory animals 7th edition.

Brain Dissections

Brain regions were dissected as described in Chapter 1. For these experiments, the amygdala dissection included the central nucleus and basolateral and basomedial nuclei.

Immunoblot

Immunoblots were conducted as detailed in the Methods section of Chapter 1.

Immunohistochemistry

Preprodynorphin was used as a marker for D_1R/dynorphin MSNs to determine the localization of ΔFosB/FosB following repeated THC administration in this MSN population.

Refer to Chapter 1 for incubation and washing methods. Slides were incubated overnight at 4°C in PBS containing 2.5% normal donkey serum and antibodies against preprodynorphin (1:500; guinea-pig) and FosB/ΔFosB (1:500; sc-48/rabbit). Refer to Chapter 1 for capturing methods. Images were taken at 40 X magnification and the number of cells that were positive for DAPI was counted. Then, the numbers of cells that contained FosB/ΔFosB-ir + dynorphin-ir or FosB/ΔFosB-ir alone were counted. ~40-50 cells per image for 4 separate animals per treatment group were counted and averaged together.

Assessment of in vivo responses

The measures of nociception, body temperature, spontaneous activity and catalepsy were done as described in Chapter 2. Baseline measures were assessed for all behaviors, and then separate groups of mice were injected (i.p.) with 70 mg THC or vehicle. Locomotor suppression was determined 20 minutes after THC injection and measures for catalepsy, antinociception and hypothermia were assessed 30, 60, 120 and 180 minutes after injection, based on the published time course for these cannabinoid-mediated affects (Andersson et al., 2005; Wiebelhaus et al., 2012). Hyperreflexia was also assessed (Dewey 1986; Patel 2001) and defined as "popcorning" or an exaggerated movement due to auditory or tactile cues.

Statistical Analysis

For all experiments, data were analyzed with Prism® version X (GraphPad Software, San Diego, CA). For comparisons of ΔFosB expression in D_1R antagonist studies, one-way ANOVAs were performed with Bonferroni post-hoc test. For co-localization studies, the number of cells containing either FosB/ΔFosB-ir + dynorphin-ir or FosB/ΔFosB-ir alone was normalized to the total number of DAPI-containing cells. One-way ANOVA and Bonferroni post-hoc test

were used to determine significance. For comparison of ΔFosB expression in DARPP-32 knockout mice, a two-way ANOVA was used with Bonferroni post-hoc test. For comparisons in the development of tolerance to hypothermia and antinociception, a repeated measures ANOVA was used with Bonferroni post-hoc test. For comparisons of catalepsy and locomotor activity, a two-way ANOVA was used with Bonferroni post-hoc test. For hyperreflexia, a z-test was used with a Bonferroni adjustment. Data are represented as % of appropriate controls ± SEM, % MPE ((test latency - baseline) /(total length of test)] X 100) ± SEM. Significance was determined with $p < 0.05$.

3.3 Results

SCH23390 blocks THC-mediated induction of ΔFosB

Mice received the D_1R antagonist SCH23390 or saline prior to administration of THC or vehicle during the 6.5 days of treatment, and ΔFosB expression was measured 24 hours after the last injection. Data were first assessed to determine whether pretreatment with SCH23390 altered ΔFosB expression in vehicle-treated mice. ΔFosB-ir was significantly increased by 55% ± 15% in the nucleus accumbens of SCH23390/vehicle compared to saline/vehicle treated mice ($p < 0.05$). Treatment with SCH23390/THC increased ΔFosB expression by 77% ± 21 % compared to saline/vehicle control mice ($p < 0.01$) (Figure 3.2 C), but there was no significant difference in ΔFosB-ir between SCH23390/vehicle and SCH23390/THC-treated groups. There were no significant differences between mice pretreated with saline or SCH23390 in the other regions examined (Figure 3.2). Because SCH23390 administration induced ΔFosB-ir in the nucleus accumbens, subsequent data are presented as the percent of ΔFosB-ir in the respective vehicle controls. The effect of THC on ΔFosB expression was determined by comparing saline

pretreated THC- and vehicle-treated mice. Repeated THC administration significantly increased ΔFosB expression compared to saline/vehicle control in the prefrontal cortex by 80% ± 12% ($F_{3,28}$, $p < 0.05$; Figure 3.1 A), in caudate-putamen by 64% ± 17% ($F_{3,28}$, $p < 0.01$; Figure 3.1 B), in nucleus accumbens by 49% ± 9% ($F_{3,28}$, $p < 0.05$; Figure 3.1 C) and in amygdala by 64% ± 24% ($F_{3,28}$, $p < 0.05$, Figure 3.1 D). Pretreatment with SCH23390 blocked THC-mediated ΔFosB induction in all four regions examined, and the levels of ΔFosB-ir did not significantly differ between SCH23390/vehicle and SCH23390/THC-treated mice in any region. These data indicate that D_1Rs are necessary for THC-mediated induction of ΔFosB in these forebrain regions.

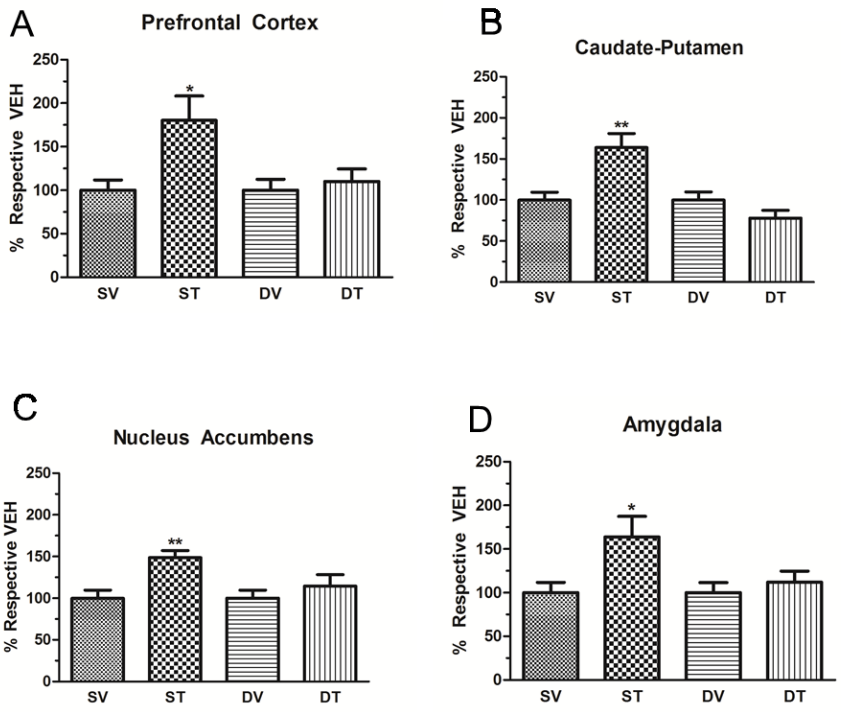


Figure 3.1 Pretreatment with the D_1R antagonist SCH23390 blocked THC-mediated ΔFosB induction in the prefrontal cortex, caudate-putamen, nucleus accumbens and amygdala following repeated THC administration. Graphs show ΔFosB-ir expressed as % respective saline/vehicle and SCH23390/vehicle controls ± SEM in A) prefrontal cortex, B) caudate-putamen, C) nucleus accumbens and D) amygdala. Repeated THC administration alone significantly increased ΔFosB induction in the prefrontal cortex, caudate-putamen, nucleus accumbens and amygdala, which was blocked by pretreatment with SCH23390. One-way ANOVAs were performed to determine significance with Bonferroni post-hoc test * $p < 0.05$ compared to saline/vehicle treated mice. SV = saline/vehicle, ST = saline/THC, DV = SCH23390/vehicle, DT = SCH23390/THC

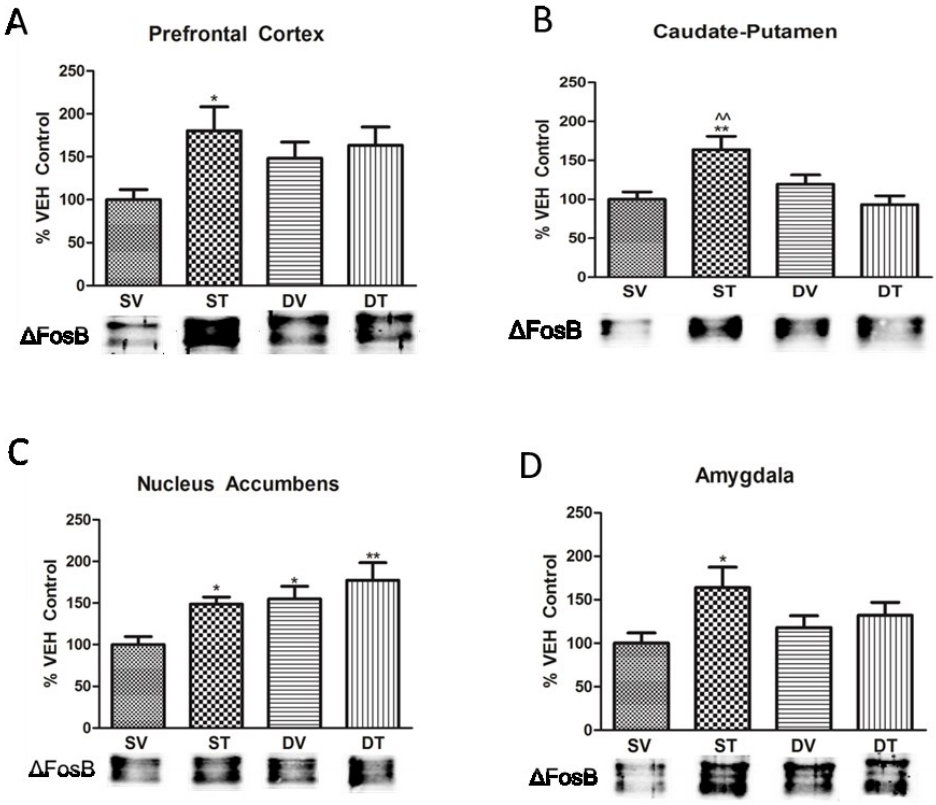


Figure 3.2 Repeated administration of SCH23390/vehicle significantly increased ΔFosB in the nucleus accumbens. Graphs representing ΔFosB-ir expressed as % saline/vehicle mice ± SEM in A) prefrontal cortex, B) caudate-putamen, C) nucleus accumbens and D) amygdala with representative immunoblots. In the nucleus accumbens, repeated SCH23390 treatment in combination with vehicle or THC treatment significantly increased ΔFosB expression compared to saline/vehicle controls by 55% ± 15% ($p < 0.05$) and 77% ± 21% ($p < 0.01$), respectively. One-way ANOVAs were performed to determine significance with Bonferroni post-hoc test * $p < 0.05$ and ** $p < 0.01$ compared to saline/vehicle controls. SV = saline/vehicle, ST = saline/THC, DV = SCH23390/vehicle, DT = SCH23390/THC

SCH39166 blocks THC-mediated induction of ΔFosB

SCH23390 administration increased ΔFosB-ir in the nucleus accumbens and can act as an agonist at $5HT_1$ and $5HT_{2c}$ receptors. Therefore mice were pretreated with another D_1R antagonist, SCH39166, to confirm the results obtained using SCH23390. ΔFosB-ir was first assessed in vehicle-treated mice to determine whether SCH39166 treatment affected ΔFosB expression. No significant differences were found in ΔFosB-ir between SCH39166/vehicle and saline/vehicle treated mice in any region examined (Figure 3.4). The effect of THC treatment on ΔFosB-ir was then determined by comparing results in brains from saline-pretreated vehicle- and THC-treated mice. Saline/THC treatment significantly increased ΔFosB expression compared to saline/vehicle control in the prefrontal cortex, by 93% ± 30% ($F_{3,28}$, $p < 0.05$; Figure 3.3 A), in caudate-putamen by 73% ± 18% ($F_{3,28}$, $p < 0.001$; Figure 3.3 B), in nucleus accumbens 58% ± 16% ($F_{3,28}$, $p < 0.001$; Figure 3.3 C) and in amygdala by 61% ± 11% ($F_{3,28}$, $p < 0.01$; Figure 3.3 D). In the nucleus accumbens, treatment with SCH39166 and THC also significantly increased ΔFosB expression compared to the saline/vehicle treatment group 38% ± 4% ($F_{3,28}$, $p < 0.05$; Figure 3.4 C). SCH39166 pretreatment blocked THC-induced ΔFosB expression in all brain regions examined, because ΔFosB-ir did not significantly differ between brains from SCH39166/THC and SCH39166/vehicle-treated mice. These results further support the hypothesis that D_1Rs are necessary for THC-mediated induction of ΔFosB in the forebrain.

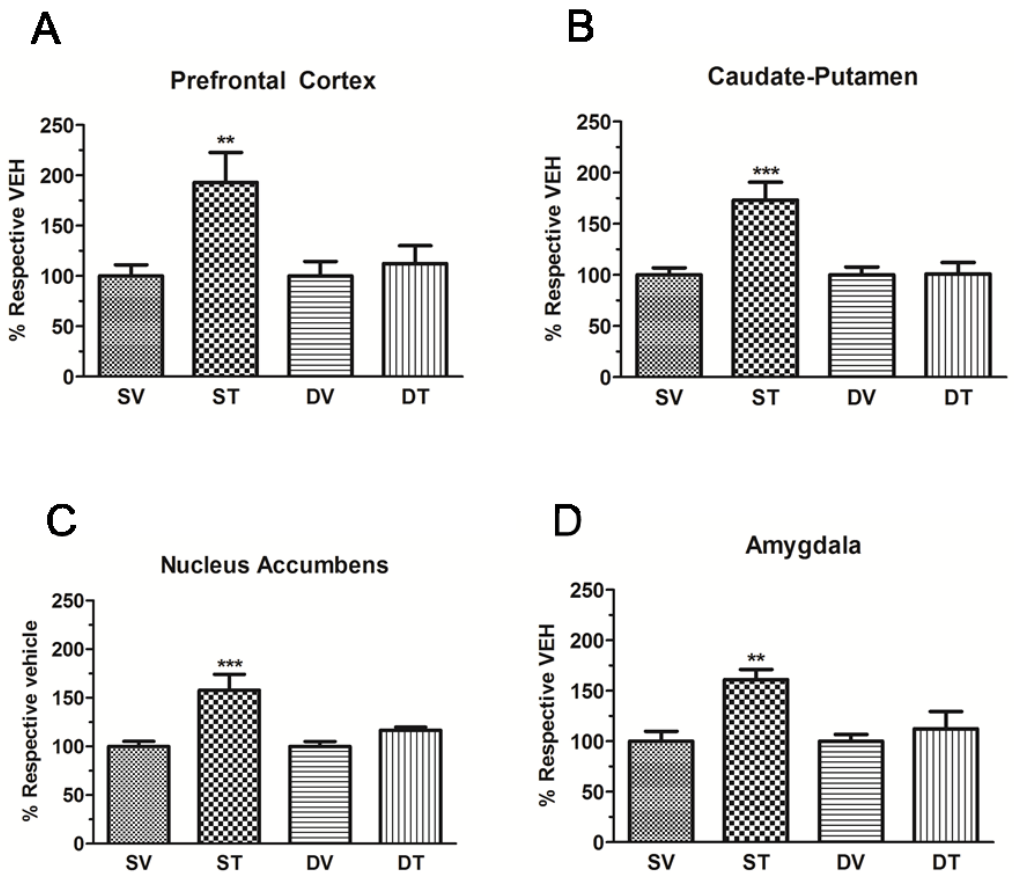


Figure 3.3 Pretreatment with the D_1R antagonist SCH39166 blocked ΔFosB induction in the prefrontal cortex, caudate-putamen, nucleus accumbens and amygdala when administered during repeated THC treatment. Graphs show ΔFosB-ir expressed as % respective saline/vehicle or SCH39166/vehicle mice ± SEM in A) prefrontal cortex, B) caudate-putamen, C) nucleus accumbens and D) amygdala. Repeated THC administration alone significantly increased ΔFosB-ir in the prefrontal cortex, caudate-putamen, nucleus accumbens and amygdala, which was blocked by pretreatment with SCH39166. One-way ANOVAs were performed to determine significance with Bonferroni post-hoc test * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared to saline/vehicle mice. SV = saline/vehicle, ST = saline/THC, DV = SCH39166/vehicle, DT = SCH39166/THC

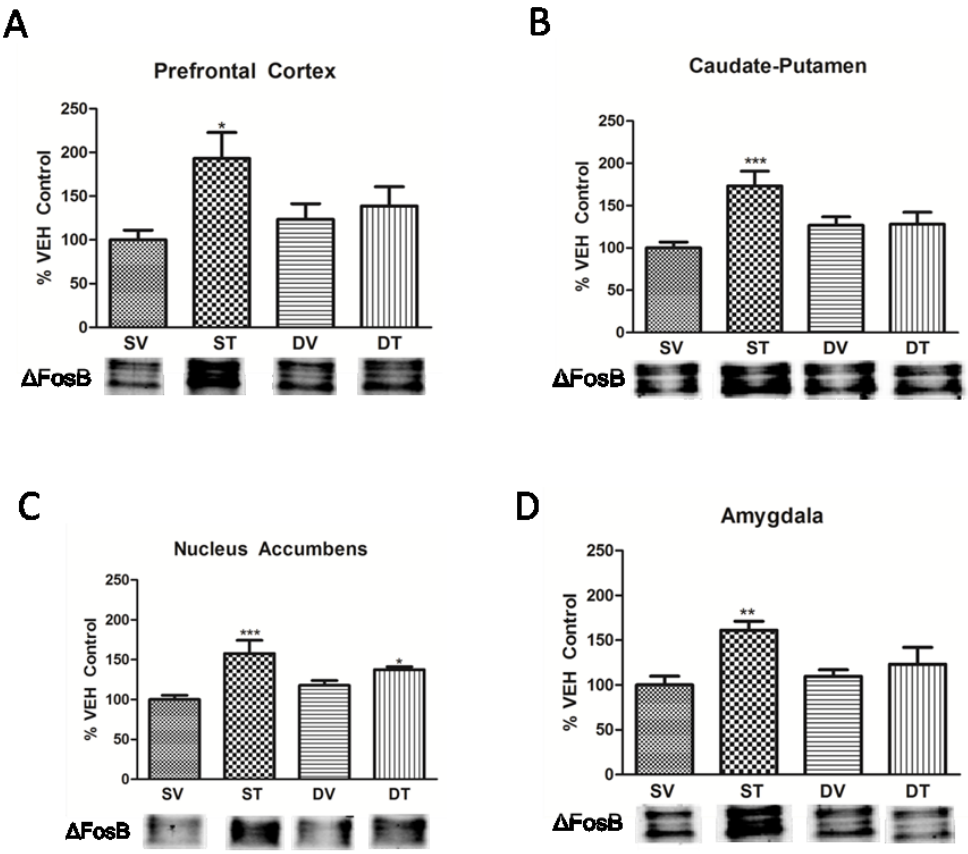


Figure 3.4 The combination of the D_1R antagonist SCH39166 and THC significantly increased ΔFosB expression in nucleus accumbens compared to saline/vehicle control mice. Graphs representing ΔFosB-ir expressed as % saline/vehicle mice ± SEM in A) prefrontal cortex, B) nucleus accumbens, C) caudate-putamen and D) amygdala with representative immunoblots. In nucleus accumbens, repeated SCH39166 treatment in combination with THC significantly increased ΔFosB expression above saline/vehicle control mice 38% ± 5% ($p < 0.05$). Data represented as % saline/vehicle control ± SEM. One-way ANOVAs were performed to determine significance with Bonferroni post-hoc test * $p < 0.05$ compared to saline/vehicle controls. SV = saline/vehicle, ST = saline/THC, DV = SCH23390/vehicle, DT = SCH23390/THC

FosB/ΔFosB positive nuclei co-localize with dynorphin-ir in striatal cells

Results showing that D_1R antagonists block THC-mediated ΔFosB induction suggest that THC induces ΔFosB in D_1R positive MSNs. However, anatomical data to support this conclusion are lacking. Therefore, dual immunohistochemistry was performed using antibodies that recognize FosB/ΔFosB and preprodynorphin, which is co-localized with D_1Rs in MSNs of the direct pathway. Dynorphin was visualized in green and FosB/ΔFosB-ir was visualized in red (Figure 3.5 and 3.6). DAPI (blue) was used to identify cell nuclei. Dynorphin diffusely stained both the dorsal and ventral striatum and appeared to be localized in striatal cells. This was confirmed by DAPI staining, which identified cell nuclei of dynorphin-ir cells. FosB/ΔFosB-ir appeared to be localized in cell nuclei, which was confirmed by DAPI staining (Figure 3.5 A and E and Figure 3.6 A and E). FosB/ΔFosB-ir positive cells were seen in brains from vehicle-treated mice in both the caudate-putamen (Figure 3.5 B) and nucleus accumbens (Figure 3.6 B). Cell counting showed that approximately half of DAPI-positive cells contained both FosB/ΔFosB-ir and dynorphin-ir in both the caudate-putamen (49% ± 3%, Figure 3.5 D and I) and nucleus accumbens (47% ± 2%, Figure 3.6 D and I). The number of dual FosB/ΔFosB-ir and dynorphin-ir cells was significantly greater than the number of cells that only expressed FosB/ΔFosB-ir (26% ± 2% in caudate-putamen and 31% ± 2% in nucleus accumbens ($p < 0.001$). Following repeated THC administration, the percent of DAPI positive cells that contained both FosB/ΔFosB-ir and dynorphin-ir did not differ from vehicle-treated mice (55% ± 2% and 52% ± 1%, caudate-putamen (Figure 3.5 G and I) and nucleus accumbens (Figure 3.6 G and I), respectively). The number of cells positive for FosB/ΔFosB-ir and dynorphin-ir cells was significantly greater than the number of FosB/ΔFosB-ir cells (26% ± 2% in caudate-putamen and 25% ± 4% in nucleus accumbens ($p < 0.001$)). In both regions, ~75%-85% of DAPI-positive

cells co-localized with FosB/ΔFosB-ir, which would suggest that FosB/ΔFosB-ir is predominantly expressed in MSNs, which represent ~95% of neurons of striatum.

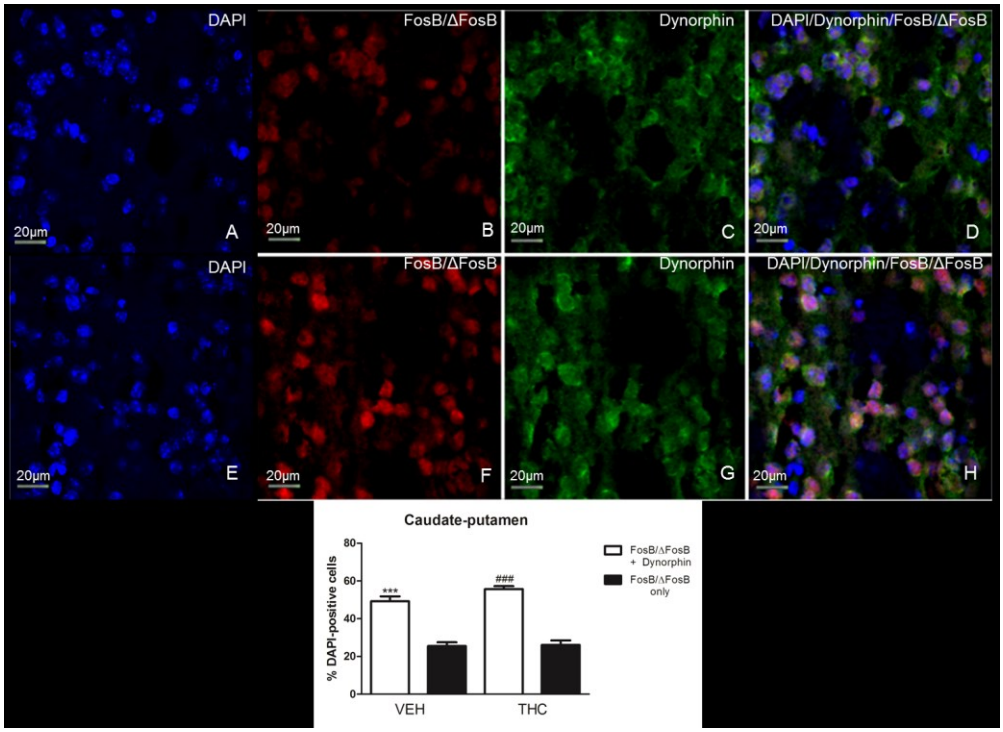


Figure 3.5 Representative images (40X) showing FosB/ΔFosB-ir (red), Dynorphin (green) and DAPI (blue) in the caudate-putamen of mice that received repeated vehicle (top row) or THC (bottom row) treatment. In both vehicle (B) and THC (F) treated mice, FosB/ΔFosB-ir was localized to the nucleus, which was visualized with DAPI (A and E), while dynorphin-ir (C, vehicle; G, THC) was localized to the cell body. (D and H) The majority of FosB/ΔFosB-ir cells were also positive for dynorphin-ir in both vehicle- and THC-treated mice (I). The number of cells positive for either FosB/ΔFosB-ir and dynorphin-ir (white bar) or FosB/ΔFosB-ir (black bar) cells as a percentage of DAPI-positive cells, were compared and results determined that a significantly higher percentage of cells contained both FosB/ΔFosB-ir and dynorphin-ir in both vehicle- and THC-treated mice ($p < 0.001$). One-way ANOVAs were performed with Bonferroni post-hoc test. *** $p < 0.001$ compared to FosB/ΔFosB-ir alone in vehicle-treated. ### $p < 0.001$ compared to FosB/ΔFosB-ir alone in THC-treated.

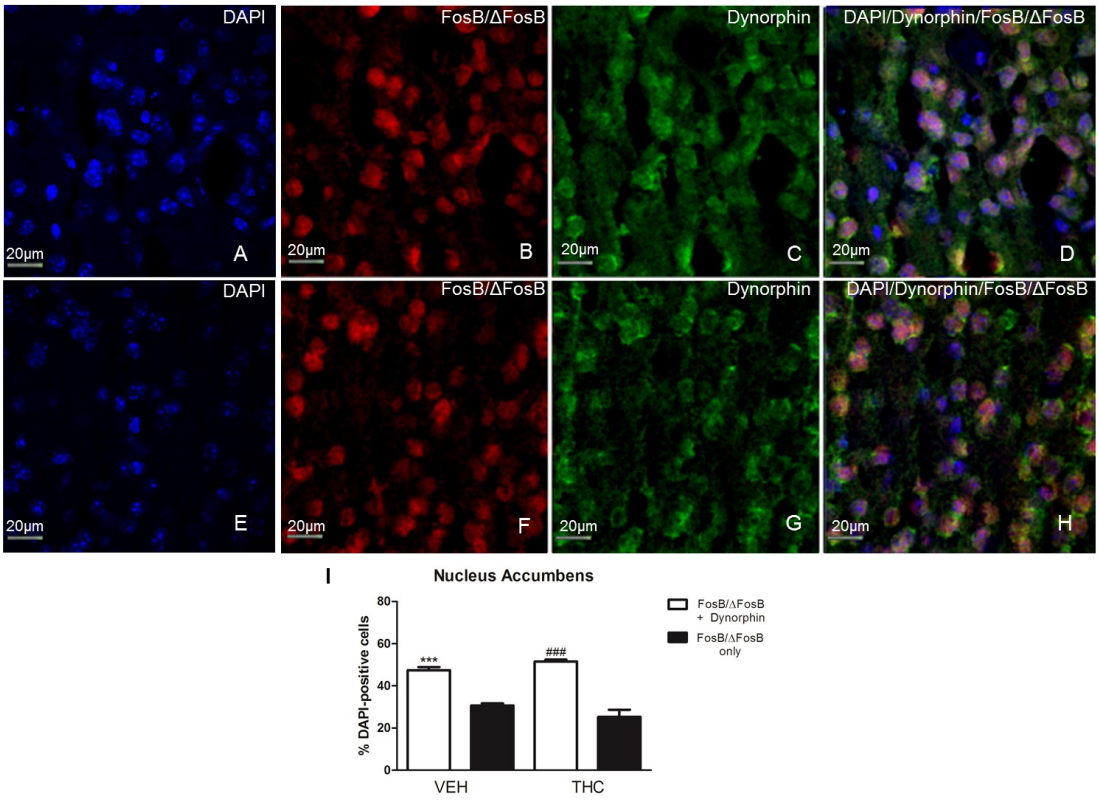


Figure 3.6 Representative images (40X) showing FosB/ΔFosB-ir (red), Dynorphin (green) and DAPI (blue) in the nucleus accumbens of mice that received repeated vehicle (top row) or THC (bottom row) treatment. In both vehicle (B) and THC (F) treated mice, FosB/ΔFosB-ir was localized to the nucleus, which was visualized with DAPI (A and E), while dynorphin-ir (C, vehicle; G, THC) was localized to the cell body. (D and H) The majority of cells positive for FosB/ΔFosB-ir were also positive for dynorphin-ir in both vehicle- and THC-treated mice (I). The number of cells positive for either FosB/ΔFosB-ir and dynorphin-ir (white bar) or FosB/ΔFosB-ir (black bar) cells as a percentage of DAPI-positive cells, were compared and results determined that a significantly higher percentage of cells contained both FosB/ΔFosB-ir and dynorphin-ir in both vehicle- and THC-treated mice ($p < 0.001$). One-way ANOVAs were performed with Bonferroni post-hoc test. *** $p < 0.001$ compared to FosB/ΔFosB-ir alone in vehicle-treated. ### $p < 0.001$ compared to FosB/ΔFosB-ir alone in THC-treated.

Acute, but not repeated, THC-mediated FosB induction is abolished in DARPP-32 knockout mice

DARPP-32 knockout and littermate wild-type mice were treated for 13.5 days with THC or vehicle and then assessed for in vivo measures by administering a single injection of THC (70 mg/kg) or vehicle. Therefore, mice of each genotype were treated as follows: repeated vehicle + acute vehicle (VEH-VEH group), repeated vehicle + acute THC (VEH-THC) and repeated THC + acute THC (70 mg/kg) (THC-THC). Mice were first tested in the *in vivo* measures, and then brains from the six groups (VEH-VEH, VEH-THC, THC-THC for DARPP-32 knockout and wild-type mice) were collected to measure ΔFosB-ir. In the caudate-putamen, A 3 X 2- way ANOVA (treatment X genotype) determined a significant main effect of both treatment ($F_{2, 36} = 68.58$ $p < 0.0001$) and an interaction ($F_{2,36} = 12.40$, $p < 0.001$) (Figure 3.7 A). ΔFosB expression did not significantly differ between VEH-VEH-treated wild type and DARPP-32 knockout mice in the caudate-putamen. An acute injection of THC in repeated vehicle-treated mice (VEH-THC) significantly increased ΔFosB expression in wild type mice (32% ± 4%, $p < 0.001$, relative to VEH-VEH wild type mice, Figure 3.7 A), but not in DARPP-32 knockout mice (4% ± 5%, relative to VEH-VEH wild type mice, Figure 3.7 A). Acute THC-induced ΔFosB-ir in wild type mice also significantly differed from ΔFosB-ir in DARPP-32 knockout mice ($p < 0.001$, Figure 3.7 A). Following repeated THC administration, ΔFosB expression was significantly increased in both wild type (50% ± 5%, $p < 0.001$, relative to VEH-VEH wild type mice) and DARPP-32 knockout (60% ± 6%, $p < 0.001$, relative to VEH-VEH wild type mice) mice. The level of ΔFosB-ir in DARPP-32 knockout mice was also significantly different from DARPP-32 knockout mice that had received only vehicle (VEH-VEH) ($p < 0.001$, Figure 3.7 A). ΔFosB

expression did not significantly differ between wild type and DARPP-32 knockout mice that received repeated THC treatment (THC-THC).

In the nucleus accumbens, a 3 X 2- way ANOVA (treatment X genotype) determined a significant main effect of both treatment ($F_{2, 36} = 13.71$ $p < 0.0001$) and genotype ($F_{1,36} = 12.04$, $p < 0.05$) (Figure 3.7 B). ΔFosB expression did not significantly differ between wild type and DARPP-32 knockout mice that received only vehicle (VEH-VEH). There was a significant difference in ΔFosB-ir between vehicle-treated wild type and DARPP-32 knockout mice that received an acute injection of THC (VEH-THC) (26 % ± 10% versus -15% ± 11%, $p < 0.05$, relative to VEH-VEH wild type mice, Figure 3.7 B). There was a significant increase in ΔFosB expression following repeated THC administration in wild type (34% ± 7%, $p < 0.05$, Figure 3.7 B), but not DARPP-32 knockout (16% ± 16%, Figure 3.7 B) mice compared to wild type vehicle-treated mice. ΔFosB expression was not significantly different between wild type and DARPP-32 knockout mice that received repeated THC treatment (THC-THC). These results show that deletion of DARPP-32 blocked ΔFosB induction produced by an acute injection of THC, but does not inhibit ΔFosB induction after repeated THC treatment in the caudate-putamen. A similar pattern was found in the nucleus accumbens; however induction of ΔFosB by repeated THC was not significant in this region.

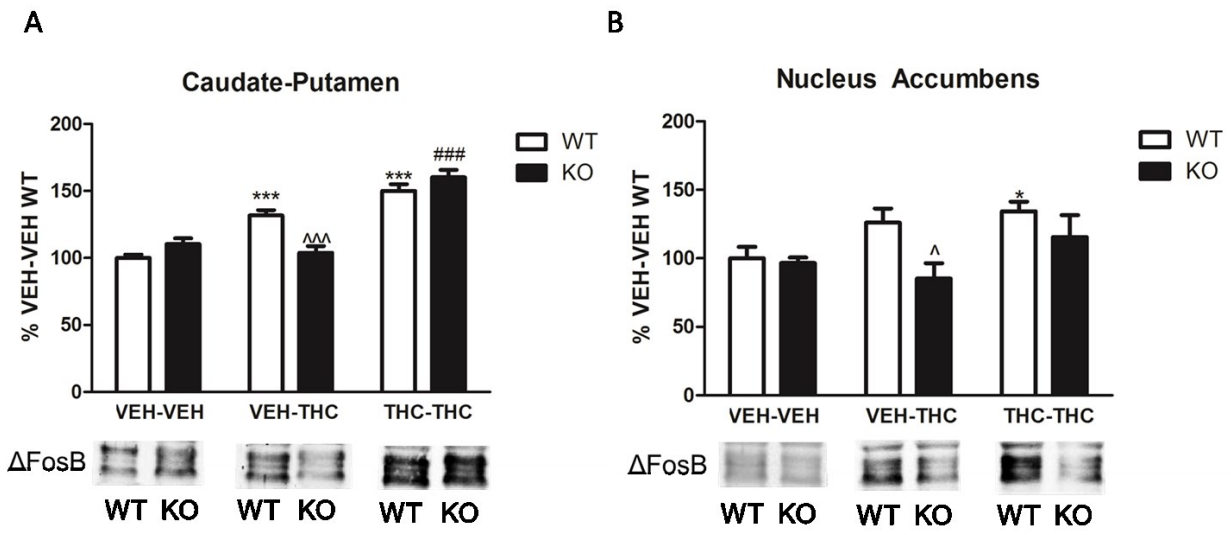


Figure 3.7 Genetic deletion of DARPP-32 attenuated induction of ΔFosB following a single injection of THC in the caudate-putamen and nucleus accumbens and attenuated induction of ΔFosB following repeated THC administration in nucleus accumbens. Graphs show ΔFosB-ir expressed as % VEH-VEH wild type mice ± SEM in A) nucleus accumbens and B) caudate-putamen. 3 X 2-way ANOVA was performed with Bonferroni post-hoc test, * $p < 0.05$ and *** $p < 0.001$ compared to VEH-VEH wild type control. ^ $p < 0.05$ and ^^^ $p < 0.001$ compared to VEH-THC wild type. ### $p < 0.001$ compared to VEH-VEH knockout.

DARPP-32 knockout mice exhibit enhanced THC-mediated locomotor suppression and greater tolerance to this effect and exhibit less THC-mediated hyperreflexia following repeated THC administration

DARPP-32 knockout mice and wild type littermate controls that received repeated THC or vehicle were assessed for THC-mediated locomotor suppression, hypothermia, antinociception, catalepsy and hyperreflexia. A separate group of mice that received repeated vehicle were challenged with vehicle to verify that there was no effect of multiple assessments on these measures (data not shown). , Acute THC administration produced significantly greater locomotor suppression in vehicle-treated DARPP-32 knockout mice as compared to wild-type mice (206 ± 13 vs. 258 ± 10 seconds immobile, wild type and DARPP-32 knockout mice, respectively, $p < 0.05$) (Figure 3.8 A). There was no significant difference in THC-mediated locomotor suppression between vehicle and THC-treated wild type mice (Figure 3.8 A). However, significantly less THC-mediated locomotor suppression was found in DARPP-32 knockout mice compared to their respective vehicle-treated control (258 ± 10 vs. 135 ± 21 seconds immobile, vehicle and THC treated, respectively, $p < 0.001$, Figure 3.8 A), indicating that tolerance had developed to this effect. DARPP-32 knockout mice also exhibited significantly less locomotor suppression than wild-type mice following repeated THC administration (187 ± 15 vs. 135 ± 21 seconds immobile, wild type and DARPP-32 knockout mice respectively, $p < 0.05$, Figure 3.8 A).

For the measure of catalepsy, comparisons were made at the 180 minute time point because mice also exhibited hyperreflexia at earlier time points (Figure 3.8 E). There was no significant difference between vehicle-treated wild type and DARPP-32 knockout mice because both genotypes remained immobile on the bar for a similar period of time after THC

administration (54 ± 2 versus 49 ± 4 seconds immobile, wild type and DARPP-32 knockout mice respectively, Figure 3.8 B). Following repeated THC administration, there was also no significant difference between wild type or DARPP-32 knockout mice (49 ± 3 versus 41 ± 4, seconds immobile, wild type and DARPP-32 knockout respectively, Figure 3.8 B). Time spent immobile on the bar did not differ between repeated vehicle-or THC-treated mice for either genotype, suggesting that tolerance did not develop for this measure. Interestingly, there was a significant difference in the percentage of mice that exhibited hyperreflexia. A significantly higher percentage of THC-treated wild type mice exhibited hyperreflexia compared to either vehicle-treated wild type mice or repeated THC-treated DARPP-32 knockout mice (Figure 3.8 E). At 30 minutes, 87.5% of wild type mice that received repeated THC administration exhibited hyperreflexia, whereas 25% of either THC-treated DARPP-32 knockout mice or vehicle-treated wild type mice exhibited hyperreflexia. The percentage of repeated THC-treated wild type mice that exhibited hyperreflexia was also greater at the 60 minute time point compared to wild type mice that received repeated vehicle and greater at the 60 and 120 minute time points compared to DARPP-32 knockout mice that received repeated THC (Figure 3.8 E).

Both vehicle-treated wild type and DARPP-32 knockout mice exhibited antinociception following acute THC administration and the time-course of the effect was similar between genotypes (Figure 3.8 C). Antinociception, measured as % MPE, was significantly decreased at all time points in THC- compared to vehicle-treated wild type and DARPP-32 knockout mice (Figure 3.8 C). Antinociception was not significantly different between repeated THC-treated wild type and DARPP-32 knockout mice over the time period examined (Figure 3.8 C). Vehicle-treated wild type and knockout mice both exhibited hypothermia following acute THC administration (Figure 3.8 D). Vehicle-treated wild type and DARPP-32 knockout mice had

similar body temperatures at 30 minutes (32.8°C ± 0.5°C versus 32.7°C ± 0.6°C, wild type and DARPP-32 knockout mice, respectively) and temperature remained stable for the remaining 120 minutes, suggesting a similar time course for hypothermia between genotypes. Body temperature was significantly higher in THC-treated mice compared to the respective vehicle-treated mice of each genotype (Figure 3.8 D). Body temperature did not significantly differ between repeated THC treated wild type and DARPP-32 knockout mice 30 minutes after THC administration (37.1°C ± 0.2°C vs. 32.0°C ± 0.3°C, wild type and DARPP-32 knockout, respectively) and values remained stable for both wild type and DARPP-32 knockout mice throughout testing (Figure 3.8 D).

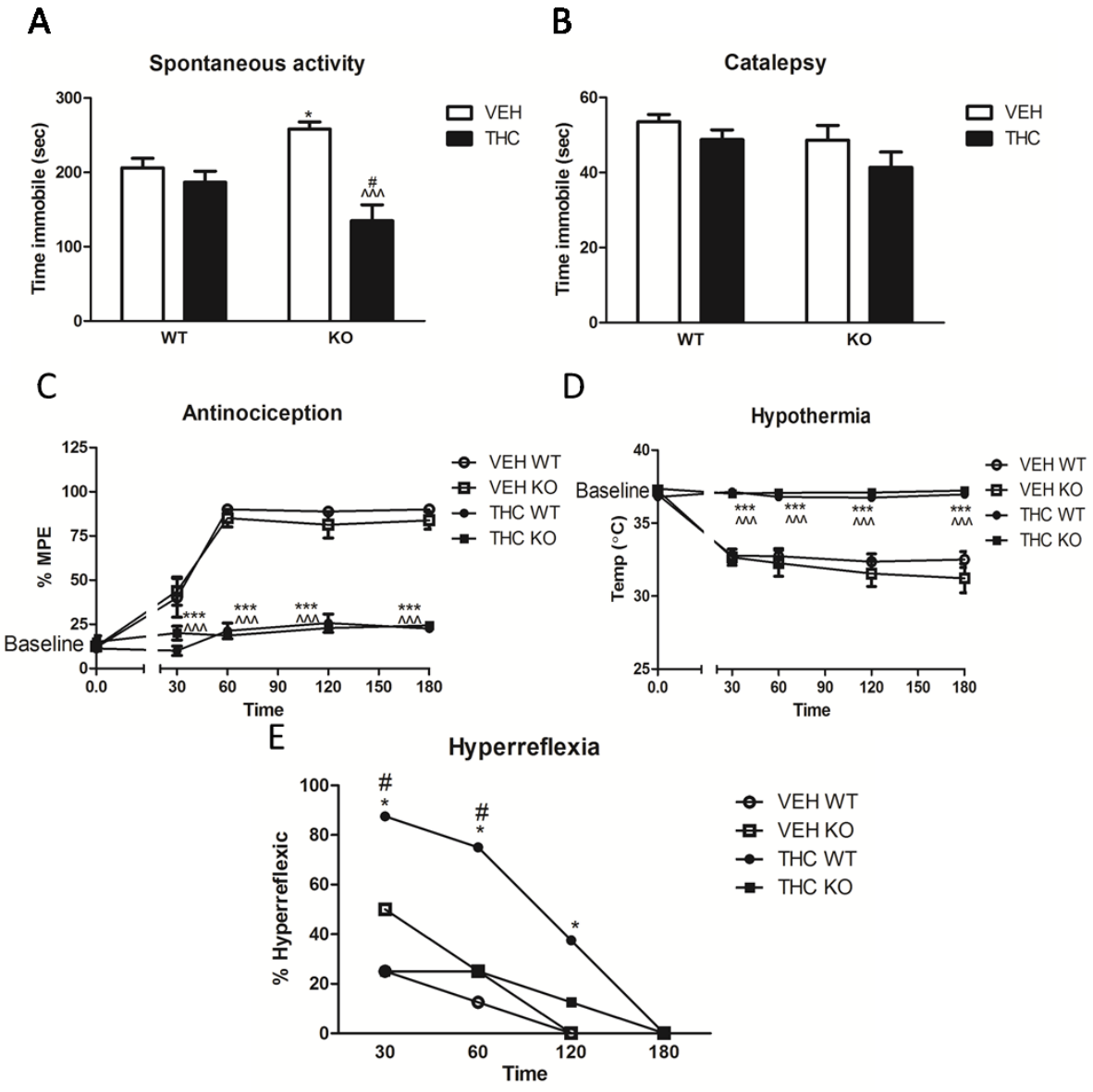


Figure 3.8 DARPP-32 knockout mice exhibited greater acute THC-mediated locomotor suppression and tolerance to THC-mediated locomotor suppression following repeated THC administration. Graphs show differences between wild type and DARPP-32 knockout mice following repeated THC administration for A) locomotor suppression, B) catalepsy, C) antinociception, D) hypothermia and E) hyperreflexia. For locomotor suppression data are presented as time immobile and catalepsy as time immobile on a bar in seconds ± SEM. Antinociception is presented as % MPE ((test latency - baseline)/(total length of test)] X 100) ±

SEM. Hypothermia presented as difference from baseline ± SEM. Hyperreflexia is represented as percent mice exhibiting hyperreflexia. For antinociception and hypothermia repeated measures ANOVA were performed with Bonferroni post-hoc test *** $p < 0.001$ compared to repeated vehicle treated wild type mice and ^^^ $p < 0.001$ compared to repeated vehicle treated DARPP-32 knockout mice. For locomotor suppression and catalepsy, two-way ANOVA was performed with Bonferroni post-hoc test * $p < 0.05$ compared to repeated vehicle treated wild type mice, ^^^ $p < 0.001$ compared to repeated THC-treated DARPP-32 knockout mice and # $p < 0.05$ compared to repeated THC-treated wild type mice. For hyperreflexia, z-tests with Bonferroni correction were performed, * $p < 0.05$ compared to repeated vehicle-treated wild type mice and # $p < 0.05$ compared to repeated THC-treated DARPP-32 knockout mice.

3.4 Discussion

The present study was conducted to investigate the role of D_1Rs and DARPP-32 in THC-mediated induction of ΔFosB and to determine the role of DARPP-32 in THC-mediated motor responses in drug naïve and THC-treated mice. A pharmacological approach was used by administering D_1R-selective antagonists, SCH23390 or SCH39166, prior to treatment with THC during the 6.5 days of treatment, and then measuring ΔFosB induction. Administration of either SCH23390 or SCH39166 blocked THC-mediated induction of ΔFosB in the prefrontal cortex, striatum, and amygdala, indicating that D_1Rs are required for THC-mediated effects on transcription via ΔFosB in these regions. Neuroanatomical studies revealed that a majority of FosB/ΔFosB positive cells in the striatum were also dynorphin positive, suggesting that ΔFosB-ir is increased mainly in D_1R-containing MSNs of the direct pathway. Studies in DARPP-32 knockout mice showed that deletion of DARPP-32 attenuated the effect of acute, but not repeated, THC on ΔFosB induction. Moreover, deletion of DARPP-32 enhanced both acute THC-mediated locomotor suppression and tolerance to this effect. Overall, these results support a role for D_1R-mediated signaling in the effects of acute and repeated THC administration.

We previously reported that THC-mediated ΔFosB induction in the caudate-putamen and nucleus accumbens was abolished in CB_1R knockout mice, demonstrating that THC induces ΔFosB in a CB_1R-dependent manner (Chapter 1). Neuroanatomical studies in which striatal sections were dual stained for CB_1Rs and ΔFosB showed that CB_1R-ir puncta surrounded ΔFosB-ir cells and also that CB_1R-ir and FosB/ΔFosB-ir were co-localized in some cells (Chapter 1). These findings suggested that activation of CB_1Rs by THC might increase ΔFosB expression both directly and via trans-synaptic events. Cannabinoids acting at CB_1Rs increase the activity of dopaminergic neurons in the substantia nigra and ventral tegmental area, leading

to increased dopamine release in the caudate-putamen and nucleus accumbens (Cheer et al., 2003; Riegel and Lupica, 2004; Wu and French, 2000). Cannabinoids can also directly modulate dopamine release at nerve terminals within the striatum (Cheer et al., 2004). Therefore, it is likely that THC-mediated dopamine release activates D_1Rs in the striatum. The results of the current study showed that THC-mediated ΔFosB induction in the striatum, as well as in the prefrontal cortex and amygdala, required D_1R activation. This finding extends our previous study by showing that D_1R antagonists also blocked ΔFosB induction in the prefrontal cortex and amygdala, which has not been shown for other drugs of abuse. Morphine-mediated induction of ΔFosB was found to be D_1R-independent in frontal cortex (Muller and Unterwald, 2005), supporting the idea that D_1Rs are involved in ΔFosB induction in non-striatal regions. Cannabinoids enhance dopamine release in the prefrontal cortex and amygdala (Polissidis et al., 2010; Polissidis et al., 2013), suggesting that CB_1R-mediated dopamine release could be a common mechanism of D_1R-mediated induction of ΔFosB in all of these forebrain regions.

CB_1Rs are located on both D_1R/dynorphin and D_2R/enkephalin MSN populations in the striatum (Hohmann and Herkenham, 2000), but pharmacological results indicate that THC induced ΔFosB primarily in D_1R/dynorphin MSNs in both the caudate-putamen and nucleus accumbens. This finding agrees with previous findings that acute THC-mediated increases in Fos-immunoreactive cells in the striatum were attenuated by administration of D_1R, but not D_2R, antagonist (Miyamoto et al., 1996). Overexpression of ΔFosB in D_1R-positive MSNs increases the rewarding properties of other drugs of abuse, including cocaine (Kelz et al., 1999) (Muschamp et al., 2012), morphine (Zachariou et al., 2006a) and naturally rewarding behaviors (Pitchers et al., 2010; Werme et al., 2002). These findings would suggest that THC-mediated motivated behaviors might also be enhanced following ΔFosB induction. THC-mediated reward

is difficult to determine in preclinical models in rodents, but has been shown in squirrel monkeys (Justinova et al., 2003; Tanda et al., 2000). THC-mediated ΔFosB induction might also enhance the effects of other psychoactive drugs. For example, nicotine self-administration (Panlilio et al., 2013) and cocaine-induced locomotor activity (Dow-Edwards and Izenwasser, 2012) were enhanced in mice that were previously exposed to THC. The current data would suggest that THC-mediated ΔFosB induction in D_1R/dynorphin MSNs is a possible mechanism underlying these observations. However, pre-exposure to THC does not increase the likelihood of self-administration of heroin (Solinas et al., 2004) or cocaine (Panlilio et al., 2007), so it is not clear whether the rewarding effects of all drugs of abuse are enhanced after pre-exposure to THC.

Previous studies have shown that administration of D_1R antagonists or genetic deletion of D_1Rs attenuated the induction of ΔFosB and other Fos family members produced by morphine or cocaine (Muller and Unterwald, 2005; Nye et al., 1995; Zhang et al., 2002). Moreover, psychomotorstimulants like cocaine and methylphenidate also induced ΔFosB in D_1R-positive striatal neurons (Hostetler and Bales, 2012; Kim et al., 2009; Nye et al., 1995). The present findings with THC support a role for D_1R-mediated ΔFosB induction with drugs that cause dopamine release within striatum. Previous studies in which SCH23390 was administered did not report a significant increase in ΔFosB induction with SCH23390 alone (Muller and Unterwald, 2005; Nye et al., 1995; Pitchers et al., 2010). This might be due to methodological differences because we pretreated twice daily, every day, whereas other studies used once-daily or intermittent drug administration. It is also possible that non-D_1R activity of SCH23390 induced ΔFosB because this effect was seen only in the nucleus accumbens and was not seen after treatment with SCH39166. SCH23390 is also a high affinity agonist for $5HT_{1c}$ (Taylor et al., 1991) and $5HT_{2c}$ (Millan et al., 2001) receptors. For example, SCH23390 blocked the

sensitization effects of 3,4-methylenedioxymethamphetamine (MDMA) via agonist activity at $5HT_{2c}$ receptors and not via D_1R antagonist properties (Ramos et al., 2005). $5HT_{2c}$ receptors are located on dopaminergic neurons of the ventral tegmental area that project to the nucleus accumbens (Bubar et al., 2011). SCH39166 is a more selective D_1R antagonist and has much lower affinity for D_2R and 5HT receptors (Alburges et al., 1992; Duffy et al., 2000; Tice et al., 1994; Wamsley et al., 1991). This might explain why SCH39166 did not significantly increase ΔFosB in the nucleus accumbens like SCH23390. The finding that SCH39166 in combination with THC increased ΔFosB expression above levels in control mice suggests that other receptors could be involved in the THC-mediated induction of ΔFosB in this region. A study that investigated THC-mediated ERK phosphorylation showed that antagonism of D_2Rs and NMDA receptors reduce ERK phosphorylation after acute THC administration, but to a lesser degree than D_1R antagonism (Valjent et al., 2001). Our data show that antagonism of D_1Rs blocked THC-mediated ΔFosB induction, but also suggest that activation of $5HT_{1/2c}$ receptors might cause induction of ΔFosB in the nucleus accumbens.

Antagonist studies showed that D_1R activation was necessary for THC-mediated induction of ΔFosB, but the signaling pathway(s) that mediate this effect has not been identified. D_1R-mediated activation of PKA leads to phosphorylation of DARPP-32 on threonine 34, which allows DARPP-32 to inhibit protein phosphatase-1 (Desdouits et al., 1995; Hemmings et al., 1984a; Kwon et al., 1997), thereby enhances the effects of PKA. Genetic deletion of DARPP-32 or point mutation of DARPP-32 at the threonine 34 site attenuates cocaine-mediated induction of ΔFosB in the nucleus accumbens but not caudate-putamen (Hiroi et al., 1999; Zachariou et al., 2006b). Results showed that deletion of DARPP-32 abolished acute THC-mediated ΔFosB induction in both the caudate-putamen and nucleus accumbens. ΔFosB is not

significantly induced following acute administration of morphine or psychostimulants (Grueter et al., 2013), but the effect of THC might be due to the large dose of THC administered (70 mg/kg) and/or the long duration of action of THC (Ashton, 2001; Whitlow et al., 2002). This difference in the pharmacokinetic properties of THC could have produced long-lasting activation of CB_1Rs and perpetuated increased expression of ΔFosB because brains were collected 24 hours after injection. Recent studies have suggested that ΔFosB might affect locomotor activity and reward-related behaviors through changes in AMPA and NMDA receptors at earlier time points than previously hypothesized (Grueter et al., 2013). Following repeated THC administration, wild type mice exhibited significant ΔFosB induction in nucleus accumbens, whereas this effect was abolished in DARPP-32 knockout mice. In contrast, THC-mediated ΔFosB induction was similar in the caudate-putamen of wild type and DARPP-32 knockout mice. These results agree with previous results showing that repeated cocaine administration in DARPP-32 knockout mice significantly increased ΔFosB expression in the caudate-putamen, but not nucleus accumbens (Hiroi et al., 1999). The finding that DARPP-32 primarily modulates acute THC- but not repeated THC-, mediated induction of ΔFosB suggests the possibility that epigenetic changes at the FosB promoter might make DARPP-32 unnecessary for further ΔFosB induction. In fact, enhanced cocaine-mediated induction of ΔFosB in cocaine-experienced animals did not depend on changes in upstream signaling factors, like ERK, which are also known to mediate ΔFosB induction (Damez-Werno et al., 2012). DARPP-32 was shown to be necessary for acute THC-mediated ERK phosphorylation in the nucleus accumbens shell (Valjent et al., 2005). ERK phosphorylation might mediate acute ΔFosB induction, but this pathway might not be necessary for ΔFosB induction after repeated THC administration. Mice with genetic deletion of Ras-GRF1, which have reduced ERK phosphorylation following D_1R activation, also exhibit

reductions in FosB/ΔFosB immunopositive cells following repeated cocaine administration in the striatum (Fasano et al., 2009). However, ERK phosphorylation was not completely blocked in Ras-GRF1 knockout mice, providing further evidence that ERK might not be necessary for ΔFosB induction following repeated drug administration.

We have previously shown an inverse correlation between ΔFosB induction and CB_1R desensitization (Chapter 1) and others have demonstrated that inhibition of PKA reduced tolerance to THC-mediated *in vivo* effects (Bass et al., 2004). Therefore, studies were performed in DARPP-32 knockout mice to determine the role that this protein might play in the development of tolerance to THC-mediated responses. Previous studies determined that mice with a mutation of DARPP-32 at the threonine 34 site that prevented its conversion to a PP1 inhibitor exhibited attenuated catalepsy following acute treatment with the cannabinoid agonist, CP55,940 (Andersson et al., 2005). Our results did not find a similar attenuation of cannabinoid-mediated catalepsy with THC. Methodological differences, as well as differences in the cannabinoid agonist administered might explain these conflicting results. The previous study used the high efficacy partial agonist CP55,940 and tested catalepsy using a tilted grid, whereas we used the partial agonist, THC, and the bar test to measure catalepsy. Additionally, the previous study used mice with a mutation at the threonine 34 site of DARPP-32, whereas mice in the current study had genetic deletion of DARPP-32. Mice also exhibited hyperreflexia until the third hour time point. At this time point, the previous study also found no difference in catalepsy. The previous authors did not report hyperreflexia, even though this response has previously been reported after CP55,940 treatment (Patel and Hillard, 2001). Following acute THC administration, DARPP-32 knockout mice did exhibit greater locomotor suppression. Furthermore, following repeated THC administration, DARPP-32 knockout mice developed

tolerance to the locomotor suppressing effects of THC whereas wild type mice did not. The lack of tolerance to the locomotor suppressing effects of THC in wild type mice was probably due to the low dose of THC administered in this study because we previously reported that mice treated with this paradigm did not exhibit desensitization in striatal regions (Chapter 1) and a one week treatment with this paradigm did not produce tolerance to this effect (McKinney et al., 2008). There were no differences in the development of tolerance to THC-mediated hypothermia or antinociception, which agrees with the expression profile of DARPP-32 because it is expressed mainly in the striatum and not in the hypothalamus or midbrain (Perez and Lewis, 1992). There was, however, a difference in the percent of mice that exhibited hyperreflexia. Vehicle-treated wild type and DARPP-32 knockout mice and THC-treated DARPP-32 knockout mice exhibited similar percentages of hyperreflexia, whereas the percentage of wild type mice that exhibited hyperreflexia following repeated THC administration was significantly higher. This is an interesting finding because hyperreflexia has been associated with activation of CB_1Rs in the cerebellum and dopamine agonists do not attenuate this effect (Patel and Hillard, 2001). The cerebellum does not contain dopaminergic projections, but DARPP-32 has been detected in this region (Schalling et al., 1990). Future studies are necessary to determine the role that DARPP-32 may play in mediating this effect and whether it is cerebellar-mediated.

These studies demonstrate a neurochemical commonality between THC and other drugs of abuse, such as cocaine and morphine, where ΔFosB induction is blocked by antagonism of D_1Rs and ΔFosB induction is primarily restricted to the D_1R/dynorphin MSNs of striatum. This similarity in the action of these drugs of abuse suggests that future therapeutic targets targeting these systems could be effective in treating polydrug use. We also found that antagonism of D_1Rs blocks THC-mediated induction of ΔFosB in the prefrontal cortex, where changes in this

region are thought to contribute to the loss of control of drug intake in addicts (Goldstein and Volkow, 2011), and the amygdala, which is proposed to mediate drug reinstatement (Stamatakis et al., 2013). In the striatum, DARPP-32 appeared to mediate acute induction of ΔFosB by THC while it has a diminished role in mediating ΔFosB induction following repeated THC administration. This suggests that different mechanisms are responsible for the acute induction of ΔFosB compared to induction of ΔFosB following repeated THC administration in striatum. DARPP-32 also plays a role in reducing tolerance to THC-mediated locomotor suppression, a behavior that is known to be resistant to tolerance in humans (D'Souza et al., 2008), suggesting that this protein could be targeted to enhance tolerance to this side-effect.

Chapter 4: Brain region-dependent differences in ΔFosB signaling following THC-challenge in THC-experienced versus drug naïve mice

4.1 Introduction

Long-term drug use produces physiological changes that are not present upon initial drug use. Some of these changes are due to the induction of transcription factors that can control multiple genes (Lazenka et al., 2013), thus altering signaling. One transcription factor thought to mediate these physiological changes is ΔFosB, a stable splice variant of FosB that is typically induced after repeated drug administration. Recent studies have determined that repeated administration of THC, the main psychoactive constituent of marijuana, induces ΔFosB in the prefrontal cortex, caudate-putamen, nucleus accumbens, amygdala and cerebellum (Perrotti et al., 2008) (Chapter 1). ΔFosB has been implicated in mediating the rewarding effects of drugs of abuse through transcriptional regulation of specific target genes (McClung and Nestler, 2003; Perrotti et al., 2008).

Since ΔFosB has a long half-life in neurons and is stable for weeks (Ulery-Reynolds et al., 2009; Ulery et al., 2006), it is proposed that it can mediate the long-term changes associated with drugs of abuse (Nestler et al., 2001). Studies in mice that received repeated cocaine administration or had genetic overexpression of ΔFosB have found that ΔFosB regulates the expression of several target genes including cyclin dependent kinase 5 (CDK5), the neuronal-specific activator of CDK5 (p35) and calmodulin-dependent protein kinase II (CAMKII) (Bibb et al., 2001a; McClung and Nestler, 2003). The expression of some of these proteins has been examined in humans, where post-mortem studies found that both ΔFosB and CAMKII were increased in the nucleus accumbens of cocaine users (Robison et al., 2013).

The long-term changes that occur following prolonged drug include increased dendritic spine formation and other cytoskeletal-dependent changes that are mediated by CDK5 (Dhavan and Tsai, 2001; Norrholm et al., 2003), and regulation by CDK5 is dependent on its coactivators: p35 and p39 (Ko et al., 2001). CDK5 produces cytoskeletal changes partly through direct phosphorylation of the microtubule associated protein, tau (Baumann et al., 1993), but also indirectly through phosphorylation of glycogen synthase kinase-3β (GSK3β) (Morfini et al., 2004), which also phosphorylates tau. CDK5 can also alter the function of the dopamine- and cAMP-regulated neuronal phosphoprotein of 32 kDA (DARPP-32) in striatal neurons by phosphorylating DARPP-32 at threonine 75 (Bibb et al., 1999). Phosphorylation at this site attenuates PKA activity and reduces dopamine type 1 receptor (D_1R) signaling (Bibb et al., 2001b). In contrast to repeated cocaine administration, acute cocaine increases phosphorylation of DARPP-32 at threonine 34 (Zachariou et al., 2006b), which enhances PKA activity. Acute administration of THC also increases phosphorylation of DARPP-32 at threonine 34 (Borgkvist et al., 2008), although levels return to baseline within one hour.

Finally, epigenetic changes play a role in long-term adaptation to prolonged drug exposure, through either enhancement of repression of gene promoters. Epigenetic changes that occur with repeated cocaine administration include changes at the DNA level through either methylation/demethylation of the C5 position of cytosines located in CpG islands or acetylation/deacetylation and methylation at histones at the promoters of genes (Anier et al., 2010; Nestler, 2013; Robison and Nestler, 2011). The following studies investigated whether there are brain region-dependent differences in the regulation of these signaling proteins following either acute or repeated THC administration. Further, it was determined whether THC

administration in mice with prior THC experience regulates these proteins differently than THC in drug naïve mice.

4.2 Materials and Methods

Materials

THC was received from the same source as in Chapter 1. The antibodies used are listed in Table 4.1. The same secondary antibodies were used as reported in Chapter 1. For RT-qPCR studies, the High Capacity cDNA Reverse Transcription Kit was purchased from Applied Biosystems Inc. (Foster City, CA) and the 2x QuantiFast® SYBR® Green PCR kit was purchased from Qiagen (Valencia, CA). All other reagent grade chemicals were obtained from Sigma Chemical Co. or Fisher Scientific.

TABLE 4.1

List of antibodies used for immunoblot studies

Antibody (animal)	Company	Dilution
α-tubulin (mouse, ab7291)	Abcam	1:20000
FosB (rabbit, sc-7203)	Santa Cruz Biotechnology	1:500
CDK5 (rabbit, sc-173)	Santa Cruz Biotechnology	1:2000
p35/p25 (rabbit)	Cell Signaling Technology	1:1000
Total ERK1 (rabbit)	Cell Signaling Technology	1:2000
pERK1 (mouse)	Cell Signaling Technology	1:2000
DARPP-32 (mouse)	Santa Cruz Biotechnology	1:2000
pT34DARPP-32 (rabbit)	Cell Signaling Technology	1:1000
pT75DARPP-32 (rabbit)	Cell Signaling Technology	1:1000
pGSK3β(mouse)	Cell Signaling Technology	1:1000
pTau (AT8, mouse)	Pierce Scientific	1:500

Drug Treatments

Male C57Bl/6J mice (Jackson Laboratories, Indianapolis, IN) 8 weeks old were used for all treatments. Mice were housed four to six per cage and maintained on a 12-hr light/dark cycle

in a temperature controlled environment (20-22°C) with food and water available ad libitum. THC (10 mg/kg) was dissolved in a 1:1:18 solution of ethanol, emulphor and saline (vehicle). Mice were injected subcutaneously with either vehicle (VEH) or THC at 07:00 and 16:00 h for 13 days. On the morning of day 14, both vehicle- and THC-treated groups of mice were divided in half and received either vehicle or 10 mg/kg THC injection to produce 4 groups: VEH-VEH, VEH-THC, THC-VEH and THC-THC. Brains were extracted 45 minutes after the final injection and dissected into appropriate regions for immunoblots (n = 8 mice per group) or RT-qPCR (n= 5-6 mice per group). The 45 minute time point was chosen because DARPP-32 phosphorylation at threonine 34 returns to baseline within one hour (Borgkvist et al., 2008) and FosB/ΔFosB mRNA is maximally induced by this time point (Damez-Werno et al., 2012). All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health guide for the care and use of Laboratory animals 7th edition.

Dissections

Regions of interest were dissected from fresh whole brains as described in Chapter 1 for immunoblots. For the globus pallidus, a cut was made directly anterior to the optic chiasm and directly posterior to the optic chiasm. The globus pallidus was isolated by removing the tissue bordered laterally by the caudate-putamen and internal capsule and dorsally by the ventral pallidum. The substantia nigra was dissected by making a first cut rostral to the mammillary bodies and a second cut rostral to the cerebellar peduncles, and then collecting tissue from the ventral aspect of the section located lateral to the mammillary bodies and ventral tegmental area and parabrachial pigmented nucleus. For RT-qPCR, regions of interest were dissected as described in Chapter 1.

Immunoblots

Immunoblots were performed as described in Chapter 1.

Real time quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from brain tissue immersed in Trizol® and were homogenized using a Powergen 125 homogenizer (Fischer Scientific). RNA (5 µg) was then converted into cDNA using a High Capacity cDNA Reverse Transcription Kit. cDNA (10 ng) was then added to 0.2 ml wells containing a master mix from the 2x QuantiFast® SYBR® Green PCR kit and specific primers at a final concentration of 0.4 µM and water was added to a final volume of 25 µl. Additional wells with no cDNA added served as no template controls (NTC) for each primer set. Samples were placed in a BioRad real-time thermocycler programed to a 2-step cycling protocol, followed by a melt curve step at the end of the reaction. Cycle threshold (Ct) values were initially normalized to ΔCt values by subtracting sample Ct values from β-actin Ct values. Data were further converted to ΔΔCt values and final mRNA quantification was calculated using the following equation: 2^(- ΔΔCt) x 100 = % mRNA expression. Primers described previously (Alibhai et al., 2007) for FosB and ΔFosB were used: FosB: Forward 5'-GTGAGAGATTTGCCAGGGTC-3' and Reverse 5'-AGAGAGAAGCCGTCAGGTTG-3', and ΔFosB: Forward 5'-AGGCAGAGCTGGAGTCGGAGAT-3' and Reverse 5' GCCCGAGGACTTGAACTTCACTCG-3'. Primers described previously for CDK5 (Hawasli et al., 2007) were used: Forward 5'-GGCTAAAAACCGGGAAACTC-3' and Reverse 5'-CCATTGCAGCTGTCGAAATA-3' A previously described β-actin primer (Grimaldi and Capasso, 2012) was also used: Forward 5'-TGTTACCAACTGGGACGA-3' and Reverse 5'GTCTCAAACATGATCTGGGTC-3'.

Data Analysis

For all experiments, data were analyzed with Prism® version X (GraphPad Software, San Diego, CA). For immunoblots and RT-qPCR, one-way ANOVAs were performed with Bonferroni post-hoc test. Significance was determined with $p < 0.05$. All one-way ANOVA data are normalized to the VEH-VEH group and presented as % VEH controls ± SEM. For comparisons of net differences from repeated treatment, data were first normalized to the VEH-VEH group and values calculated as: VEH-THC – VEH-VEH and THC-THC – THC-VEH. Significance for these data was determined with Student's t-tests with $p < 0.05$ as significance.

4.3 Results

THC administration increases ΔFosB expression in the prefrontal cortex, nucleus accumbens and caudate-putamen.

We have previously shown that repeated THC treatment induced ΔFosB when measured 24 hours after the last drug injection (Chapter 1). However, the effect of previous treatment with THC on acute THC-mediated ΔFosB induction has not been determined. Therefore, studies were conducted to determine whether ΔFosB is induced by a single injection of THC and whether previous repeated THC treatment alters that response. Repeated vehicle- (drug naïve) or THC- (THC-experienced) treated mice received a final injection of either vehicle or THC and brains were collected 45 minutes after injection to measure ΔFosB. No significant differences were found in the nucleus accumbens using one-way ANOVA, but post-hoc test determined that ΔFosB-ir was significantly different in THC-THC compared to VEH-VEH (increased by 36% ± 13% compared to VEH-VEH; $p < 0.05$, Figure 4.1 B) treated mice. One-way ANOVA in the

caudate-putamen showed a significant effect of treatment ($F_{3, 28} = 5.548$ $p < 0.01$). ΔFosB-ir was significantly increased by 46% ± 12% ($p < 0.01$, Figure 4.1 C) in THC-VEH compared to VEH-VEH-treated mice. ΔFosB-ir in VEH-THC-treated mice was also significantly different from values in VEH-THC-treated mice ($p < 0.05$, Figure 4.1 C). One-way ANOVA determined a significant difference in ΔFosB-ir between groups in the prefrontal cortex ($F_{3,28} = 8.116$, $p < 0.001$). ΔFosB-ir was significantly increased by 66% ± 6% in THC-THC-compared to VEH-VEH-treated mice ($p < 0.001$, Figure 4.1 A). ΔFosB-ir in THC-THC treated mice was also significantly different from levels in mice that received VEH-THC ($p < 0.01$, Figure 4.1 A) or THC-VEH ($p < 0.05$, Figure 4.1 A) treatment. There was no significant change in ΔFosB-ir following acute or repeated THC administration in the hippocampus, consistent with our previous studies (Figure 4.1 D). These results suggest that while ΔFosB is not induced by acute THC administration, ΔFosB-ir is increased following repeated THC administration. Further, ΔFosB induction following THC-challenge in THC-experienced animals is enhanced compared to a single administration of THC in naïve animals in the nucleus accumbens and prefrontal cortex.

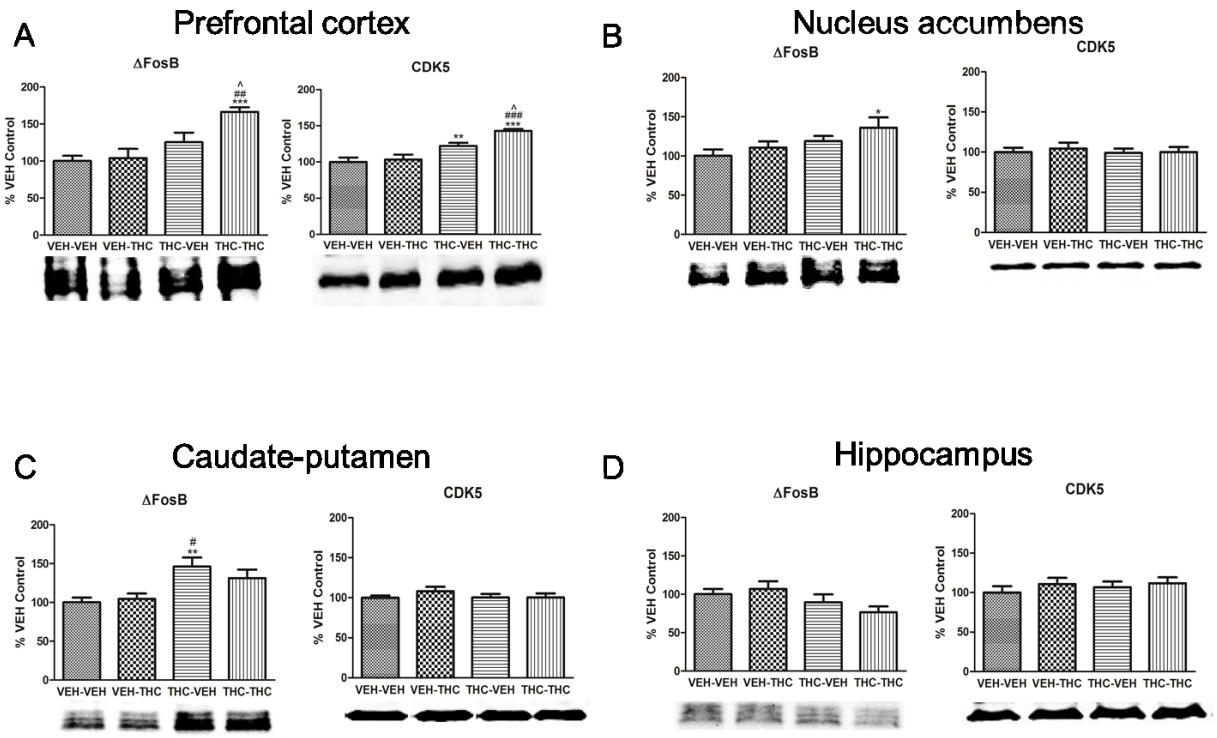


Figure 4.1 ΔFosB expression is increased following repeated THC administration in the prefrontal cortex, nucleus accumbens, caudate-putamen but CDK5 expression is only increased in the prefrontal cortex. Graphs representing ΔFosB-ir and CDK5-ir expressed as percent expression in VEH-VEH-treated control mice for (A) prefrontal cortex (B) nucleus accumbens (C) caudate-putamen and (D) hippocampus. Values are represented as % VEH-VEH controls ± SEM. Significance was determined with one-way ANOVA and Bonferroni post-hoc test * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ compared to VEH-VEH controls. # $p < 0.05$ and ## $p < 0.01$ compared to VEH-THC treated mice. ^ $p < 0.05$ compared to THC-VEH treated mice. N = 8 per group

THC administration enhances CDK5 expression in the prefrontal cortex

CDK5 has been identified as a downstream target of ΔFosB following induction by cocaine treatment, but the effect of THC treatment on CDK5 is not known. Therefore, CDK5 was measured by immunoblot in the same brain regions of the four treatment groups.

In contrast to the results with ΔFosB, there were no significant differences in CDK5-ir between any treatment groups in the nucleus accumbens (Figure 4.1 B) or caudate-putamen (Figure 4.1 C). There were also no significant differences in the expression of CDK5-ir between treatment groups in the hippocampus (Figure 4.1 D). Results in the prefrontal cortex showed a significant effect of treatment on CDK5-ir between treatment groups ($F_{3,28} = 11.59$, $p < 0.001$). CDK5-ir was significantly increased by 43% ± 3% in THC-THC-treated mice compared to VEH-VEH- ($p < 0.001$, Figure 4.1 A), VEH-THC- ($p < 0.001$) and THC-VEH-treated ($p < 0.05$) mice. CDK5-ir was also significantly increased by 22% ± 4% in THC-VEH compared to VEH-VEH- treated mice ($p < 0.05$, Figure 4.1 A). These results suggest there are brain region-dependent differences in ΔFosB-mediated regulation of CDK5 following repeated THC-administration.

Levels of FosB, ΔFosB and CDK5 mRNA and proteins differ depending on THC experience

The finding that both ΔFosB and CDK5 were increased after acute THC injection in repeated THC-treated mice suggests that these changes occur at either the level of transcription or translation. To address whether these effects occur at the level of transcription, mRNA levels of ΔFosB and CDK5 were measured in the prefrontal cortex. Because ΔFosB is a splice variant of FosB, experiments were first conducted to determine whether FosB protein is also regulated by THC. Results showed no significant difference using one-way ANOVA, but post-hoc test determined that FosB-ir was significantly increased in VEH-THC- as compared to VEH-VEH-

treated mice (79% ± 22% increase compared to VEH-VEH; $p < 0.01$, Figure 4.2 A). At the mRNA level, acute THC administration increased FosB mRNA levels by 96% ± 36% compared to VEH-VEH-treated mice ($F_{3,18} = 3.384$ $p < 0.05$, Figure 4.2 B). In contrast to FosB mRNA levels, ΔFosB mRNA levels were not increased with acute THC administration but were increased in THC-THC-treated mice (50% ± 21% compared to VEH-VEH-treated mice ($F_{3,18} = 5.0126$ $p < 0.05$, Figure 4.2 C). CDK5-ir was also enhanced in the prefrontal cortex after THC injection in VEH-treated mice, so CDK5 mRNA levels were also assessed following THC administration. Comparisons between treatment groups found no significant differences in CDK5 mRNA levels. These results suggested similar differences between protein changes and mRNA for FosB and ΔFosB depending on the drug experience of the animals. FosB mRNA/protein (Figure 4.3 A) were increased after THC injection in VEH-treated mice and ΔFosB mRNA/protein (Figure 4.3 B) were increased after THC injection in THC-treated mice. Comparisons of CDK5 mRNA/protein were not similar, however, a comparison of CDK5 mRNA expressed as a net difference from either repeated vehicle or repeated THC showed that CDK5 mRNA expression significantly differed depending on the drug experience of the animal. Mice that received THC challenge following repeated vehicle treatment (VEH-THC) had a decrease of 19% ± 16% in CDK5 mRNA, while mice that received THC-challenge following repeated THC administration (THC-THC) had an increase of 31% ± 14% in CDK5 mRNA ($p < 0.05$, $df = 9$, Figure 4.3 C). Although there was no significant difference between CDK5-ir following these treatments, there was a trend towards increased CDK5 expression ($p = 0.058$).

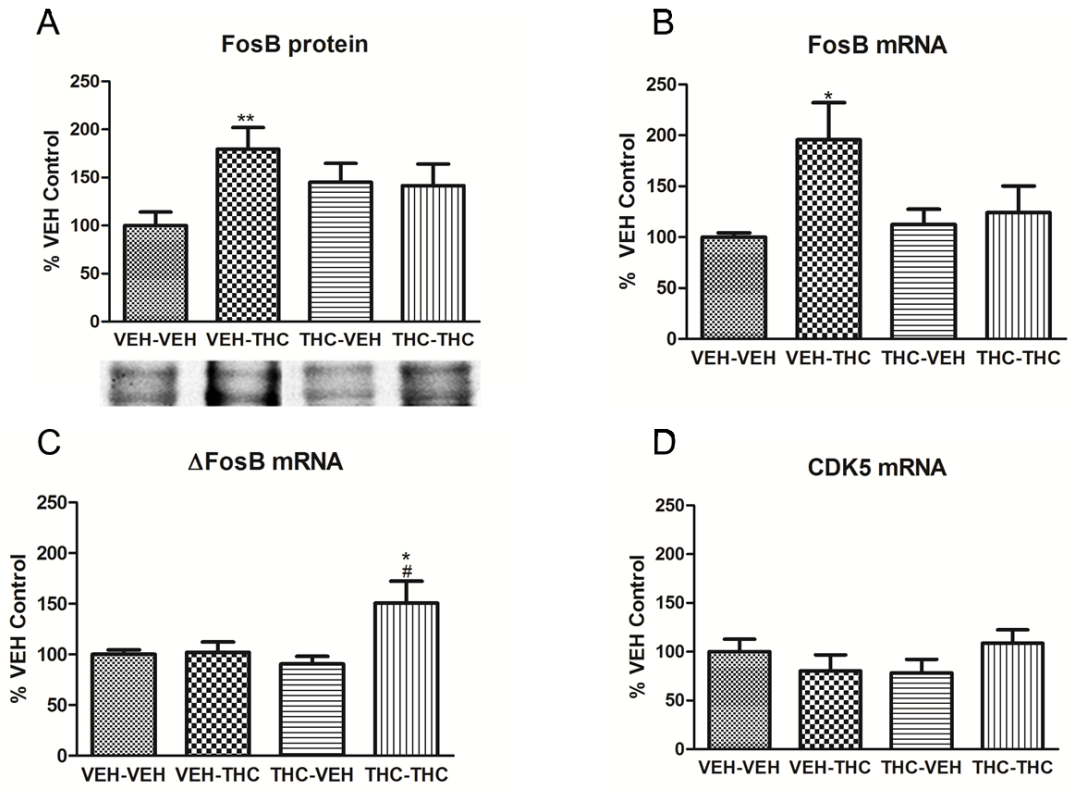


Figure 4.2 FosB mRNA/protein is increased in the prefrontal cortex in VEH-THC treated mice, whereas ΔFosB mRNA is increased following THC-THC treatment. (A) Graph representing FosB-ir in the prefrontal cortex as percent expression in VEH-VEH-treated controls ± SEM. Graphs representing mRNA levels in prefrontal cortex expressed as VEH-VEH-treated controls ± SEM for (B) FosB, (C) ΔFosB and (D) CDK5. A: One-way ANOVA with Bonferroni post-hoc test ** $p < 0.01$, N = 8 per group. N = 5-6 for mRNA, N = 8 for protein.

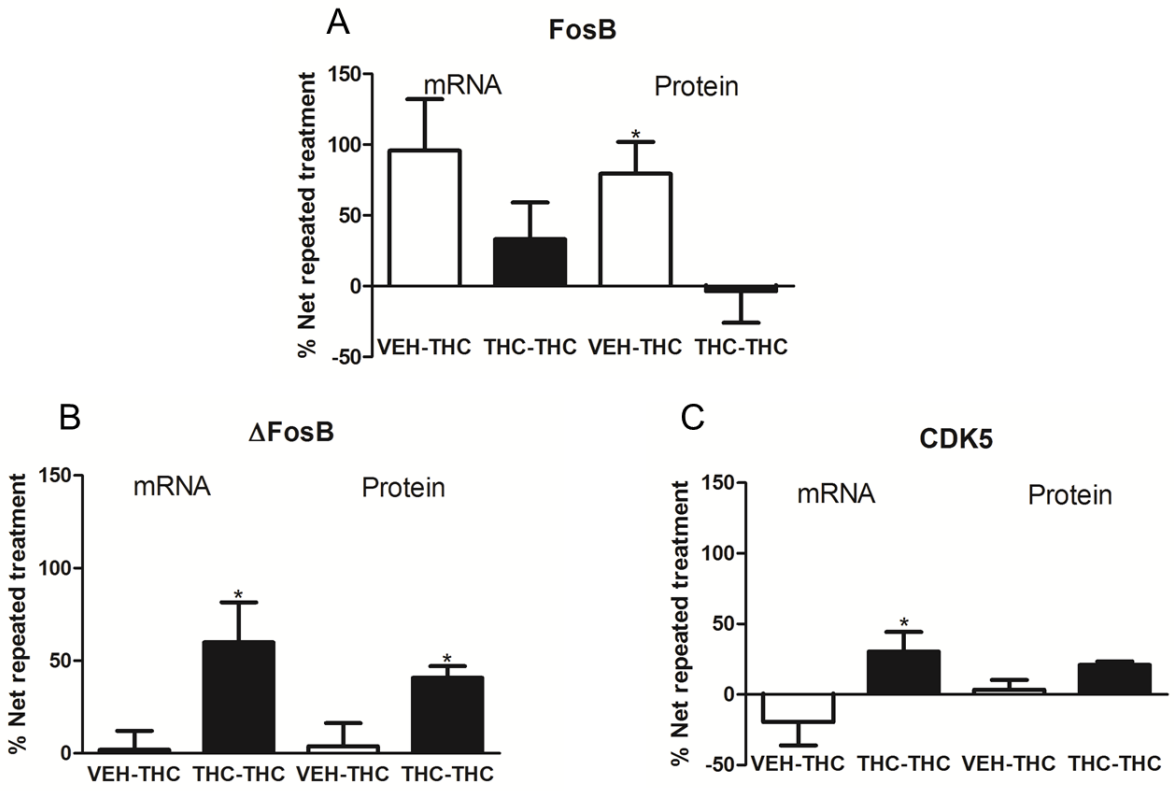


Figure 4.3 ΔFosB mRNA/protein is enhanced in prefrontal cortex following THC-challenge in THC-experienced mice. Comparisons were made between mRNA/protein expression following THC-challenge in both drug naïve and THC-experienced mice in prefrontal cortex for (A) FosB, (B) ΔFosB and (C) CDK5. Data presented as the net difference in mRNA/protein expression for mice that received THC-challenge following repeated vehicle treatment (VEH-THC) and THC-challenge in following repeated THC treatment (THC-THC). Student's t-test * $p < 0.05$ compared to net repeated treatment in VEH-THC group.

Increased phosphorylation of ERK1, but not DARPP-32, occurs in the prefrontal cortex following THC administration in THC-experienced mice

The enhanced induction of ΔFosB found in the prefrontal cortex following THC injection in repeated THC-treated mice could occur due to changes in signaling proteins upstream of ΔFosB. ΔFosB induction can be regulated by phosphorylation of ERK1 at Thr202/Tyr204 and/or DARPP-32 at threonine 34. Phosphorylation of ERK1 and DARPP-32 was determined by measuring phosphorylation levels/ total protein levels. ERK1 phosphorylation was significantly increased by 53% ± 10% in the prefrontal cortex of THC-THC-treated mice compared to VEH-VEH-treated mice ($p < 0.05$, Figure 4.4), but there was no significant difference in ERK1 phosphorylation in VEH-THC- compared to VEH-VEH-treated mice. Phosphorylation of DARPP-32 at threonine 34 did not significantly differ between any treatment groups in the prefrontal cortex (Table 4.2). Phosphorylation of ERK1 and DARPP-32 was also determined in the caudate-putamen, a region in which THC-THC treatment did not enhance ΔFosB induction. There was no significant change in the phosphorylation of either ERK1 (data not shown) or DARPP-32 at threonine 34 (Table 4.2) for any treatment condition. These results suggest that enhanced ΔFosB induction in the prefrontal cortex could be mediated by phosphorylation of ERK1.

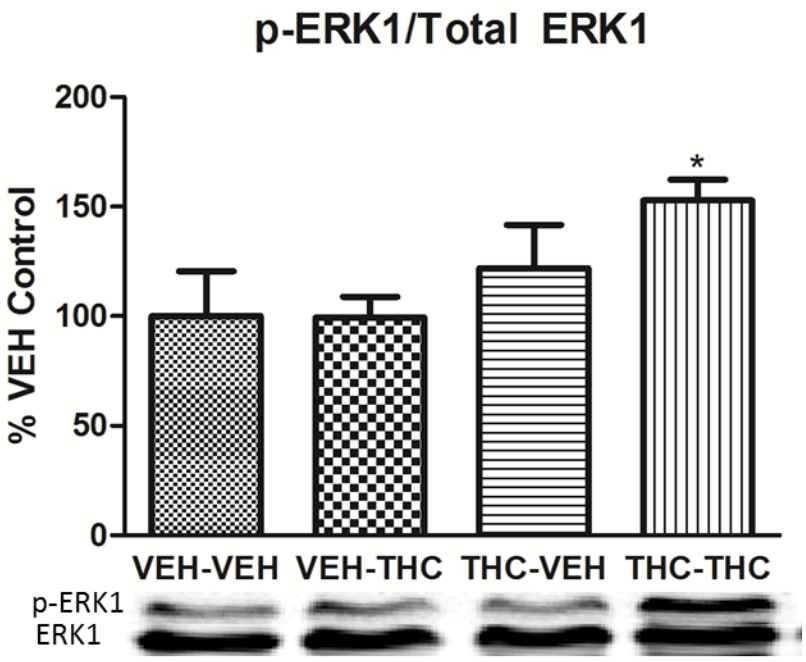


Figure 4.4 ERK1 phosphorylation is significantly increased in prefrontal cortex following THC-challenge in THC-experienced mice. Graph representing pERK1-ir/Total ERK-ir in the prefrontal cortex as percent expression in VEH-VEH-treated controls ± SEM. One-way ANOVA followed by Bonferroni post-hoc test, * $p < 0.05$. N = 8 per group

Expression of p35/p25 and tau phosphorylation are increased in the prefrontal cortex following THC injection in THC-experienced mice

CDK5 phosphorylates several targets, including DARPP-32 at threonine 75 (Bibb et al., 1999), tau protein at Ser202/Thr205 (Hashiguchi et al., 2002) and GSK3β at Ser9 (Morfini et al., 2004) when it is dimerized with either p35 or its cleaved form, p25. Therefore, increases in CDK5, p35 and p25 could lead to an increase in phosphorylation of these proteins. In the prefrontal cortex, significant differences in p35-ir were found between treatment groups ($F_{3,28}$ = 7.196, $p < 0.01$, Figure 4.5 A). Expression of p35 was significantly increased by 21% ± 6% in THC-THC-treated compared to VEH-VEH-treated ($p < 0.001$, Figure 4.5 A) or VEH-THC-treated ($p < 0.01$, Figure 4.5 A) mice. Expression of p35 was also significantly increased by 14% ± 2 % ($p < 0.05$, Figure 4.5 A) in THC-VEH- compared to VEH-VEH-treated mice. Based on these results, levels of p25, the cleavage product of p35, were measured. For p25, although there were no significant differences by one-way ANOVA, post-hoc test determined that THC-VEH-treated mice had significantly increased p25-ir (33% ± 9 %, $p < 0.05$, Figure 4.5 B) compared to VEH-VEH-treated mice. THC-THC-treated mice also had significantly increased p25 expression (29% ± 8%, $p < 0.05$, Figure 4.5 B) compared to VEH-VEH-treated mice.

Studies were then conducted to determine whether increased expression of CDK5 and p35/p25 occurred in conjunction with changes in the phosphorylation of target proteins in the prefrontal cortex. Phosphorylation of the Ser202/Thr205 site of tau was significantly increased by 33% ± 8% in THC-THC-treated mice compared to VEH-VEH-treated mice ($p < 0.05$, Figure 4.5 C). There were no other significant differences in the phosphorylation of tau between groups. Phosphorylation of the Ser9 site of GSK3β was significantly decreased by 38% ± 6 % and 38% ± 5% in THC-VEH ($p < 0.001$, Figure 4.5 D) and THC-THC ($p < 0.001$, Figure 4.5 D), treated

mice, respectively, compared to VEH-VEH-treated mice. This suggests that repeated THC administration decreased phosphorylation of GSK3β at Ser9 and that these levels remained decreased after THC-challenge. There were no significant differences in the phosphorylation of DARPP-32 at threonine 75 for any treatment (Table 4.3). These data show that increased CDK5 expression is associated with increased phosphorylation of tau in THC-THC-treated mice, whereas decreased phosphorylation of GSK3β was found in THC-VEH- and THC-THC-treated mice.

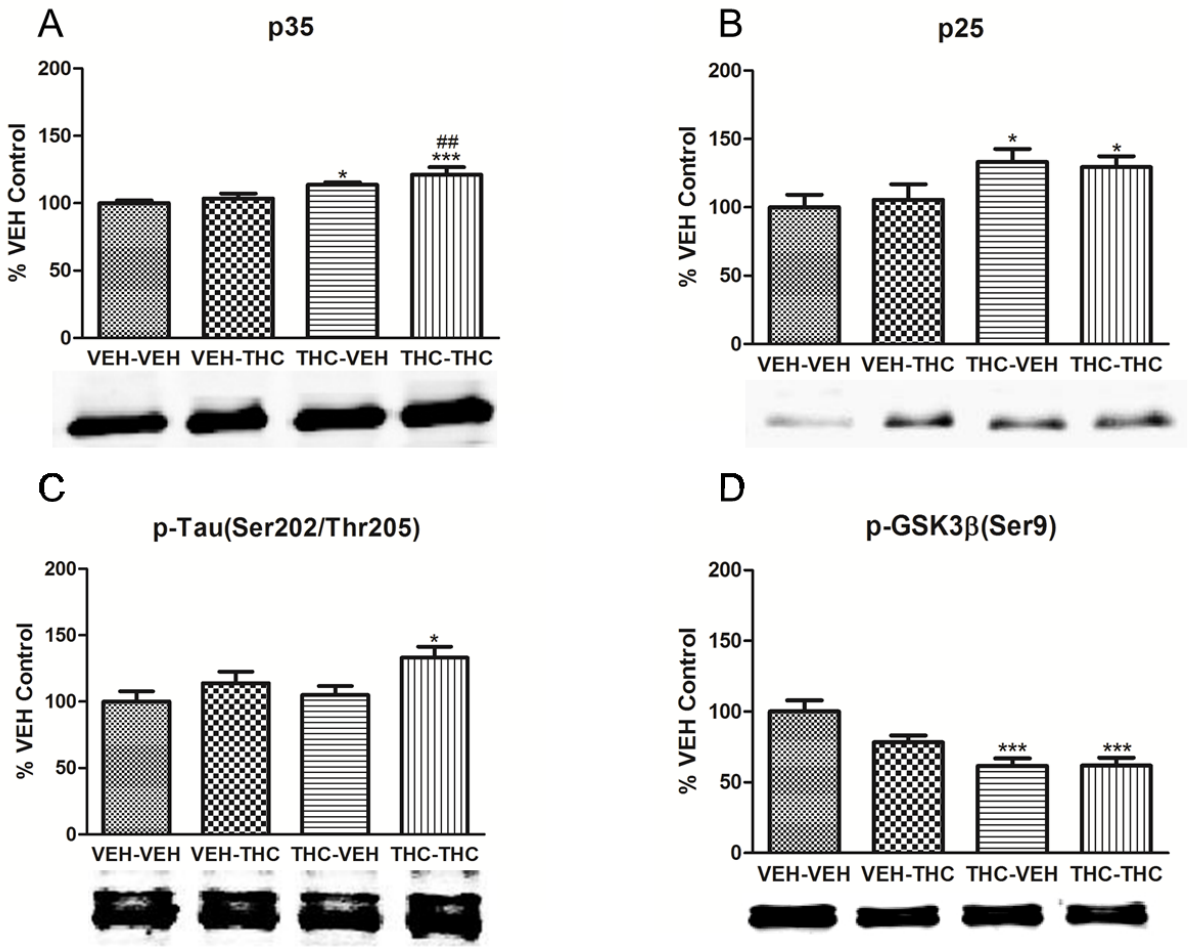


Figure 4.5 p35 and p25 expression are increased in the prefrontal cortex following repeated THC administration whereas pTau is increased and pGSK3 is decreased. (A) p35 expression (B) p25 expression (C) phosphorylation of tau at Serine 202/Threonine 205 and (D) GSK3β phosphorylation at serine 9. Values represented as % VEH-VEH controls ± SEM. One-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$ and ***$p < 0.001$ compared to VEH-VEH controls. ## $p < 0.01$ compared to THC-VEH administration. N = 8 per group

Expression of p35 is reduced in the caudate-putamen and substantia nigra of THC-THC-treated mice

Analysis of signaling proteins related to ΔFosB and CDK5 in the prefrontal cortex showed that expression of p35/p25 could be regulated by THC treatment. In order to fully assess these signaling pathways and determine the regional profile of THC-mediated regulation of these pathways, expression of p35/p25 was measured in additional forebrain regions. Expression of DARPP-32 was also assessed because D_1Rs in these regions are required for THC-mediated ΔFosB induction (Chapter 3). In the nucleus accumbens, there were no significant differences between any of the treatment groups for expression of p35, p25 (Figure 4.6 A) or phosphorylation of DARPP-32 at either threonine 34 or threonine 75 (Table 4.2 and 4.3). In the caudate-putamen, one-way ANOVA determined a significant difference ($F_{3, 28} = 3.108$, $p < 0.05$) in p35-ir between the VEH-THC (increased by 26% ± 15%) and THC-THC (decreased by 15% ± 5%) compared to VEH-VEH-treated mice ($p < 0.05$, Figure 4.6 B). Expression of p35 in THC-THC-treated mice was also significantly different ($p < 0.05$, Figure 4.6 B) from THC-VEH-treated mice (17% ± 9% increase compared to VEH-VEH-treated mice). There were no significant differences in p25 levels between any of the groups tested. There were no significant differences in the phosphorylation of DARPP-32 at either the threonine 34 or threonine 75 site between any of the groups in the caudate-putamen (Table 4.2 and 4.3). Levels of p25 and p35 were also measured in the hippocampus, but there were no significant differences in expression between the treatment groups (Figure 4.6 C). Expression of DARPP-32 was not detectable in the hippocampus with 50µg of total protein loaded. Overall, these results show that p35 is regulated by THC treatment only in the caudate-putamen and that DARPP phosphorylation is not affected by these THC treatments in the nucleus accumbens or caudate-putamen.

Results in the caudate-putamen showed that ΔFosB and p35 were regulated by THC treatment, although the other proteins examined were not affected. CB_1Rs on striatal MSNs are predominantly expressed on axon terminals in the globus pallidus and substantia nigra, suggesting that THC-mediated regulation of signaling might occur in these projection regions. ΔFosB expression was not assessed because the globus pallidus and substantia nigra contain primarily efferent projections from the caudate-putamen and not the cell bodies of origin where FosB would be expressed. In the globus pallidus, there were no significant differences in CDK5-ir, p35-ir or p25-ir (Figure 4.7 A) between the treatment groups. There were also no significant differences in CDK5-ir in the substantia nigra of any of the THC-treated groups (Figure 4.7 B). For p35-ir in substantia nigra, there was no significant difference by one-way ANOVA, but post-hoc test determined a significant difference between THC-VEH- (26% ± 15% increase) and THC-THC-treated (17% ± 3% decrease) compared to VEH-VEH-treated mice ($p < 0.01$, Figure 4.7 B). For p25, one-way ANOVA showed a significant difference between treatments ($F_{1,28} = 3.507$ $p < 0.05$, Figure 4.7 B), and post-hoc test determined a significant difference between VEH-THC- (13 ± 10% decrease) and THC-VEH-treated mice (27% ± 11% increase) compared to VEH-VEH-treated mice. There were no significant differences in the phosphorylation of DARPP-32 at either the threonine 34 or threonine 75 site in the globus pallidus or substantia nigra (Table 4.2 and 4.3). These results showed that none of the proteins examined was regulated by THC in the globus pallidus. However, repeated THC treatment with THC injection reduced p35-ir in the substantia nigra, which is similar to results in the caudate-putamen. In the substantia nigra, expression of p25 was regulated differently following a single injection of THC compared to repeated THC administration.

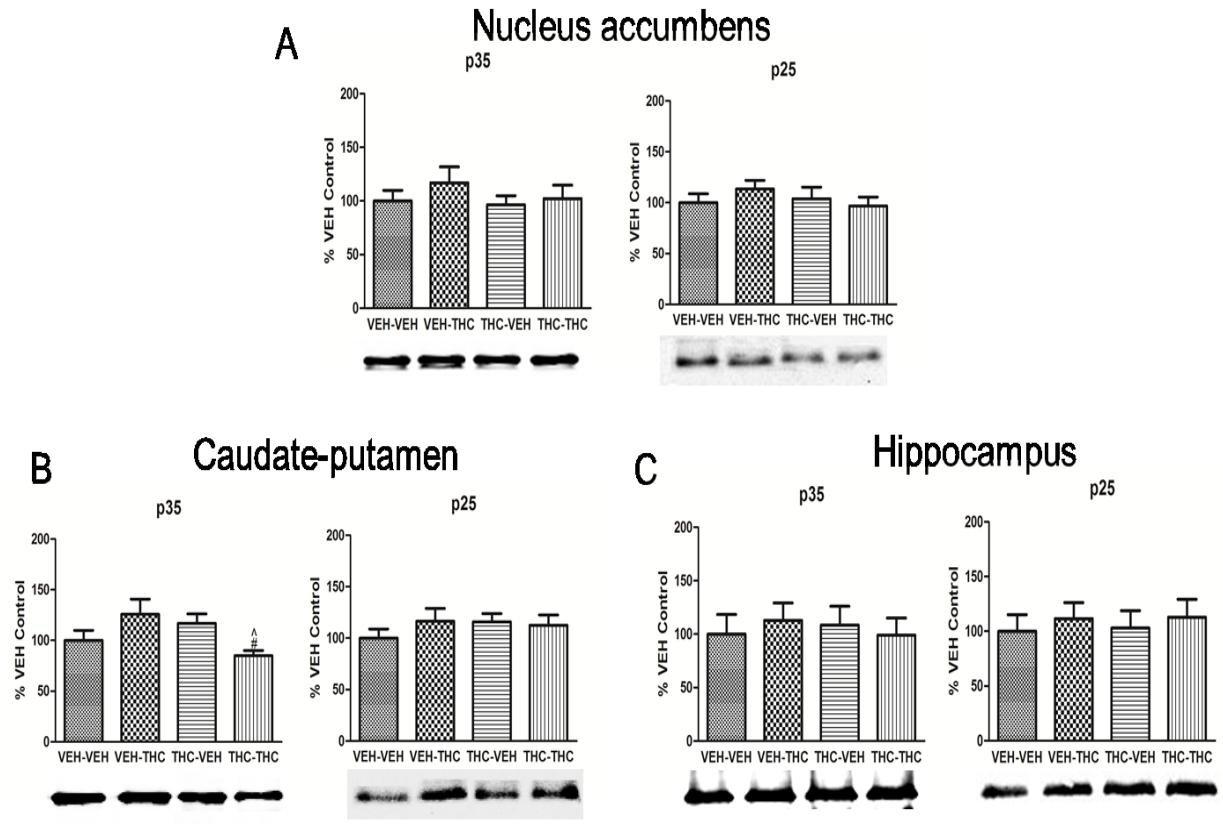


Figure 4.6 p35 expression is reduced in the caudate-putamen of THC-THC-treated mice. Graphs representing p35-ir and p25-ir as percent expression in VEH-VEH-treated controls ±SEM for (A) nucleus accumbens (B) caudate-putamen and (D) hippocampus. Values are represented as % VEH-VEH controls ± SEM. Significance was determined with one-way ANOVA and Bonferroni post-hoc test. # $p < 0.05$ compared to THC-VEH administration. ^ $p < 0.05$ compared to THC-VEH administration. N = 8 per group

THC challenge in THC-experienced mice decreases phosphorylation of DARPP-32 at threonine 34 in cerebellum

For ΔFosB, there was a significant difference between treatment groups in the cerebellum based on one-way ANOVA ($F_{1,28} = 14.98$, $p < 0.001$). Post-hoc test determined a significant increase in ΔFosB-ir in THC-VEH- (36% ± 4%, $p<0.01$, Figure 4.7 C) and THC-THC-treated mice (58% ± 7%, $p<0.001$, Figure 4.7 C) compared to VEH-VEH-treated mice. ΔFosB expression was also significantly increased in THC-VEH-treated ($p < 0.05$, Figure 4.7 C) and THC-THC-treated mice ($p < 0.001$, Figure 4.7 C) compared to VEH-THC-treated mice. CDK5-ir, p35-ir and p25-ir were not significantly different between treatments (Figure 4.7 C); however, there was a significant decrease in phosphorylation of DARPP-32 at threonine 34 in THC-THC-treated mice compared to VEH-VEH-treated mice (5.02% ± 0.37% vs. 3.91% ±0.28%, ratio of T34DARPP32/Total DARPP-32 (Table 4.2). There were no significant differences between treatment groups in the phosphorylation of DARPP-32 at threonine 75 (Table 4.3).

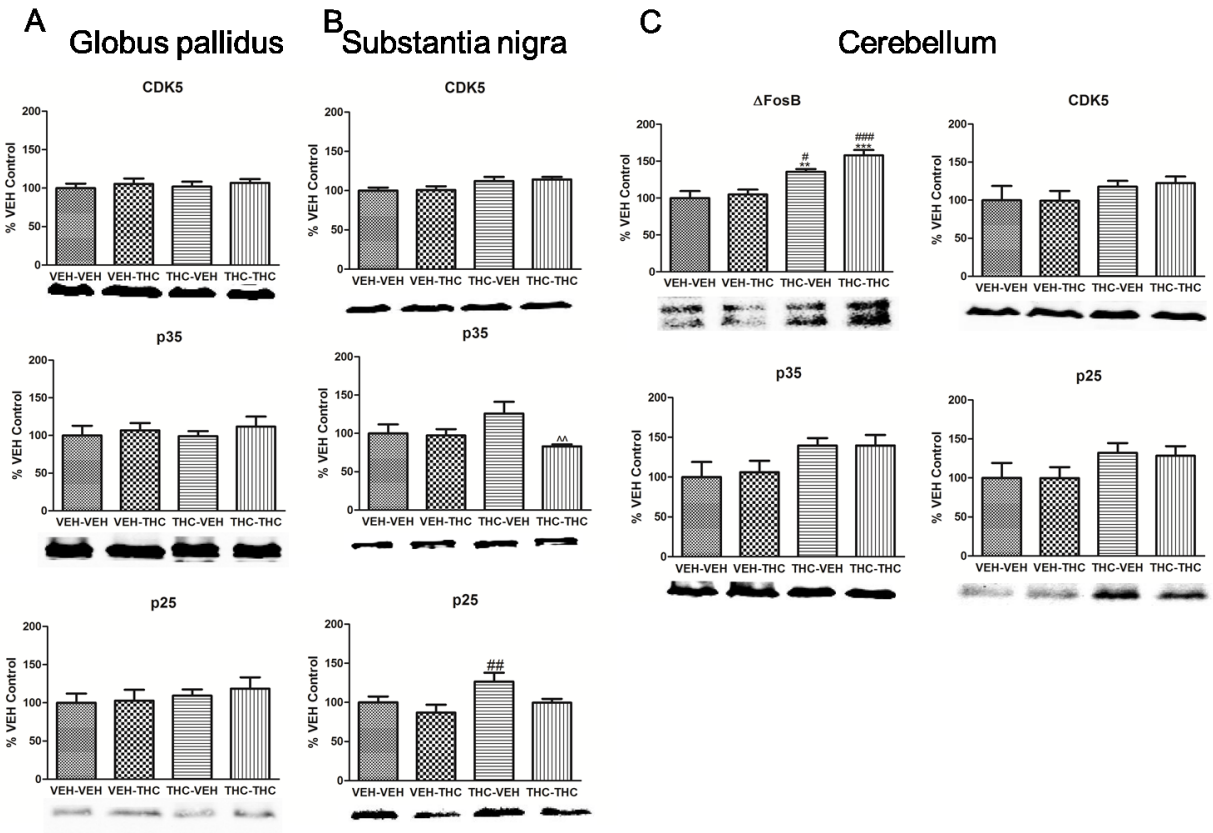


Figure 4.7 Expression of p25 was increased following repeated THC administration, whereas p35 wass decreased after THC injection in THC-experienced mice in substantia nigra. (A) CDK5-ir, p35-ir and p25-ir in the globus pallidus (B) CDK5-ir, p35-ir and p25-ir in the substantia nigra, (C) CDK5-ir, p35-ir and p25-ir in the cerebellum. Values represented as % VEH-VEH controls ± SEM. One-way ANOVA with Bonferroni post-hoc test. ** $p < 0.01$ and ***$p < 0.001$ compared to VEH-VEH controls. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to THC-VEH-treated mice. ^^ $p < 0.01$ compared to THC-VEH-treated mice. N = 8 per group

TABLE 4.2

Immunoblot results for phosphorylation of DARPP-32 at threonine 34/total DARPP-32

Brain Region	VEH-VEH	VEH-THC	THC-VEH	THC-THC
Prefrontal cortex	5.15 ± 1.09	4.34 ± 0.99	6.26 ± 1.11	4.28 ± 0.79
Caudate-putamen	23.00 ± 3.86	18.75 ± 2.47	23 ± 2.78	19.56 ± 2.39
Nucleus accumbens	19.71 ± 2.40	19.72 ± 2.13	19.20 ± 1.40	19.62 ± 1.50
Globus pallidus	23.20 ± 3.37	22.22 ± 3.35	22.72 ± 3.41	26.63 ± 3.31
Hippocampus	NA	NA	NA	NA
Substantia Nigra	22.86 ± 3.38	23.51 ± 4.22	22.57 ± 2.87	20.45 ± 2.03
Cerebellum	5.02 ± 0.38	4.20 ± 0.43	4.96 ± 0.36	3.91 ± 0.28*

Total DARPP-32-ir and DARPP-32-ir phosphorylated at threonine 34 (T34-DARPP-32) were measured in brain region homogenates as described in Methods. Results are expressed as T34-DARPP-32/total DARPP-32 * 100% ± SEM. * $p < 0.05$ different from vehicle-vehicle controls by one-way ANOVA and Bonferroni post-hoc test, N = 8 per group.

TABLE 4.3

Immunoblot results for phosphorylation of DARPP-32 at threonine 75/total DARPP-32

Brain Region	VEH-VEH	VEH-THC	THC-VEH	THC-THC
Prefrontal cortex	19.18 ± 2.27	19.63 ± 4.39	22.74 ± 5.26	21.36 ± 2.88
Caudate-putamen	9.94 ± 0.83	8.89 ± 0.72	10.21 ± 0.68	10.05 ± 0.83
Nucleus accumbens	14.79 ± 1.23	15.69 ± 1.02	14.80 ± 0.81	16.06 ± 1.52
Globus pallidus	13.34 ± 0.75	13.71 ± 0.41	14.71 ± 1.30	15.37 ± 1.05
Hippocampus	NA	NA	NA	NA
Substantia Nigra	3.36 ± 0.29	3.18 ± 0.35	3.48 ± 0.49	3.45 ± 0.48
Cerebellum	43.04 ± 3.74	48.33 ± 5.14	42.60 ± 4.60	45.51 ± 5.65

Total DARPP-32-ir and DARPP-32-ir phosphorylated at threonine 75 (T75-DARPP-32) were measured in brain region homogenates as described in Methods. Results are expressed as T75-DARPP-32/total DARPP-32 * 100% ± SEM, N = 8 per group.

4.4 Discussion

The present study compared THC-mediated induction of ΔFosB and its target proteins in drug naïve and THC-experienced mice to determine the effect of prior THC treatment on THC-mediated transcription. Protein expression was measured in forebrain regions that mediate the development of drug abuse and addiction (Koob and Volkow, 2010). In the prefrontal cortex, caudate-putamen and nucleus accumbens, repeated THC administration increased ΔFosB expression, in agreement with previous studies performed in our laboratory (Chapter 1). ΔFosB expression was also assessed in the cerebellum, because this brain region is thought to mediate extrapyramidal effects of cannabinoids (Castane et al., 2004; Patel and Hillard, 2001). Studies also determined that ΔFosB is not induced following acute THC injection, which is consistent with other studies that tested acute administration of morphine (Nye and Nestler, 1996) and cocaine (Nye et al., 1995).

THC-mediated ΔFosB induction was measured at both 45 minutes and 24 hours after THC injection, whereas previous studies assessed ΔFosB at 24 hours after THC-administration. Results showed that there are brain region-dependent differences in the induction of ΔFosB following THC injection in THC-experienced mice. After acute administration of THC, it was determined that ΔFosB expression did not change in prefrontal cortex, nucleus accumbens, caudate-putamen, hippocampus and cerebellum. However, repeated THC administration did increase ΔFosB expression in the prefrontal cortex, nucleus accumbens, caudate-putamen and cerebellum, but not in hippocampus. In the prefrontal cortex, ΔFosB induction was enhanced in THC-experienced mice compared to drug naïve mice that received THC injection. A previous study showed that cocaine administration enhanced ΔFosB protein/mRNA expression, but not

FosB protein/mRNA expression, in the nucleus accumbens of cocaine-experienced mice that received a challenge of cocaine following 28 days of withdrawal (Damez-Werno et al., 2012). Although this effect was not seen in nucleus accumbens after THC-treatment, it was seen in prefrontal cortex. ΔFosB mRNA/protein expression was enhanced in THC-experienced mice that received THC challenge, supporting the idea that THC-experience alters induction of ΔFosB produced by THC injection. Damez-Werno et al (2012) showed that dimethylation of histone H3 at lysine 9 (H3K9me2) and increased stalled RNA polymerase II (Pol II) binding may have contributed to the enhancement of ΔFosB induction, but the phosphorylation of ERK was not involved (Damez-Werno et al., 2012). In the current study, ERK1 phosphorylation was enhanced in the prefrontal cortex of THC-experienced mice that received THC injection, which is a possible mechanism that could underlie the enhanced induction of ΔFosB. Genetically modified mice that have reduced ERK phosphorylation also exhibited reductions in ΔFosB expression following repeated cocaine administration (Besnard et al., 2011; Fasano et al., 2009), suggesting a role for ERK phosphorylation in ΔFosB induction. Increased DARPP-32 phosphorylation at threonine 34 can also regulate ΔFosB induction (Zachariou et al., 2006b); however, there was no change in phosphorylation of DARPP-32 at this site in the current study. We have previously reported that DARPP-32 is not necessary for THC-mediated induction of ΔFosB in the striatum (Chapter 3), which suggests that DARPP-32 is most likely not necessary for enhancement of ΔFosB induction.

ΔFosB transcriptionally regulates the expression of CDK5 and p35 (Bibb et al., 1999; Chen et al., 2000b; Kumar et al., 2005; Peakman et al., 2003), therefore these proteins were assessed in brains from the same treatment groups. In the prefrontal cortex, expression of CDK5 and p35 were increased following repeated THC administration, but not by acute THC

administration, supporting a role for ΔFosB in regulating these proteins. In the prefrontal cortex, there was a similar enhancement of CDK5 expression as was found for ΔFosB. Therefore, CDK5 mRNA levels were measured and it was determined that CDK5 mRNA expression was differed following THC injection in drug naïve (decreased CDK5 mRNA expression) and THC experienced (increased CDK5 mRNA expression) mice. These results suggest that enhanced ΔFosB induction also leads to enhanced CDK5 expression through ΔFosB-mediated regulation of transcription. CDK5 expression was not increased in the nucleus accumbens or caudate-putamen, suggesting that although THC induces ΔFosB, it does not appear to regulate CDK5 expression in these regions. This finding is different from studies with cocaine and in mice overexpressing ΔFosB in the striatum (Bibb et al., 2001a), suggesting that THC negatively regulates ΔFosB-mediated transcription of CDK5 in the caudate putamen and substantia nigra.

ΔFosB also regulates expression of p35, which dimerizes with CDK5 and facilitates its kinase function. In the nucleus accumbens and cerebellum, p35 expression did not change with repeated THC administration, suggesting that ΔFosB does not regulate p35 expression in these regions following THC administration. In the caudate-putamen, p35 expression differed between drug naïve and THC-experienced mice that received THC injection. Acute THC administration actually increased p35 expression, suggesting that a different transcription factor might regulate p35 induction in the caudate-putamen. In fact, early growth response protein 1 (EGR1), also known as zif268 and krox-24, has been implicated in the induction of p35 (Utreras et al., 2011) and is induced in the caudate-putamen by acute THC administration (Mailleux et al., 1994). In THC-experienced mice, the decrease in p35 expression could be due to either increased calpain-mediated cleavage of p35 to p25 (Kusakawa et al., 2000) or through the proteasome pathway as a result of phosphorylation of p35 (Kerokoski et al., 2002; Patrick et al., 1998; Saito et al., 1998).

It would appear that the latter is more likely because p25 expression did not increase in the caudate-putamen following THC injection in THC-experienced mice. There was a similar effect for p35 in the substantia nigra, where repeated THC administration increased p35 expression, but THC challenge in THC-experienced mice decreased p35. D_1R/dynorphin MSNs in the caudate-putamen project to the substantia nigra, therefore it is possible that repeated THC administration might increase trafficking of p35 from these neurons to axonal projections in the substantia nigra. It is possible that p35 is also increased in neuronal cells of the substantia nigra. Similar to the caudate-putamen, the cleavage of p35 to p25 does not explain the decrease of p35 expression in the substantia nigra following THC injection in THC-experienced mice. However, there was an increase in p25 expression in the substantia nigra following repeated THC administration, suggesting that THC mediates increased cleavage of p35 to p25 in this region. There was no change in CDK5, p35 or p25 expression in the globus pallidus, suggesting that the same signaling responses that occur in D_1R/dynorphin MSNs do not occur in dopamine type 2 receptor (D_2R)/enkephalin MSNs. This would agree with our previous findings that ΔFosB induction in caudate-putamen is primarily restricted to neurons that express dynorphin (Chapter 3).

Expression of both p35 and p25 increased in the prefrontal cortex, which would increase the kinase activity of CDK5 (Kusakawa et al., 2000; Tsai et al., 1994). Three substrates of CDK5: DARPP-32, GSK3β and tau protein were assessed for phosphorylation levels to determine if increases in p35/p25 would increase CDK5 kinase activity. Phosphorylation of tau at Ser202/Thr205 was increased in the prefrontal cortex of THC-experienced mice that received THC injection, whereas DARPP-32 phosphorylation at Thr75 was unchanged and GSK3β phosphorylation of Ser9 was decreased. Although the increase in phosphorylation of tau would suggest an increase in CDK5 kinase activity, CDK5 activity assays are necessary to assess CDK5

activity. Phosphorylation of tau, DARPP-32 and GSK3β involve complex signaling pathways, so it is not clear why THC injection in THC-experienced mice did not increase phosphorylation of all three substrates. The finding that tau was phosphorylated in the prefrontal cortex after repeated THC administration is interesting because previous studies have suggested that synthetic cannabinoids (WIN55,212-2 and arachidonyl-2-chloroethylamide) are neuroprotective in Alzheimer's disease-related mouse models (Aso et al., 2012). The current results suggest that THC might not be neuroprotective because hyperphosphorylation of tau is actually a symptom of Alzheimer's disease and THC-mediated phosphorylation of tau could exacerbate this condition (Pettegrew et al., 1987).

DARPP-32 phosphorylation was unchanged in most brain regions following either acute or repeated THC administration. Previous studies in the striatum found that acute administration of either CP55,940 (Andersson et al., 2005) or THC (Borgkvist et al., 2008) increased phosphorylation of DARPP-32 at threonine 34 in caudate-putamen and nucleus accumbens of mice. Other studies have found that acute administration of THC in rats increased DARPP-32 phosphorylation at threonine 34 in the prefrontal cortex (Polissidis et al., 2010). The same dose of THC and the same strain of mice were used in the current study as Borgkvist et al. (2008); however, we measured DARPP-32 phosphorylation at 45 minutes. Borgkvist et al. (2008) showed that phosphorylation of DARPP-32 at threonine 34 was maximal at 30 minutes and was gone by one hour. Our studies would suggest that the threonine 34 site of DARPP-32 is dephosphorylated back to baseline levels by 45 minutes. The finding that repeated THC administration did not increase phosphorylation of DARPP-32 at threonine 75 in the nucleus accumbens, caudate-putamen or cerebellum, is likely due to the lack of increase in CDK5, p35 and p25 expression in those brain regions. Phosphorylation of DARPP-32 at threonine 34 was

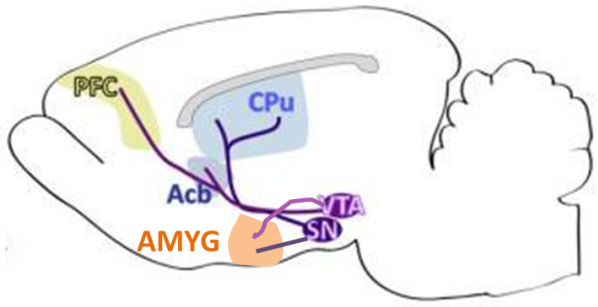
decreased in the cerebellum following THC injection in THC-experienced mice, suggesting that THC modulates DARPP-32 phosphorylation in this region.

Studies with other drugs of abuse, like cocaine, have focused on signaling changes in the nucleus accumbens, whereas THC administration produced few changes in the nucleus accumbens. However, the current study demonstrates that the neurochemistry of the prefrontal cortex changes dramatically with administration of THC. Meta-analysis of neuroimaging studies in adolescent and adult human cannabis users found that alterations in frontal cortex volumes and cerebral blood flow following both THC administration and during memory-related tasks were common in many studies (Batalla et al., 2013), suggesting an important role for this region in continued marijuana use. ΔFosB appears to be an important regulator of these signaling changes because CDK5 and p35 expression were increased in prefrontal cortex. Moreover, repeated THC administration regulated other signaling cascades, including increases in ERK1 phosphorylation, decreases in GSK3β phosphorylation at Ser9 and increases in tau phosphorylation. Maladaptation of the prefrontal cortex, manifested as alterations in delta and gamma oscillations, is consistently found in schizophrenia patients (Curley and Lewis, 2012). Positron emission topography (PET) studies, using the CB_1R-specific ligand [^{11}C]JHU75528, determined that CB_1R levels are increased in the frontal cortex, caudate and putamen and globus pallidus, among others, suggesting that CB_1Rs might mediate these changes in gamma oscillations (Wong et al., 2010). A higher percentage of schizophrenic patients also abuse marijuana compared to populations of healthy individuals, and it has been suggested that marijuana use exacerbates disease progression (Bossong and Niesink, 2010; Weiser and Noy, 2005). The changes in signaling found in prefrontal cortex following repeated THC administration might offer insights into possible mechanisms underlying this observation.

The results of these studies suggest that repeated THC treatment alters signaling pathways such that THC injection produces very different effects in THC-experienced versus drug naïve mice. These studies are consistent with reports showing that cocaine-mediated increases in CDK5 and p35 could result from the induction of ΔFosB following repeated drug administration. However, THC-mediated signaling changes occurred predominantly in the prefrontal cortex, whereas cocaine-mediated signaling changes were found in the nucleus accumbens. These findings suggest that drug-induced changes in signaling are both drug- and brain region-dependent.

Conclusions and Perspectives

TABLE 5.1
Summary of major findings in this dissertation



Brain Region	ΔFosB induction/ CB1R-/ D1R-dependent?	Does ΔFosB reduce CB1R desensitization?	Does ΔcJun enhance CB1R desensitization?	CDK5 and p35 expression	DARPP-32 phosphorylation changes?	DARPP-32 necessary for ΔFosB induction?	p-ERK-1
Prefrontal cortex	Yes*/ND/ Yes *↑ induction with THC-experience	ND	No	CDK5 and p35↑ repeated THC	No	ND	↑ repeated THC
Caudate-putamen	Yes/Yes/Yes	Yes, in D1R/dynorphin MSN population	Yes	p35 ↑ acute THC and ↓ repeated THC	No	Yes for acute No for repeated	No change
Nucleus accumbens	Yes/Yes/Yes	No	No, but reduces desensitization in VTA	No	No	Yes for acute contribute with repeated	ND
Amygdala	Yes/ND/ Yes	Yes	No	ND	ND	ND	ND

Abbreviations: PFC, prefrontal cortex, CPu, caudate-putamen, Acb, nucleus accumbens, AMYG, amygdala, VTA, ventral tegmental area, SN, substantia nigra

The thesis chapters, contained herein, addressed the role of the transcription factor, ΔFosB, in regulating both CB_1R signaling and adaptation following repeated THC administration and some of the possible mechanisms involved in THC-mediated induction of ΔFosB. It was hypothesized that ΔFosB would reduce CB_1R desensitization and contribute to the brain region-dependent differences in CB_1R desensitization that occur following repeated THC administration. Studies in Chapter 2 were designed primarily to address the relationship between ΔFosB and CB_1R desensitization/downregulation and tolerance. However, the finding that THC-mediated induction of ΔFosB was more regionally widespread than had been previously determined (Perrotti et al., 2008), suggests that this transcription factor could play an important role in other physiological changes following repeated THC administration. Major findings from these dissertation studies include: 1) that THC-mediated induction of ΔFosB in the caudate-putamen and nucleus accumbens is CB_1R-dependent 2) that THC-mediated induction of ΔFosB is D_1R-dependent in the prefrontal cortex, caudate-putamen, nucleus accumbens and amygdala 3) that overexpression of ΔFosB in D_1R/dynorphin containing MSNs of the striatum reduce CB_1R desensitization in their respective output nuclei, and 4) that the FosB promoter is primed in the prefrontal cortex such that THC challenge in THC-experienced mice enhances ΔFosB induction.

These studies investigated possible mechanism(s) that might underlie brain region-dependent differences in CB_1R desensitization/downregulation. Studies in Chapter 1 showed an inverse region-dependent correlation between CB_1R desensitization and ΔFosB induction. It was determined that regions like the caudate-putamen and nucleus accumbens exhibited significant ΔFosB induction in the absence of CB_1R desensitization, whereas the hippocampus exhibited significant CB_1R desensitization without ΔFosB induction following repeated THC

administration. In Chapter 1, studies utilizing mice with genetic deletion of CB_1Rs determined that the induction of ΔFosB following repeated THC administration was dependent on CB_1R expression in the caudate-putamen and nucleus accumbens, and that CB_1Rs were located on axonal terminals surrounding ΔFosB positive cells and within the cell bodies of ΔFosB positive cells. These studies provided evidence that, following repeated THC administration, CB_1Rs were necessary for ΔFosB induction and that ΔFosB could modulate CB_1R signaling. These studies did not address whether CB_1Rs located on astrocytes may also play a role in ΔFosB induction or whether ΔFosB is induced in astrocytes following repeated THC administration CB_1Rs are expressed by astrocytes and function to support neuronal cell viability (Stella, 2010).

Based on the inverse regional correlation between ΔFosB and CB_1R desensitization determined in Chapter 1, studies in Chapter 2 were designed to determine whether overexpression of ΔFosB could regulate CB_1R desensitization following repeated THC administration. To test this hypothesis, mice overexpressing ΔFosB or ΔcJun, a dominant negative inhibitor of ΔFosB, were assessed after repeated THC treatment. One group of mice overexpressed ΔFosB primarily in the D_1R/dynorphin MSN population of the striatum, which project to the substantia nigra (cell bodies of origin in the caudate-putamen) and to the ventral tegmental area (cell bodies of origin in the nucleus accumbens). These mice also overexpressed ΔFosB in the hippocampus and parietal cortex. The other group of mice overexpressed ΔcJun in both the D_1R/dynorphin and D_2R/enkephalin MSN populations, which project to the globus pallidus (cell bodies of origin in the caudate-putamen) and to the ventral pallidum (cell bodies of origin in the nucleus accumbens). These mice also overexpressed ΔcJun in the hippocampus and parietal cortex. Based on our studies in Chapter 1, it was predicted that overexpression of ΔFosB would reduce CB_1R desensitization in the caudate-putamen, nucleus accumbens, ventral

tegmental area and substantia nigra. However, it was determined that overexpression of ΔFosB only reduced CB_1R desensitization in the substantia nigra and ventral tegmental area. The finding that overexpression of ΔFosB did not reduce CB_1R desensitization in the caudate-putamen and nucleus accumbens is likely due to the limited overexpression of ΔFosB in only the D_1R-positive population of MSNs in these regions. The caudate-putamen and nucleus accumbens also receive inputs from the cortex, amygdala, hippocampus and thalamus, which express CB_1Rs. Therefore, significant desensitization in these CB_1R populations may have masked attenuation of CB_1R desensitization in these regions. Although ΔFosB is significantly increased in the hippocampus, this region did not exhibit reduced CB_1R desensitization. This finding likely reflects the lack of THC-mediated ΔFosB induction previously shown in the hippocampus (Chapter 1). This is evidenced by our findings in Chapter 4, where ΔFosB did not cause induction of CDK5or p35 in the striatum. These results suggest that ΔFosB could regulate different signaling proteins in a brain region-dependent manner that leads to regulation of CB_1R desensitization. Inhibition of ΔFosB-mediated transcription by overexpression of ΔcJun enhanced CB_1R desensitization in the caudate-putamen, consistent with our hypothesis. The difference between these results and those in ΔFosB overexpressing mice might reflect the fact that ΔcJun is overexpressed in both the D_1R/dynorphin and D_2R/enkephalin MSN populations. ΔcJun overexpression did not enhance desensitization in substantia nigra. It is possible that ΔcJun also inhibited the transcriptional regulation of other Fos family members, which are known to regulate the expression of different signaling proteins. The results could also be due to the dose of THC administered (10-30-60 mg/kg). It could be that the level of ΔFosB produced by this THC dose is not sufficient to reduce CB_1R desensitization, whereas ΔFosB overexpression induces a higher level of protein induction. These studies focused only on the

effect of ΔFosB overexpression on CB_1R desensitization in the striatum, but not regions like prefrontal cortex and amygdala where ΔFosB is also induced by THC. Future studies should test whether overexpression of ΔFosB in the prefrontal cortex and amygdala would reduce desensitization in these regions. These studies would help support our correlation model proposed in Chapter 1. It is also important to note that the mice overexpressing ΔFosB were on a mixed C57BL/6J and FVB genetic background, whereas the mice overexpressing ΔcJun were on an FVB genetic background. Future studies could address this issue by overexpressing ΔFosB or ΔcJun using viral vectors in the same mouse strain. This could be an especially important consideration for in vivo studies assessing the effect of ΔFosB on THC-mediated effects. Results of studies in both Chapter 1 and Chapter 2 suggest that ΔFosB does not regulate CB_1R desensitization in the hippocampus. However, overexpression of ΔcJun inhibited CB_1R desensitization in this region, suggesting that other Fos family members could regulate CB_1R desensitization in the hippocampus. Using mouse models with overexpression of other Fos family members, like c-Fos, could determine if c-Fos regulates CB_1R desensitization. Using viral vectors with siRNA, to knockdown c-Fos expression, would serve as a complement to this study.

The rewarding effects of most drugs of abuse are associated with enhanced dopamine release in the shell of the nucleus accumbens (Pontieri et al., 1995). Most drugs of abuse also induce ΔFosB in the nucleus accumbens following repeated administration (Perrotti et al., 2008). Studies in Chapter 1 showed that THC, which enhances dopamine release (Wu and French, 2000), also induces ΔFosB in the nucleus accumbens, as well as prefrontal cortex, caudate-putamen and basolateral amygdala (Polissidis et al., 2010). Further, THC-mediated induction of ΔFosB is both CB_1R- and D_1R-mediated in the nucleus accumbens and caudate-putamen and

D_1R-mediated in prefrontal cortex and amygdala. Although it is not certain whether ΔFosB is a necessary component for the switch from occasional drug use to addiction, the results of these studies provide evidence that modulation of D_1Rs would modulate the induction of ΔFosB and could alleviate marijuana dependence. These results also highlight the need to focus on additional brain regions that contribute to addiction since ΔFosB is induced in the prefrontal cortex and amygdala. These regions appear to be important for drug craving and drug-cued memory/reinstatement, respectively (Goldstein and Volkow, 2011; Stamatakis et al., 2013).

The results showing that THC-mediated ΔFosB induction is blocked by D_1R antagonists and that the majority of ΔFosB is expressed in D_1R/dynorphin MSNs of the striatum are somewhat surprising since CB_1Rs are found on both D_1R/dynorphin and D_2R/enkephalin MSNs (Hohmann and Herkenham, 2000). Further, evidence would suggest that CB_1Rs and D_2Rs can dimerize (Wager-Miller et al., 2002) and that pharmacological inhibition or genetic deletion of D_2Rs or A_{2A} receptors (which are also located in D_2R MSNs and purported to dimerize with CB_1Rs) blocks cannabinoid-mediated phosphorylation of DARPP-32 at threonine 34 (Andersson et al., 2005; Borgkvist et al., 2008). One explanation, supported by these dissertation studies, is that DARPP-32 might not be necessary for ΔFosB induction following repeated THC administration under the conditions tested in these studies. However, ΔFosB induction produced by an acute administration of a 70 mg/kg dose of THC was abolished in DARPP-32 knockout mice. It is possible that this dose of THC could produce acute induction of ΔFosB in the D_2R/enkephalin MSN population through a DARPP-32-dependent mechanism. One caveat to this interpretation is that blockade of D_1Rs also inhibits THC-mediated phosphorylation of DARPP-32 at threonine 34 (Borgkvist et al., 2008). Therefore, it is also possible that CB_1R/D_2R mediated signaling could enhance dopamine release and activate D_1Rs, which is one mechanism

through which THC-mediated ΔFosB induction occurs. However, it is still not clear if dimerization of CB_1Rs and D_2Rs could regulate the induction of ΔFosB in the D_2R/enkephalin medium spiny neuron population. The role of dimerization of these receptors in the induction of ΔFosB could be tested through simultaneous treatment of CB_1R and D_2R agonists. Another caveat to this finding is that compensatory adaptations might occur in mice with global, lifelong deletion of DARPP-32. Future studies could address this possibility using conditional DARPP-32 knockout mice with temporally and spatially restricted DARPP-32 deletion. The finding that genetic deletion of DARPP-32 also enhanced tolerance to the locomotor suppressing effects of THC suggests that these mice may also have brain region-dependent differences in CB_1R desensitization. This finding was similar to results showing that enhanced tolerance the locomotor suppressing effects of THC were found in mice with attenuated CB_1R desensitization in the substantia nigra (through overexpression of ΔFosB) and enhanced CB_1R desensitization in the caudate-putamen (through overexpression of ΔcJun). It is not clear if these changes in CB_1R desensitization are directly responsible for enhanced tolerance; however, measuring desensitization in DARPP-32 knockout mice might offer further evidence for whether differences in desensitization in these regions might be mediating this enhanced tolerance. Therefore, it is likely that brain region-dependent differences in CB_1R desensitization contribute to this finding. It is also possible that genetic deletion of DARPP-32 produces adaptations in CB_1R signaling downstream of G-protein activation, perhaps at the effector level, which might explain the finding that DARPP-32 knockout mice also display increased locomotor suppression following acute THC administration. Future studies are necessary to determine whether there are brain region-dependent differences in CB_1R-mediated G-protein activity in drug naïve mice and CB_1R desensitization following repeated THC administration between DARPP-32 knockout and

wild-type. Autoradiographic studies, as performed in Chapter 2, would be appropriate in testing this hypothesis.

There were also brain region-dependent differences in the regulation of CDK5 and p35, proteins that are transcriptionally regulated by ΔFosB (Bibb et al., 2001a). Although ΔFosB expression was increased in the prefrontal cortex, caudate-putamen and nucleus accumbens after repeated THC administration, CDK5 and p35 expression were only increased in the prefrontal cortex. This differs from previous studies that showed that cocaine-mediated ΔFosB induction is associated with increased expression of both CDK5 and p35 in the nucleus accumbens (Bibb et al., 2001a). This highlights one major difference between these different drugs of abuse and could explain some of the preclinical rodent data that suggests that THC is not rewarding, whereas cocaine is consistently found to be rewarding under these preclinical conditions (Tanda and Goldberg, 2003). The lack of changes in CDK5 and p35 expression in the nucleus accumbens of THC-treated mice could be due to degradation of the proteins because they are not as stably expressed as ΔFosB. Studies were performed to address this possibility by measuring protein levels at both 24 hours (at which time ΔFosB would still be elevated due to its stability) and 45 minutes (to determine if CDK5 and p35 expression levels were elevated at earlier time points but degraded by 24 hours) after THC challenge. Based on the results in the prefrontal cortex, one conclusion is that CDK5 and p35 are continuously regulated by ΔFosB since both CDK5 and p35 were elevated at the 24 hour time point. However, additional studies would be needed to determine if CDK5 and p35 are also stable by using radiolabeled amino acids and measuring the time course of CDK5 and p35 degradation. Assessment at earlier time points could address the possibility that expression of CDK5 and p35 is increased within 30 minutes, but rapidly degraded by the 45 minute time point.

The other interesting finding is that THC-experience appeared to prime the FosB promoter because THC challenge produced induction of ΔFosB that was not present in drug naïve mice. This finding supports the importance of determining epigenetic factors that may occur with long-term drug use and suggests the necessity of targeting these factors for drug abuse treatment (Renthal and Nestler, 2008). These therapies would have to target and reverse epigenetic changes to provide effective treatment. Currently, there are no clinically approved therapies available for altering epigenetic effects (Renthal and Nestler, 2008). The studies in Chapter 4 also provided evidence for the selective regulation of p35 in D_1R/dynorphin MSNs because the regulation of this protein by THC was similar in the caudate-putamen and substantia nigra, whereas there was no effect in globus pallidus, which receives inputs from the D_2R/enkephalin MSN population. Future studies are necessary to determine whether regulation of p35 is restricted to the D_1R/dynorphin MSN population, as it would suggest further differences in the regulation of these two MSN populations following THC administration. Studies similar to Chapter 3 could be performed to determine if antagonism of either D_1Rs or D_2Rs blocks this effect. Finally, although CDK5, p35 and p25 were increased in the prefrontal cortex, only one target of CDK5, tau, exhibited increased phosphorylation as predicted. Functional assays that measure the kinase activity of CDK5 are necessary to determine whether CDK5 activity also increased in the prefrontal cortex and to determine if the lack of phosphorylation of targets of CDK5 was due to other factors. Understanding these signaling changes may also help elucidate possible mechanisms for marijuana-mediated exacerbation of the progression of schizophrenia, a disorder that is hypothesized to be heavily influenced by maladaptive cortical oscillations (Curley and Lewis, 2012), which may relate to the regulation of neurotransmission by CB_1Rs. New therapies are necessary for the treatment of schizophrenia

because only approximately 50% of patients that receive current medications achieve sustained remission of positive and negative symptoms (Galderisi et al., 2013). Understanding how THC may exacerbate these symptoms could provide insight into designing therapeutic strategies that might alleviate these symptoms.

One consistent finding of the studies in this dissertation is that repeated THC administration produces specific brain region-dependent induction of ΔFosB. Although the role that ΔFosB plays in drug abuse is not completely understood, this thesis suggests that both CB_1Rs and D_1Rs are involved in its induction by THC. The brain region-dependent induction of ΔFosB, however, does not necessarily translate into similar ΔFosB-mediated regulation of transcription, because the expression of well-defined targets of ΔFosB differed among brain regions. However, it is important to remember that these results were determined using a limited scope of THC treatment paradigms and time courses. Again, these studies did not address whether ΔFosB is expressed exclusively in neurons and whether the findings discussed above could be due to induction of ΔFosB in astroctyes (Stella, 2010). It is possible that ΔFosB differentially regulates protein expression in neurons and astrocytes. Future studies will need to identify the protein targets that are regulated by ΔFosB, and in which cell types, to determine if they regulate CB_1R desensitization and whether they contribute to the rewarding effects of drugs of abuse. Further immunohistochemical characterization could be used to address this question. Although it is not clear whether ΔFosB is a necessary regulator of CB_1R desensitization, these studies suggest that it could contribute to CB_1R desensitization in certain brain circuits. Future studies could further investigate the brain regions in which ΔFosB regulate CB_1R desensitization using virally-mediated overexpression of ΔFosB, or through use of small molecules that inhibit ΔFosB. The ability to design cannabinoid-based therapeutics by maximizing their clinical utility

while minimizing their side effects requires understanding these brain region-dependent differences in signaling. This is an important consideration for patients with long-term disorders such as epilepsy and multiple sclerosis, for which THC has shown promise in treating, who will need to function in their daily lives. If decrements in motor coordination impact their ability to drive, for instance, then THC treatment would not be entirely beneficial. Further, inhibition of ΔFosB might be useful for treating marijuana dependence based on preclinical evidence that ΔFosB contributes to the rewarding effects of drugs of abuse.

These results have implications for developing drugs that could mitigate some of the negative side effects of THC and enhance its therapeutic utility. Recently, Nestler and collaborators have developed small molecules that could inhibit the function of ΔFosB by screening small molecules for their ability to prevent ΔFosB from binding to a modified CDK5 promoter (Wang et al., 2012). Nucleic acid aptamers provide another strategy for producing selective targets that could inhibit ΔFosB transcription (Li et al., 2013). The strategy of blocking ΔFosB transcription could be used to enhance CB_1R desensitization in the caudate-putamen and enhance the development of tolerance to THC-mediated motor impairment. This is based on results in caudate-putamen that showed overexpression of ΔcJun, which also inhibits ΔFosB transcriptional regulation, enhanced CB_1R desensitization and tolerance to locomotor suppression. Tolerance to motor impairment does not develop as readily as tolerance to other THC-mediated effects in human marijuana users (D'Souza et al., 2008), and motor impairment is a potential concern for the performance of day to day activities in patients. This would suggest that introducing a small molecular inhibitor in combination with THC could enhance tolerance to its motor impairing effects, and improve diving safety in patients treated with cannabinoids for long periods of time. Targeting transcriptional regulation of other Fos family members could

also mitigate the memory impairing effects of cannabinoids (Nestor et al., 2008). Similar small molecules and aptamers could be produced to block the Fos family members that may contribute to THC-mediated CB_1R desensitization in hippocampus, which could mitigate memory-impairing effects. Targeting ΔFosB may also help those who are dependent on marijuana. The findings of this dissertation have further characterized the brain region-dependent differences in the receptors /signaling proteins that modulate THC-mediated induction of ΔFosB. It has also elucidated a role for ΔFosB and other Fos family members in modulating CB_1R signaling and provided evidence for brain region-dependent differences in the transcriptional regulation of ΔFosB following repeated THC administration. These results provide insights into the therapeutic potential of targeting ΔFosB for mitigating the long-term side effects of THC.

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VITA

PUBLICATIONS:

Lazenka, M.F., Selley D. E. and Sim-Selley, L.J. ΔFosB induction correlates inversely with CB_1 receptor desensitization in a brain region- dependent manner following repeated Δ^9-THC administration. Neuropharmacology, in press.

REVIEWS:

Lazenka, M.F., Selley, D. E. and Sim-Selley, L.J. (2013). Brain Regional differences in CB1 receptor adaptation and regulation of transcription. Life Sci. *92*, 446-452.

CONFERENCE POSTER PRESENTATIONS:

Sim-Selley, L.J., **Lazenka, M.F.**, Nestler, E.J. and Selley, D.E. (2013) Repeated cannabinoid administration induces ΔFosB and sensitizes mu opioid receptor activity in the nucleus accumbens. American Society for Pharmacology and Experimental Therapeutics.

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M. F. Lazenka, D. E. Selley, and L. J. Sim-Selley (2011). Brain region-dependent correlation between ΔFosB induction and the desensitization and downregulation of CB1 receptors following repeated THC administration. Carolina Cannabinoid Collaborative.

AWARDS:

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2007	Psi Chi Regional Research Award (Southeastern Regional Convention)

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