ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF PHARMACY

Involvement of the Endocannabinoid System in Modulating the Neurobehavioral Effects of *Catha edulis* (Vahl) Endl. (Khat) in Mice, Implication for Diseases Associated with Dopamine Dysregulation

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A dissertation submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University in partial fulfillment for the requirements of the Degree of Doctor of Philosophy in Pharmacology

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June 2020

Declaration

This is to certify that the PhD thesis prepared by Berhanu Geresu Kibret entitled "**Involvement of the Endocannabinoid System in Modulating the Neurobehavioral Effects of *Catha edulis* (Vahl) Endl. (Khat) in Mice, Implication for Diseases Associated with Dopamine Dysregulation**" and submitted for the fulfillments for the Degree of Doctor of Philosophy in Pharmacology complies with the regulation of the University and meets the accepted standards with respect to originality and quality.

Examiners

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Publications

- Geresu B, Canseco-Alba A, Sanabria B, Lin Z, Liu Q-R, Onaivi E, Engidawork E (2019). Involvement of CB2 Receptors in the Neurobehavioral Effects of *Catha edulis* (Vahl) Endl. (Khat) in Mice. Molecules. 24: 3164.
- Geresu B, Onaivi E, Engidawork E (2016). Behavioral evidence for the interaction between cannabinoids and *Catha edulis* F. (Khat) in mice. Brain Res. 1648: 333-338.

Abstract

Several lines of experimental evidence on wild type and cannabinoid receptor manipulated animals have shown the role of the endocannabinoid system on the effect of psychoactive substances, including opioids,nicotine and cocaine. Since the mesocorticolimbic dopaminergic pathway plays a vital role in mediating some of the behavioral effects of both khat and cannabinoids, studying the interaction of the endocannabinoid system and khat would provide an insight in the identification of drug targets and development of new pharmacologic approaches to treatment of central nervous system disorders associated with dopamine dysregulation.The objective of this study was therefore to investigate the involvement of the endocannabinoid system in modulating the neurobehavioral effects of khat in mice.A battery of behavioral tests including Y-maze, elevated plus maze and locomotor activity were used to assess behavioral effects. In addition, immunohistochemistry and reverse transcriptase polymerase chain reaction (RT-PCR) technique were employed to investigate tyrosine hydroxylase immunoreactivity and expression of dopamine transporter mRNA, respectively. The experiments were performed using adult male BALB/c albino, C57BL/6J and DAT-*Cnr2*cKO mice. The BALB/c albino mice were used for acute studies, whereas, the C57BL/6J and DAT-*Cnr2* mice were used for sub-acute studies. In the acute study khat extract and the different drugs were administered as a single dose, however, in the sub-acute study khat and drugs were administered once per day for seven consecutive days.Khat extract was administered in a dose of 300 mg/kg; WIN-55,212-2 (non-selective cannabinoid receptor agonist), 1 mg/kg; JWH133 (cannabinoid type 2 receptor agonist), 5 mg/kg; AM251 (cannabinoid type 1 receptor antagonist), 1 mg/kg and AM630 (cannabinoid type 2 receptor antagonist); 1 mg/kg. The results show that, acute administration of khat with WIN-55,212-2, enhanced locomotor activity, anxiolytic and working memory related behavior of

khat.Sub-acute co-administration of khatwith JWH133reducedhyperlocomotor behavior of khat, however, cell type specific deletion of cannabinoid receptor type 2 on dopaminergic neuronsincreased the effect of khat on locomotor activity. Furthermore,khat attenuated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)induced motor deficits, which is enhanced by JWH133. JWH133 didn't alter the effect of khat on tyrosine hydroxylase immunoreactivity and dopamine transporter mRNA expression when given together with khat. Taken together, the results suggest that the endocannabinoid system modulates the neurobehavioral effects of khat, where, cannabinoid type 1 receptors negatively modulate the neurobehavioral effects of khat but cannabinoid type 2 receptors selectively interact with khat-mediated effectswhich could be utilized as therapeutic target in central nervous system movement disorders associated with dopamine dysregulation.

Keywords: khat, Y-maze, elevated plus maze, endocannabinoid system, tyrosine hydroxylase, dopamine transporter, locomotor activity, MPTP, JWH133,WIN-55,212-2.

Acknowledgments

I would first like to acknowledge my advisor, Prof EphremEngidawork. His support throughout my graduate career has been invaluable. He has encouraged my development scientifically and beyond. I hope to embrace science and life with the enthusiasm that he does.I am also grateful for the assistance I received from my second advisor Prof Emmanuel Onaivi. His helpful guidance and support during my stay at his laboratory at William Paterson University of New Jersey, USA is highly acknowledged. He provided much time and effort to my project and always encourages me to go forward when situations drag me back. I would also like to thank Prof Patricia Tagliaferro, Prof Emmanuel's wife, for allowing me to stay at their house in New Jersey and for her invaluable advice throughout my stay in William Paterson University. My heartfelt gratitude also goes to my collaborator DrPritesh Kumar who helped me through provision of chemicals for my study and for his advice.

Also I owe a debt of gratitude to Ana, Branden and the late Norman (my condolences for his family and relatives on their sudden and tragic loss) for their help and support and providing training with molecular biology techniques during my stay at William Paterson University.

This work would not be possible without the student worker support for maintenance of mice in the animal laboratory by the Dean of the College of Science and Health at William Paterson University, DrVenkat Sharman and to the Department of Pharmacology and Clinical Pharmacy of School of Pharmacy at Addis Ababa University.

A special thanks is extended to my wife, Enat, for her consistent advice and guidance and for taking care of our kids while I was doing my PhD.She always given me faith in my abilities, and her constant encouragement and love is the reason I push myself to accomplish my goals.

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List of abbreviations

AC	Adenylatecyclase
CB1Rs	Cannabinoid type-1 receptors
CB2Rs	Cannabinoid type-2 receptors
CBRs	Cannabinoid receptors
cKO	Conditional knockout
CNS	Central nervous system
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DAT	Dopamine transporter
eCBs	Endocannabinoids
ECS	Endocannabinoid system
GPCR	G-protein coupled receptors
MAP	Mitogen activated protein
MAO-B	Monoamine oxidase type B
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
PFC	Prefrontal cortex
SNc	Substantianigra pars compacta
TH	Tyrosine hydroxylase
TRPV	Transient receptor protein vanilloid
VTA	Ventral tegmental area
WT	Wild type
Δ^9-THC	Delta 9-tetrahydrocannabinol

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1. Introduction

1.1. Overview of *Catha edulis* (Vahl) Endl. (Khat)

Khat is a name generally used for *Catha edulis* (Vahl) Endl., a dicotyledonous evergreen shrub of the family *Celastraceae*(Fig. 1). The khat tree has a slender bole and white bark. In Yemen, the trees range from 1 to 10m in height, while in Ethiopian highlands they can reach heights of 18 m(Al-Hebshi and Skaug, 2005; Al-Motarreb et al., 2002). The use of khat has traditionally been confined to the regions where khat is grown. In recent years, however, the economic importance and consumption of khat leaves have increased dramatically which had allowed a much wider distribution (Feyissa and Kelly, 2008). The habit of khat chewing is largely confined to inhabitants of the countries of Eastern Africa and South-Western Arabia. In Yemen approximately 80% of adult men in the major cities and 90% of adult men in the villages of regions in which khat is produced are regular chewers (Mela and McBride, 2000).

Figure 1.Leaves of *Catha edulis* (Vahl) Endl(khat) plant.

Cultivation of khat

Khat is cultivated by farmers across a wide geographical area: the southern shores of the Red Sea, the Southern parts of the Arabian Peninsula, the mountains of the Yemen Arab Republic, and Eastern and Southern African regions, including the Harar Plateau of Ethiopia, the Meru districts of Kenya, the Jima district of Ethiopia, Tanzania, Uganda and Zimbabwe (McKee, 1987).

Pharmacology of khat

The constituents of khat vary with the geographical location of the plant. Fresh khat leaves contains alkaloids of the phenylalkylamine type (basic fraction) known as khatamines, more than 14 alkaloids of the sesquiterpene polyester type (weakly basic fairly lipophilic fraction) known as cathedulins, large amount of polyphenols (including tannins and flavonoid glycosides) and volatile oil, triterpenes, sterols, amino acids, ascorbic acid and sugar alcohols (Dhaifalah and Santavý, 2004).

Cathinone is the main psychoactive alkaloid of fresh khat leaves and has the same indirect sympathomimetic mechanism of action as amphetamine. Therefore, cathinone may be called, like khat, the "natural amphetamine". (+)-Norpseudoephedrine and (-)-norephedrine (cathines) are much less active than cathinone. Both of khat's major active ingredients cathine and cathinone are phenylalkylamines. Cathinone and cathine have a very similar molecular structure to amphetamine (Al-Hebshi and Skaug, 2005).

During chewing, the alkaloids from khat leaves are effectively liberated with about 80% of cathinone and cathine, and over 90% of norephedrine. The absorption of the constituents of khat is said to have two phases, the first being at the buccal mucosa, plays a major role in the absorption of alkaloids. The second phase is following swallowing of the juice, at the stomach and/or small intestine (Toennes et al., 2003).

A study done on five volunteer healthy adults by Halket et al. (1995)revealed that the euphoric effects of khat start after about 1h of chewing. Blood levels of cathinone start to rise within 1h and peak plasma levels are obtained 1.5-3.5 h after the onset of chewing. The study also showed that maximum plasma levels ranged from 41-141 ng/ml (mean 83 ng/ml) after a 1h chewing dose of 60 g fresh khat leaves per subject (cathinone: 0.8-1 mg/kg body weight). Cathinone was barely detectable at 7.5 h and not detectableafter 24 h.

A double blind controlledstudy by Widler et al. (1994) on six drug-naive volunteers receiving a single dose of khat corresponding to 0.8 mg/kg body weight showed that maximal plasma concentrations of cathinone (127±53 ng/ml) were attained after 127±30 min. The terminal elimination half-life was 260±102 min. Maximal plasma concentrations of norephedrine (110±51 ng/ml) and norpseudoephedrine (89±49 ng/ml were observed after 200±134 and 183±73 min, respectively (Fig. 2).

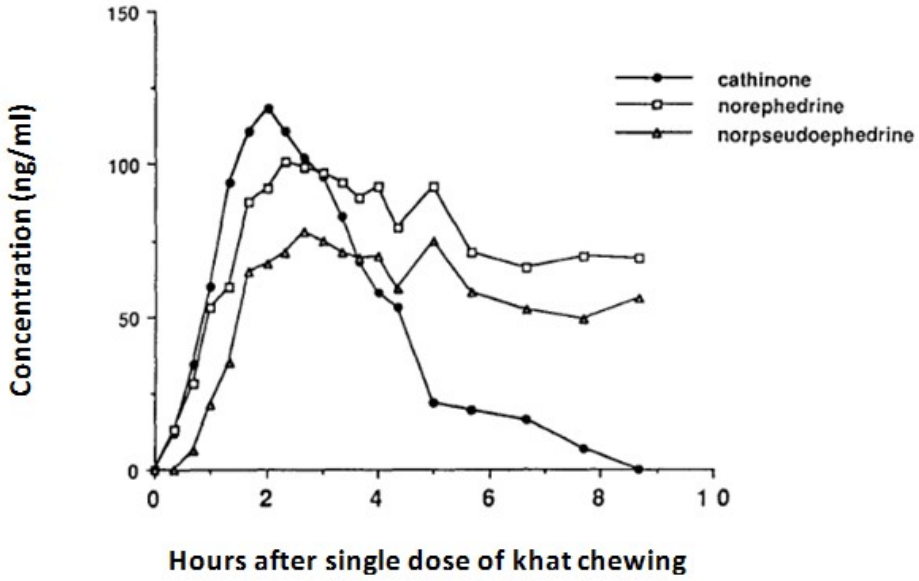

Figure 2. Mean plasma concentrations of cathinone, norephedrine, and norpseudephedrine after chewing standardized khat leaves (equivalent to 0.8 mg cathinone/kg body weight). The data represent mean values of six subjects.Adapted from Wilder et al., 1994.

The main metabolite of cathinone was identified as (–)-norephedrine. Metabolism is rapid and occurs during first passage through the liver. Only 2% of administered cathinone was found unchanged in the urine. In humans, norephedrine and norpseudoephedrine are slowly absorbed and excreted almost unchanged in urine (Brenneisen et al., 1986). The enzymes involved in the metabolism of khat have not yet been characterized, although it is predicted from the metabolic pathways of amphetamines and synthetic cathinones that major cytochrome P450 (CYP) might be involved (Aklillu et al., 2002). A recent study hypothesized the potential role of CYP2D6 in the metabolism of cathinone (Bedada et al., 2018).

There is a body of evidence that accounts for the central nervous system (CNS) effects of khat. The effects observed following khat consumption are generally of central stimulation and include euphoria, excitation, anorexia, increased respiration, hyperthermia, analgesia and increased

sensory stimulation.Cathinone has a more rapid and intense action compared with cathine due to its higher lipid solubility which facilitates access into the CNS (Patel, 2000). The stimulatory effect of khat is perceived as an increase in alertness and energy and relief from fatigue. Indeed, these effects have been reproduced in rats after oral administration of different concentrations of khat(Hassan et al., 2007).

1.2. Cannabinoids and the endocannabinoid system

The *Cannabis sativa* plant (Fig. 3) has been exploited for medicinal, agricultural and spiritual purposes in diverse cultures over thousands of years. Cannabis has been used recreationally for its psychotropic properties, while effects such as stimulation of appetite, analgesia and anti-emesis have led to the medicinal application of cannabis. Indeed, reports of medicinal efficacy of cannabis can be traced back as far as 2700 BC, and even at that time reports also suggested a neuroprotective effect of the cultivar. The psychoactive component of cannabis resin, Δ^9-tetrahydrocannabinol (Δ^9-THC), was first isolated in 1964 and at least 70 other structurally related 'phytocannabinoid' compounds have since been isolated. The development of synthetic cannabimimetic drugs has aided in the pharmacological characterization of an endogenous system which responds to cannabis. However, it was the serendipitous identification of a cannabinoid G-protein coupled receptor (GPCR) at which cannabinoidcompounds are active in the brain, which heralded an explosion in endocannabinoid research (Scotter et al., 2010).

Figure 3.Leaves of the *Cannabis sativa* plant.

The endocannabinoid system (ECS) is a signaling system composed of cannabinoid receptors (CBRs), endogenous ligands or endocannabinoids (eCBs) for these receptors and proteins involved in the formation and deactivation of these endogenous ligands(Scotter et al., 2010).

Endocannabinoids

Endocannabinoids are lipid mediators that exert most of theirfunctions by binding and activating CBRs. The two most thoroughly studied endocannabinoids are arachidonic acid derivatives, the *N*-acylethanolamine*N*-arachidonoylethanolamine (anandamide) and themonoacylglycerol, 2-arachidonoylglycerol.Their production from cell membrane lipid precursors is activity-dependent, and their actions are terminated via hydrolysis by specific lipases(Alhouayek and Muccioli, 2012).

It is generally accepted that anandamide is generated by calcium dependent enzymatic transfer of arachidonic acid from the *sn*-1 position of membrane phospholipids to the primary amine of phosphatidylethanolamine to form *N*-arachidonoylphosphatidylethanolamine, followed by hydrolysis to give anandamide. Multiple mechanisms and putative anandamide biosynthetic enzymes have been suggested, including i) direct liberation of anandamide byan *N*-acyl phosphatidylethanolamineselective phospholipase D enzyme; ii) sequential *O*-deacylation of *N*-

arachidonoylphosphatidylethanolamineby the lyso(*N*-arachidonoylphosphatidylethanolamine)-lipase α-β hydrolase 4 and cleavage of the phosphodiester bond by the glycerophosphodiesterase; iii) *O*-deacylation of *N*-acyl phosphatidylethanolamineby phospholipase A2 and hydrolysis of the phosphodiester bond by a lyso- phospholipase D enzyme and finally, iv) conversion of *N*-arachidonoylphosphatidylethanolamineto phospho-anandamide by a phospholipase C-like enzyme followed by dephosphorylation by the tyrosine phosphatase PTPN22 or the inositol 5′ phosphatase SHIP (Liu et al., 2008).

As shown in Fig. 4, 2-arachidonyl glycerol is synthesized from arachidonoyl-containing diacylglycerol species by *sn*-1-specific diacylglycerol lipase-α and -β. Characterization of diacylglycerollipase(–/–) mice confirmed a primary role for diacylglycerol lipase-α in 2-arachidonyl glycerol formation in the brain and diacylglycerol lipase-β in peripheral tissues such as the liver. Diacylglycerolprecursors are themselves synthesized from membrane phospholipids with most evidence suggesting that the major 2-arachidonyl glycerol biosynthetic pathway is hydrolysis of *sn*-2 arachidonoyl phosphatidylinositol 4,5-bisphosphate species by phospholipase C-β (Hashimotodani et al., 2005).

After cellular uptake, anandamide and 2-arachidonyl glycerol are subject to metabolism by the fatty acid amide hydrolase and monoacylglycerol lipase, respectively. In addition, anandamide and 2-arachidonyl glycerol have been shown to undergo oxidation by cyclooxygenase-2 and the 12- and 15-lipoxygenases. Catalytic degradation/modification of anandamide and 2-arachidonyl glycerol not only serves as a mechanism for the augmentation of cellular uptake and cessation of extracellular signaling but also regulates the intracellular signaling events of these two endocannabinoids(Di Marzo, 2006).

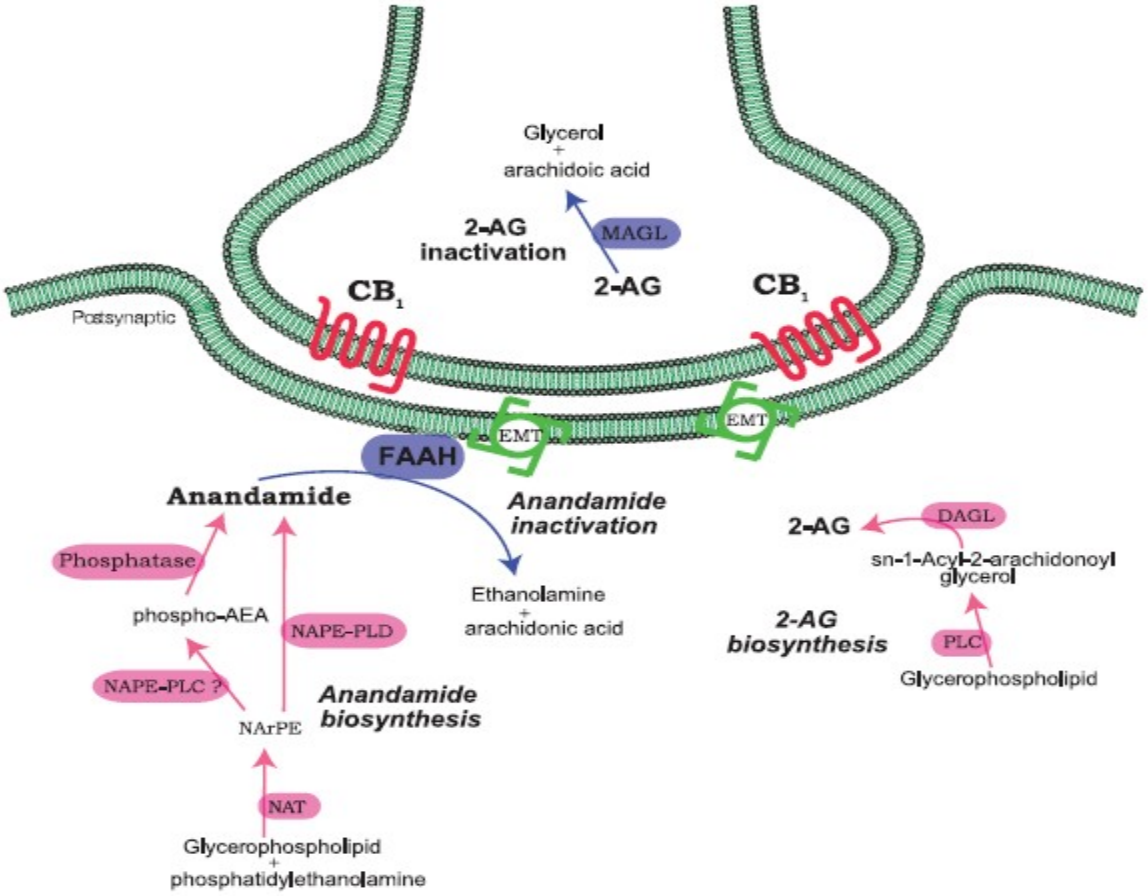

Figure 4. Schematic representation of biosynthesis and inactivation of the endocannabinoids.Adapted from Pál Pacher et al. (2006). EMT, endocannabinoid membrane transporter; MAGL, monoacylglyceride lipase; DAGL, DAG lipase; AEA, anandamide; NArPE, *N*-arachidonylphosphatidylethanolamine; NAT, *N*-acyltransferase.

Cannabinoid receptors

Two CBRs have been identified, the cannabinoid type 1 receptors (CB1Rs) and the cannabinoid type 2 receptors (CB2Rs). They differ in signaling mechanisms and tissue distribution. Activation of CBRs causes inhibition of adenylatecyclase (AC), thus inhibiting the conversion of ATP to cyclic AMP. Other mechanisms have also been observed, e.g. interaction with certain ion channels. Both CB1Rs and CB2Rs belong to the large family of the GPCRs (Pertwee and Ross, 2002).

Various studies have mapped the localization of CBRs in tissues and at a subcellular level, and these have been critical to our understanding of the effects of cannabinoids in disease. CB1Rs are expressed in both the CNS and periphery. They are the most abundant GPCRs in the brain, with high expression levels in the basal ganglia nuclei and moderately high expression in the hippocampus, cerebellum and neocortex. At the subcellular level, CB1Rs have been localized to pre-synaptic terminals, and is found at significantly higher levels on GABAergic than glutamatergic neurons in various brain regions (Scotter et al., 2010).

CB2Rs are found in particular abundance in peripheral organs with immune function, including macrophages, spleen, tonsils, thymus and leucocytes, as well as the lung and testes. Previously it is believed that CB2Rs are found only in the periphery. In contrary to these previous thoughts (Poso and Huffman, 2008), a large body of recent evidence demonstrates CB2Rs expression in the brain with their presence detected on microglia and neurons in the hippocampus, striatum and brain stem (Brusco et al., 2008; Van Sickle et al., 2005). CB2Rs are expressed in mouse brain dopamine neurons and are involved in drug reward, synaptic plasticity, drug addiction, eating disorders, psychosis, depression, and autism spectrum disorders(Liu et al., 2017).

Two orphan GPCRs have recently emerged as potential non-CB1/CB2 GPCRs for endocannabinoids. These are GPR55, which is reportedly activated by various plant and synthetic cannabinoids, and GPR119, suggested to be a receptor for oleoylethanolamide(Overton et al., 2006).

GPR55 was identified as an orphan GPCR in the purinergic subfamily, most closely related to two other orphan GPCR's, GPR35 and GPR23, and the purinoceptor P2Y5. The human GPR55 gene and its mRNA transcripts have been detected in the caudate nucleus and putamen, but not in the hippocampus, thalamus, pons, cerebellum, and frontal cortex. GPR119 is expressed predominantly in the pancreas and gastrointestinal tract (Fredriksson et al., 2003). It is coupled to Gαs in insulin-producing β-cells of the pancreatic islets, and functions as a glucose dependent insulinotropic receptor. Its targeting was suggested to be useful for the development of potent, orally active, small-molecule insulin-releasing compounds. One of these, AR231453, increases cAMP accumulation via Gαs coupling to AC, and insulin release, and enhances glucose-dependent insulin release in vivo, while improving oral glucose tolerance in wild-type mice but not in GPR119-deficient mice (Chu et al., 2008).

Cannabinoid receptor signaling

CB1 and CB2 receptors couple primarily to the Gi/o subtypes of G protein and their signaling is remarkably complex. Although coupling to AC through Gi/o usually results in inhibition of cyclase activity through the release of Giα isoforms, cannabinoids can also stimulate isoforms 2, 4, or 7 of AC via the release of βγ subunits (Rhee et al., 2002). Activation of AC also occurs when CB1 and dopamine D2 receptors are simultaneously activated, probably as a result of heterodimerization of these two types of receptors. Although direct evidence for the coupling of

CB1 receptors to Gq/11 had until recently been lacking, the agonist WIN-55,212-2, but not other cannabinoids, has recently been reported to increase intracellular calcium in cultured hippocampal neurons and in human embryonic kidney 293 cells via coupling to Gq/11 proteins (Lauckner et al., 2005).

Receptor dimerization may facilitate such coupling, which may account for CB1-mediated mobilization of intracellular calcium in NG108-15 neuroblastomaglioma cells. Cannabinoids can also inhibit different types of calcium channels and activate certain potassium channels via G protein βγ subunits. Cannabinoids can activate members of all three families of multifunctional mitogen-activated protein (MAP) kinases, including p44/42 MAP kinase as well as Jun-terminal kinase and activate the phosphatidylinositol-3-kinase pathway. These effects could be via G protein activation or pathways independent of G proteins via other adaptor proteins. Another G protein-independent pathway activated by cannabinoids involves G protein-coupled receptor kinase-3 and β-arrestin-2, which are required for desensitization, but not for internalization, of CB1Rs, and the related development of tolerance. Cannabinoids can also regulate the activity of phosphatases, as exemplified by the CB1-mediated regulation of calcineurin (protein phosphatase 2β) or the activation of MAP kinase phosphatase 1, which plays an important role in the anti-inflammatory action of anandamide(Pal Pacher et al., 2006).

Different structural classes of cannabinoid receptor agonists have the unique ability to activate different signaling cascades which, in turn, influence agonist efficacy. Using an *insitu* receptor/G protein reconstitution technique, CB1Rs were found to efficiently couple and activate both Gi and Go, whereas CB2Rs only activated Go. Furthermore, the efficacy of a given agonist was different whether CB1Rs coupled to Gi or Go, demonstrating agonist-selective G protein signaling (Glass and Northup, 1999). Prather et al. (2000)found that the aminoalkylindol

agonistWIN-55,212-2 activated different Giα subunits with markedly different potencies. Even more striking is the recent finding that demonstrates cannabinoid agonist-selective activation of different Giα subunits. A possible practical implication of such findings is that unique therapeutic profiles may be achieved through the use of different agonists for the same receptor, and such profiles may differ from one target tissue to the other, depending on the pattern of G protein subunit expression.

At least part of this agonist selectivity in G protein activation may be related to the existence of distinct binding sites on CB1Rs for different classes of ligands, as documented by site-directed mutagenesis and molecular modeling studies. These studies indicate that a K3.28A mutation in the third transmembrane domain caused a more than 1000-fold loss in affinity and loss of efficacy for anandamide and nonclassic cannabinoids, without affecting the affinity for WIN-55,212-2. In contrast, mutations at different sites in the third, fifth, and sixth transmembrane helices (F3.36A, W5.43A, and W6.48A) affected the binding of WIN-55,212-2 and SR141716, but not anandamide(McAllister et al., 2003).

Another important feature of cannabinoid signaling in the brain is the lack of correlation between the density of CB1Rs in a given brain region and the efficiency of receptor coupling, as determined by GTPγS binding, which may explain why functionally important responses can be triggered in brain regions with very sparse CB1R expression, such as the brainstem or the hypothalamus(Jamshidi and Taylor, 2001). Selley et al. (2001)have shown that the reduction in CB1R density in CB1 heterozygote mice was compensated for by an increase in receptor/G protein coupling efficiency for some, but not other, agonists. Although the underlying mechanisms for such compensation are not clear, differences in the degree of receptor multimerization, or changes in signal amplification are possibilities. Recent observations indicate

that a considerable proportion of the psychomotor effect of cannabinoids can be accounted for by a signaling cascade in striatal projection neurons involving protein kinase A-dependent phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), achieved via modulation of dopamine D2 and adenosine A2A transmission. This represents a unique form of amplification of CB1 signaling, as phosphorylation of DARPP-32 at Thr-34 amplifies downstream signaling via inhibition of protein phosphatase-1 (Mackie, 2005).

The mechanisms of endocannabinoid signaling in the nervous system differ considerably from those of the classic neurotransmission systems (e.g., cholinergic, amino-acidergic, and monoaminergic). In the classic model of neurotransmission, depolarization of the presynaptic neuron by an action potential results in the release of neurotransmitters, which then traverse the synaptic cleft to bind and activate their cognate receptors on the postsynaptic neuron. In contrast, eCBsignaling appears to occur via a retrograde mechanism (Fig. 5), where stimulation of the postsynaptic neuron triggers the biosynthesis of endocannabinoids, which are released and transported by poorly understood mechanisms to activate CB1Rs expressed primarily on the presynaptic terminal. CB1 activation of Gi/o proteins initiates a signaling cascade that regulates calcium and potassium channels and ultimately suppresses further neurotransmitter release. In this model, eCB signaling modulates transmission efficiency by facilitating communication from the postsynaptic to the presynaptic neuron. As CB1 activation acts to inhibit neurotransmission, the ultimate outcome of eCBsignaling depends on the nature of the participating cells (Howlett, 2005).

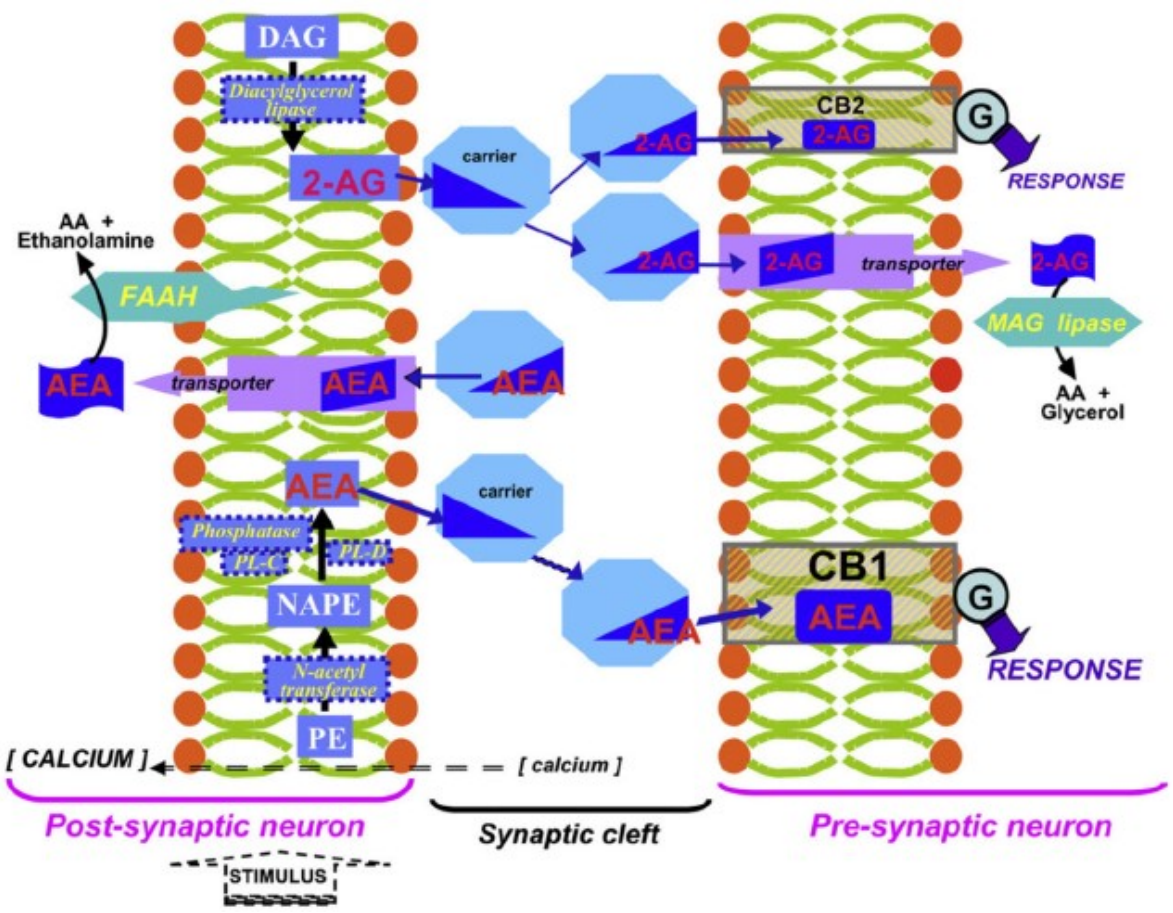

Figure 5.Retrograde cannabinoid signaling.Adapted from Vemuri et al. (2008). In response to a stimulus that induced intracellular calcium increase, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized in post-synaptic neurons. The endocannabinoids are then released from postsynaptic neurons and traverse the synaptic cleft (aided, perhaps, by carrier proteins) to presynaptic neurons, where they function as retrograde messengers by binding to cannabinoid receptors: AEA has the greater affinity for the CB1 receptor, whereas 2-AG binds preferentially to the relatively minute population of CB2 receptors in the CNS. Presynaptic monoacylglycerol (MAG) lipase inactivates 2-AG, and post-synaptic fatty acid amide hydrolase (FAAH) inactivates AEA.

Electrophysiological experiments have yielded data that strongly support the hypothesis that increases in intracellular calcium caused by strong depolarization of postsynaptic hippocampal pyramidal cells or cerebellar Purkinje cells rapidly trigger the biosynthesis and non-vesicular release of eCBs. These are then thought to act through presynaptic CB1Rs to inhibit the presynaptic release of GABA from hippocampal neurons (depolarization-induced suppression of inhibition) or of glutamate from cerebellar climbing fibers that originate in the inferior olive or from parallel fibers of cerebellar granule cells (depolarization-induced suppression of excitation). It is noteworthy that whilst depolarization-induced suppression of excitation should provide a negative feedback mechanism for damping down high synaptic activity, depolarization-induced suppression of inhibition is expected to exacerbate intense synaptic activity (Pertwee and Ross, 2002; Sidhpura and Parsons, 2011).

1.3. Behavioral effects of khat and cannabinoids

Anxiety-like behavior

The open field simultaneously provides measures of locomotion, exploration and anxiety. In the test, increased number of centre square frequencies and duration are indicative of low anxiety. On the other hand, reduced line crossings and rearing frequencies are reflective of a higher level of anxiety (Podhorna and Brown, 2002).In a study done to enumerate the effect of single and daily khat on locomotor behavior in mice, khat had mixed effects on the levels of anxiety depending on the parameter looked at. Based on line crossing, the results are indicative of enhanced exploration and reduction of anxiety in mice in a dose-dependent manner. On the other hand, the result of the effect of khat on rearing and center square frequencies are indicative of enhancement of anxiety levels, with high doses increasing it while low doses reducing the

anxiety. The effect of khat on anxiety and locomotor activity is modulated by dopamine and serotonin(Kimani et al., 2008). A study done to evaluate the acute effects of hydro-alcoholic khat extract on locomotor activity and anxiety behavior in mice revealed that, khat extract significantly enhanced anxiogenic behavior progressively following the initial 15 - 20 min anxiolytic effect (Gebremariam et al., 2017).

Cannabinoids are able to display both anxiogenic- and anxiolytic-like effects depending upon doses, animal models, specific test conditions, and strains (Onaivi et al., 1990). Overall, low doses of cannabinoid agonists usually induce an anxiolytic-like effect, whereas higher doses cause the opposite response. Some results suggest that the ECS is involved in the control of emotional behavior via CB1Rs. Neuroanatomical studies showed that this receptor is expressed at high levels in brain regions involved in the control of fear and anxiety, such as the basolateral amygdala, the anterior cingulate cortex, the prefrontal cortex (PFC), and the paraventicular nucleus of the hypothalamus (Tsou et al., 1998). Furthermore, both exogenous and endogenous cannabinoids have been found to activate the hypothalamic–pituitary–adrenal axis, the neuroendocrine system implicated in responses to emotional stress. Acute intracerebroventricular administration of anandamide or Δ^9-THC stimulates the release of adreno-corticotropin hormone and corticosterone, probably via a central mechanism, which involves the secretion ofcorticotropin releasing factor (Wenger et al., 1997). This result suggests that hypothalamic–pituitary–adrenal axis activation plays an important role in the mediation of cannabinoid–induced anxiogenic patterns.

Cognition and memory

Kimani and Nyongesa (2008)reported that khat has differential effects on learning and memory task in mice depending on dose, with low dose having no effect on learning but impairing memory, whereas high dose impairs learning but improves memory. In a study done to evaluate the effect of exposure to crude khat on learning and memory using a host of behavioral paradigms, acute, subacute, and subchronic exposure tokhat does not have any effect in learning and long-term memory. However, subchronic exposure to khat significantly decreased short-term memory, without any effect in long-term memory (Mohammed et al., 2014).Bogale et al., (2016)also showed that subchronic oral administration of khat reduced cognition and impaired short term and long term memory in mice. A study done to investigate the effect of acute exposure to crude khaton spatial learning and memory in mice using multiple T-maze test revealed that, khat did not show any improvement in learning and memory compared to placebo at all doses (Assefa et al., 2018).

Administration of both synthetic and phytocannabinoids, including Δ^9-THC, WIN-55,212-2, and CP 55,940, impair working memory and short-term memory through a CB1Rs-mediated mechanism in rats (Braida and Sala, 2000; Egashira et al., 2002). The cellular and molecular mechanisms underlying learning and memory deficits produced by cannabinoids, and the role of eCBsin such mechanisms, have been investigated in a number of studies using *in vivo* and *in vitro* systems. *In vitro* experiments indicated that cannabinoids and eCBs produce persistent changes in memory-related neuronal activity. The dense localization of CB1Rs in the hippocampus and amygdala, which play an important role in learning and memory, may

represent the anatomical substrate for cannabinoids to influence mnemonic processes (Gerdeman and Lovinger, 2003).

Δ^9-THC impairs memory in rodents and monkeys tested in a variety of experimental procedures (radial maze, instrumental discrimination tasks, Morris water maze). The effects exerted by CBR agonists, including Δ^9-THC, WIN-55,212-2, CP 55,940, and anandamide, are reversed by the CB1R antagonist, rimonabant, providing evidence for the involvement of CB1Rs-related mechanisms. Although eCBs mimic the pharmacological effects of cannabinoids, experiments carried out by the latter group have shown that anandamide impairs memory consolidation in random bred mice, and exerts genotype-dependent influences on memory in inbred strains of mice. The mechanism by which cannabinoids and eCBs influence learning and memory may be by directly acting on the CB1Rs in the hippocampus or through modulation of the release of other neurotransmitters, such as glutamate and acetylcholine (Castellano et al., 2003).

Locomotor activity

Once cathinone was identified as an active constituent of khat, there have been investigations of its effect on animal behavior, particularly on locomotor activity. Subcutaneous administration of cathinone in rats markedly increased spontaneous locomotor activity of the animals. It was reported that the potency of cathinone was almost comparable with (+)-amphetamine (Banjaw et al., 2003; Kimani and Nyongesa, 2008). The occurrence of strong behavioral sensitization after repeated intermittent oral administration of khatleaves or cathinone in rats was demonstrated in several studies. The rats developed sensitization for locomotor activity, rearing, upward and downward sniffing, and turning after oral administration of the extract which was also observed with cathinone and amphetamine (Banjaw and Schmidt, 2005; Connor et al., 2002). Geresu and

Engidawork (2010) reported that, acute and subacute administration of khat at 200 and 300 mg/kg dose as well as amphetamine (50 mg/kg) produced a consistent improvement in motor performance in mice.

It is now well accepted that the control of movement is one of the more relevant physiological role of the eCBs in the brain. Synthetic, plant-derived and endogenous cannabinoids have powerful actions on motor activity in animals. In fact, these effects are bidirectional, depending on the dose. Large doses of cannabinoid reduced motor activity in a variety of behavioral tests and even produced strong catalepsy, whereas low doses stimulated motor activity as indicated by hyperlocomotion in intact animals, and ipsilateral circling in rats with unilateral 6-hydroxydopamine lesion of the substantianigra(Chaperon and Thiébot, 1999; Fernández-Ruiz et al., 2002).

Likewise, low doses (0.01 mg/kg) of anandamide enhanced, and moderate or high doses (10-100 mg/kg) reduced motor activity in rodents. However, although the overall pharmacological activity of eCBs is similar to that of exogenous cannabinoids, there are also differences, and it is clear that anandamide has partial effects for some behavioral components(Sulcova et al., 1998). Moreover, when different routes of anandamide administration were compared, a complex pattern of full and partial agonist activities was observed. Additional behavioral differences include the inhibition by very low doses (0.0001-0.01 mg/kg) of anandamide and docosahexaenylethanolamide, a synthetic endocannabinoid-like compound, but not Δ^9-THC, of the pharmacological effects of conventional doses of Δ^9-THC. There is also evidence that, rimonabant prevented the motor effects of CBR agonists (Chaperon and Thiébot, 1999).

Since the functional neuronal expression of CB2Rs has been a subject of controversy and debate, their role in CNS effects of cannabinoids was not well studied. A recent study using dopamineneuron cell type-specific CB2R conditional knockout (cKO) mice showed that CB2Rs in dopamine neurons are involved in motor function, and their deletion releases the “brake” on psychomotor activity and results in continuous spontaneous hyperactivity(Liu et al., 2017).

1.4. Dopaminergic signalingin behavioral effects of khat and cannabinoids

Dopamine has prominent involvement in core behavioral processes including motor control, motivation, learning, and memory and contribution to several neuropsychiatric disorders like Parkinson’s disease, schizophrenia, and drug addiction (Iversen et al., 2010).

It is synthesized from tyrosine by the ratelimiting enzyme tyrosine hydroxylase (TH), to produce L-DOPA which is quickly decarboxylated by L-aromatic acid decarboxylase to dopamine. Intraneuronaldopamine is accumulated into synaptic vesicles by the vesicular monoamine transporter-2. Dopamine released into the extracellular space exerts its physiological functions via activation of G protein-coupled D1-like and D2-like dopamine receptors. Finally, dopamine in the extracellular space is subject to dilution by diffusion and metabolic degradation; however the major route of clearance from the extracellular space in the striatum/nucleus accumbens is the rapid recycling of the neurotransmitter back into dopaminergic terminals by the Na^{+}/Cl^{-} dependent plasma membrane dopamine transporter (DAT). Recycled dopamine in the dopaminergic terminals is then stored in the large intracellullar storage pool available for subsequent re-release (Sotnikova et al., 2005).

The major groups of dopaminergic neurons are classified as A8-A17 (Fig. 6). These groups are functionally divided in to four main groups, each of which has activity with a unique set of physiological and psychological effects (Andrade, 2010). The mesencephalic or midbrain dopaminergic neurons comprised of groups A8-A10, the diencephalic dopaminergic neurons comprised of groups A11-A15, dopaminergic neurons in the olfactory bulb (A16), and the dopaminergic neurons located in the retina (A17)(Melis and Argiolas, 1995).

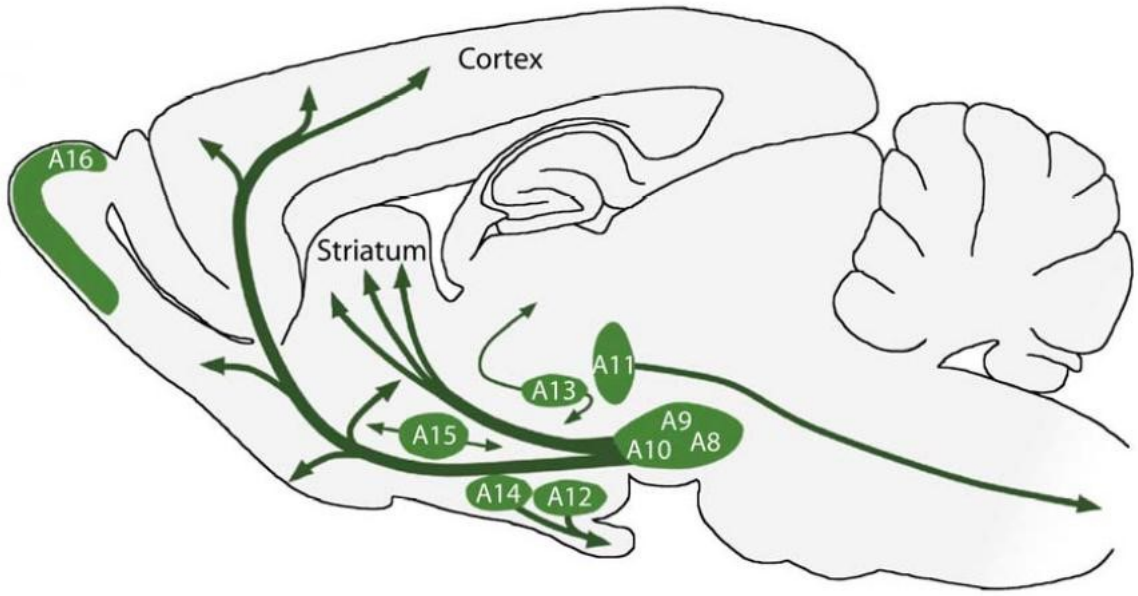

Figure 6. Distribution of dopamine neuron cell groups in rat brain. Adapted from Melis and Argiolas, 1995.The dopaminergic neurons in the midbrain of the rat are located in three cell groups: nucleus A8 cells in the retrorubral field, nucleus A9 cells in the substantianigra, and nucleus A10 cells in the ventral tegmental area and related nuclei.

The mesencephalic, or midbrain, dopaminergic system is further sub-divided into three separate pathways, the nigrostriatal, mesolimbic, and mesocortical pathways, all of which originate from the A8-A10 cell groups. These dopaminergic neurons originate in several neighboring mid brain nuclei, including the substantianigraparscompacta (SNc; A9) and ventral tegmental area (VTA; A10). The nigrostriatal dopaminergic pathway consists of neurons whose cell bodies originates primarily from the A9 group of SNc and to a lesser degree fromA10 neurons of the VTA and terminate in to the dorsal striatum structures including the caudate, putamen, and globuspallidus, and is important in the regulation and coordination of locomotor activity(Ungerstedt, 1976).

Unlike the nigrostriatal pathway, majority of the neurons that make up the mesolimbic dopaminergic pathway originate from the A10 neurons of the VTA, with fewer neurons originating from the A8 and A9 groups, and project to the nucleus accumbens, amygdala, and olfactory tubercle. In addition to its role in the regulation of affect, emotion, and locomotor activity, the mesolimbic dopaminergic pathway has also been implicated in reward and pleasure, and is often referred to as the "reward pathway" of the brain. The mesocortical dopaminergic pathway projects to various areas of the PFC, including the orbitofrontal, medial, dorso lateral and cingulate regions (Abi-dargham and Moore, 2003). The mesocortical dopaminergic neurons appear to be important for social behavior, working memory, attention, and executive function (Bubser and Schmidt, 1990; Floresco and Magyar, 2006; Sawaguchi and Goldman-Rakic, 1994).

Studies showthat the behavioral effect of cathinone in animals is believed to be mediated by the dopaminergic system, similar to amphetamine (Al-Hebshi and Skaug, 2005; Feyissa and Kelly, 2008; Fleckenstein et al., 1999). Radio-labelling studies in rabbits have shown that cathinone increases the level of dopamine in a dose-dependent fashion in the nucleus accumbens and striatum, suggesting its role in dopaminergic neurotransmission (Mela and McBride, 2000).

Indeed, similarly to amphetamine, cathinone inhibits the activity of dopamine, noradrenalin and serotonin transporters, as observed at synaptic level in the striatum of rats treated with the alkaloid (Al-Hebshi and Skaug, 2005; Fleckenstein et al., 1999). Unlike cathinone, there is little evidence in existing literature on the role of the dopaminergic pathway in the behavioral effects of khat. Earlier studies revealed that dopamine has been shown to have modulatory effects onkhatinduced stereotyped movement, locomotor activity, aggression and sexual arousal and the possible involvement of the dopaminergic system on these measures(Abdulwaheb et al., 2007; Banjaw et al., 2006; Connor et al., 2002; Geresu et al., 2016).

The involvement of the ECS inbrain functions is likely the consequence of its capability to interact with specific neurotransmittersin several brain regions. Dopamine is one of the neurotransmitters thathasbeen more frequently linked to the action of cannabinoidswithin the CNS. This can be applied to the caseof those dopaminergic neuronal subpopulations, whosecell bodies are located in the reticular formation of the midbrain (e.g., SNcand VTA), and that project to different forebrain structures, namely, the caudate-putamen (nigrostriatalpathway), and the nucleus accumbens/PFC complex (mesocorticolimbic pathway). Both neuronal systems would exert a regulatory action on differenteffector neurons in these structures, then influencing processes such as the control of movement and variouscognitive functions, respectively, effects that are among the most relevant pharmacological actions of cannabinoids(Fernández-Ruiz and González, 2005; Gerdeman and Fernández-Ruiz, 2008).

Cannabinoids effects on dopamine transmission are frequently indirect and exerted by either postsynaptic or presynaptic mechanisms. The abundance of CB1Rs in GABAergic, glutamatergic or opioidergicprojections located in the closest vicinity of dopaminergic neurons facilitates such indirect action. This is also supported by data showing that midbrain

dopaminergic neurons, which do not contain CB1Rscan, however, produce and release eCBligands from their somas and dendrites, then facilitating the retrograde signaling function of these molecules via CB1Rsreceptors in excitatory and inhibitory synapses(Fernández-Ruiz et al., 2010).

Despite that most of cannabinoid effects on dopamine transmissionseem to be GABA- and/or glutamate-mediated, studies have provided an additional mechanism available for those eicosanoid-derived cannabinoids that have demonstrated some affinity for the transient receptor potential vanilloid (TRPV)-1 receptor (e.g., anandamide, AM404 or *N*-arachidonoyl-dopamine). These receptors are molecular integrators of nociceptive stimuli, abundant on sensory neurons, but they have been found in dopaminergic neurons within the basal ganglia too, thus enablinga direct action of certain cannabinoids on dopamine function(Starowicz et al., 2007).

In fact, there is evidence demonstrating that endovanilloid and dopamine signaling systems are closely linked in the regulation of various neurobiological processes including the control of movement. Activation of CB2Rs and the TRPV-1 receptors located in nigrostriatal dopaminergic neurons allowa direct regulation of dopamine transmission by specific cannabinoids. In addition, CB1Rs form heteromers with dopaminergic receptors which provide another pathway to direct interactions between both systems, in this case at the postsynaptic level. Interaction of cannabinoids with dopaminergic transmission in the basal ganglia is likely to have important effects on dopamine-related functions in these structures (García et al., 2016).

1.5. Rationale for the study

Several lines of experimental evidence on wild type (WT) and cannabinoid receptor manipulated animals have shown the role of the ECS on the effect of psychoactive substances, including opioids (Scavone et al., 2013), nicotine (Viveros et al., 2006; Werling et al., 2009) and cocaine (Xi et al., 2011). So far, there is no evidence for involvement of the ECS in the behavioral effects of khat. In this study, the involvement of the ECS on the neurobehavioral effect of khat was investigated in DAT-*Cnr2*cKO mice with selective deletion of CB2Rs in dopamine neurons, C57BL/6J WTand BALB/c albino mice following concomitant administration of khat and the CB2R agonists. In addition, the behavioral effect of sub-acute exposure of khat was investigated in C57BL/6J mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). There is no literature on the effect of khat on MPTP-induced damage to mouse dopaminergic neurons. Accordingly, the present study investigated whether khat has a protective effect on dopaminergic neurons or not.

Dysregulation of the dopaminergic pathway may cause different dopamine related CNS disorders including PD and psychosis. It is well established that dopamine neurotransmission in both dorsal and ventral striatum is essential for normal locomotor functions, and progressive degeneration of dopamine neurons in these areas is a known cause of PD. PD is a progressive neurodegenerative disease with an unknown pathogenesis, and the loss of substantianigra dopaminergic neurons is characteristic of its lesions (Sotnikova et al., 2005).

In PD, there is a good correlation between severity of motor symptoms, particularly bradikynesia and rigidity and dopamineloss in the striatum and thus treatment has focused almost exclusively on the replacement of dopamine. Levodopa, a precursor to dopamine, gained Food & Drug Administration approval for the treatment of PD in 1970. To date, levodopa remains the most effective and well tolerated dopamine-replacing agent, and contributes significantly to improvements in the quality of life of patients with PD. The strong evidence supporting the use of levodopa means that it is currently considered the gold standard for the symptomatic treatment of the motor features of PD(Grandas et al., 1998; Sethi, 2010).

Despite the strong heritage of levodopa as the gold standard therapy for PD, its use is restricted by several limitations including, 'dopa resistant' motor symptoms (postural abnormalities, freezing episodes, speech impairment), 'dopa resistant' non-motor signs (autonomic dysfunction, mood and cognitive impairment), and/or drug related side effects (especially psychosis, motor fluctuations, and dyskinesias)(Ogawa, 1994; Salat and Tolosa, 2013; Thanvi and Lo, 2004).Consequently, there is a need for further research including identifying new therapeutic options and drug targets for the treatment of PD and other neurodegenerative disorders.

The majority of previous studies have focused on the adverse effects of khat and very few reports exist on the beneficial aspects of thisplant. An impediment to the development of khatbased medications has been its socially unacceptable psychoactive effects. However, the abuse potential may be limited through the use of preparations with controlled composition and selective effect and the careful selection of dose and route of administration. So far, only one preclinical study (Geresu and Engidawork, 2010)attempted to assess the potential of khat in the treatment of motor dysfunctions in mice. Unlike khat, there are numerous preclinical and clinical

studies that investigate the promising effects of cannabinoids in neurodegenerative disorders like PD (Fernández-Ruiz et al., 2010).The use of khat and/or cannabinoids that modulate systems like the dopaminergic path way may result in novel therapeutic approaches in a number of CNS diseases associated with dopamine dysregulation for which current conventional treatments do not fully address patients' needs.

2. Objectives

2.1. General objective

- To investigate the involvement of the endocannabinoid system in modulating the neurobehavioral effects of khatin mice.

2.2. Specific objectives

- To study the effect of khat alone or with cannabinoid ligands on short term memoryupon acute administration in WT mice.
- To evaluate the effect of khat alone or with cannabinoid ligands on anxiety like behavior upon acute administration in WT mice.
- To study the effect of khat alone or with cannabinoid ligands on locomotor activity upon acute and sub-acute administration in WT mice.
- To studythe effect of khat alone or with cannabinoid ligands on locomotor activity upon sub-acute administration in DAT-*Cnr2*cKO mice.
- To evaluate the effect ofkhat alone or with cannabinoid ligands on neuroprotection upon sub-acute administration in MPTP lesioned mice.
- To examine TH immunoreactivity ofkhat alone or with cannabinoid ligands upon sub-acute administration in WT mice.
- To quantify DATmRNA gene expression of khat alone or with cannabinoid ligands upon sub-acute administration in WT mice.

3. Materials and Methods

3.1. Drugs and chemicals

All chemicals were of analytical grade. The non-selective cannabinoid receptor agonist, WIN-55,212-2; the CB2R agonist, JWH133; the CB1R antagonist, AM251; and the CB2R antagonist, AM630; chloroform and diethyl ether were purchased from Sigma-Aldrich Chem. Co. (St. Louis, Mo, USA) and MPTP was purchased from Cayman Chem. Co. (Ann Arbor, MI, USA).

3.2. Animals

The experiments were performed using adult male BALB/c albino, C57BL/6J and DAT-*Cnr2* cKOmice (20 - 30 g body weight) obtained from the Animal house of Addis Ababa University and William Paterson University. The BALB/c albino mice were used for acute studies, whereas, the C57BL/6J and DAT-*Cnr2* mice were used for sub-acute studies. The animals werehoused in plastic cages with standard wood chip bedding under controlled room temperature (25±2°C) and light-dark (12:12 hour) conditions with free access to food and water.The generation of DAT-*Cnr2* cKO mice, genotyping and RNAscope*in situ* hybridization has been described elsewhere (Liu et al., 2017). Briefly, we generated *Cnr2*-floxed mice that were crossed with DAT-*Cre*mice, in which *Cre*recombinase expression is under DAT promoter control to ablate *Cnr2* gene in midbrain DA neurons of DAT-*Cnr*2 cKO mice. The experimental procedures followed the Guide for the Care and Use of Laboratory Animals (IACUC) and were approved by both Addis Ababa University and William Paterson University animal care and use committee.

3.3. Collection and extraction of khat

Bundles of khat shoots and small branches were purchased fresh at a local market in Aweday, 515 km east of Addis Ababa, Ethiopia, which is one of the common natural habitats. The fresh bundles were packed in plastic bags and transported in an ice box to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University. The fresh leaves were then immediately kept in a deep freezer (-20 ºC).

Khat extractionwas performed as described elsewhere (Admassie and Engidawork, 2011). Briefly, the leaves were finely chopped with knife in a dark room, weighed by electronic digital balance and placed in an Erlenmeyer flask containing organic solvents; diethyl ether and chloroform in a 3:1 ratio. Enough volume of the solvent was added so as to cover the crushed plant material in the flask. The flask was wrapped with aluminum foil and the contents were continuously stirred using a rotary shaker at 120 rpm (New Brunswick Scientific Co, USA) for 24 h. It was then filtered using Whatman No.1 filter paper (90 mm diameter, Whatman Ltd, England) and concentrated in a hood for 24 h. The concentrated extract was then poured on a flat container and subjected to freeze drying using a lyophilizer (OPERAN Lyophilizer, KOREA). The percentage yield was found to be 1% and the extract was kept in a tightly sealed container in a deep freezer at -20ºC until use at School of Pharmacy, Addis Ababa University laboratory or its transportation in an ice bag to Biology Department, William Paterson University, USA.

3.4. Grouping and dosing of animals

The experimental design is shown in Fig. 7 below. The experiment is composed of acute and sub-acute studies. In the acute study khat extract and the different drugs were administered as a single dose, however, in the sub-acute study khat and drugs were administered once per day for seven consecutive days.The BALB/c albino mice were assigned to six groups (n=6 animals per group). Group I (CTR) received Tween 80 and served as a control. Group II (KHAT) treated with khat (300 mg/kg). Group III (WIN) received the non-selective cannabinoid receptor agonist, WIN-55,212-2 (1 mg/kg). Group IV (KHAT+WIN) pretreated with khat and then WIN-55,212-2 (1 mg/kg) 40 minafter khat administration. Group V (KHAT+AM251) pretreated with khat and then with the CB1R antagonist, AM251 (1 mg/kg) after 40 min. Group VI (KHAT+AM630) pretreated with khat and then with the CB2R antagonist, AM630 (1 mg/kg) after 40 min. The various doses for the agents were selected based on previous reports (Geresu and Engidawork, 2010; Hayase, 2013; Onaivi et al., 1990).Khat was weighed, mixed with Tween 80 to a predetermined concentration, vortexed continuously and administered orally via a feeding needle as a fine suspension. WIN-55,212-2, AM251 and AM630 were weighed and diluted with Tween 80 till the required concentration and administered intraperitoneally. Final volumes of khat or drugs were all adjusted to a uniform 1.0 ml with Tween 80 and administered as a single dose in the acute study. The vehicle was given to the control animals in the same volume and for same duration as khat or the drugs.

The C57BL/6J WT type mice were grouped into eight groups (n=6 animals per group). Group I (CTR) received the vehiclemade of tween, DMSO, and saline solution (1:2:7) and served as a control. Group II (KHAT) received khat (300 mg/kg). Group III (JWH133) received the CB2R

agonist JWH133 (5 mg/kg). Group IV (JWH133+KHAT) pretreated with khat (300 mg/kg) and then JWH133 (5 mg/kg) after 40 min of khat treatment. Group V (MPTP)injected with MPTP (25 mg/kg). Group VI (JWH133+MPTP) pretreated with JWH133 (5 mg/kg) 45 min prior to MPTP (25 mg/kg) injection. Group VII (KHAT+MPTP) pretreated with khat (300 mg/kg) 45 min prior to MPTP (25 mg/kg) injection. The last group (JWH133+KHAT+MPTP) was pretreated with a combination of JWH133 (5 mg/kg) and khat (300 mg/kg) 45 min prior to MPTP (25 mg/kg) injection. The DAT-*Cnr2* cKO mice were grouped into two (n=6 animals per group). Group I (CTR) received the vehiclemade of tween, DMSO, and saline solution (1:2:7) and served as a control. Group II (KHAT) received khat (300 mg/kg).The various doses were selected based on previous reports (Geresu and Engidawork, 2010; Liu et al., 2017). Khat, JWH133 and MPTP were weighed, mixed with the vehicle made of tween, DMSO, and saline solution (1:2:7) to a predetermined concentration, vortexed continuously and administered intraperitonially (i.p.) in a volume of 10 ml/kg body weight daily for seven consecutive days. The vehicle was given to the control animals in the same volume and for same duration as khat or the drugs.

Through-out all the experiments khatsample containers including syringes were covered with aluminum foil to avoid light decomposition. Khat and all drugs were made up fresh in the vehicle to a predetermined concentration before administration.

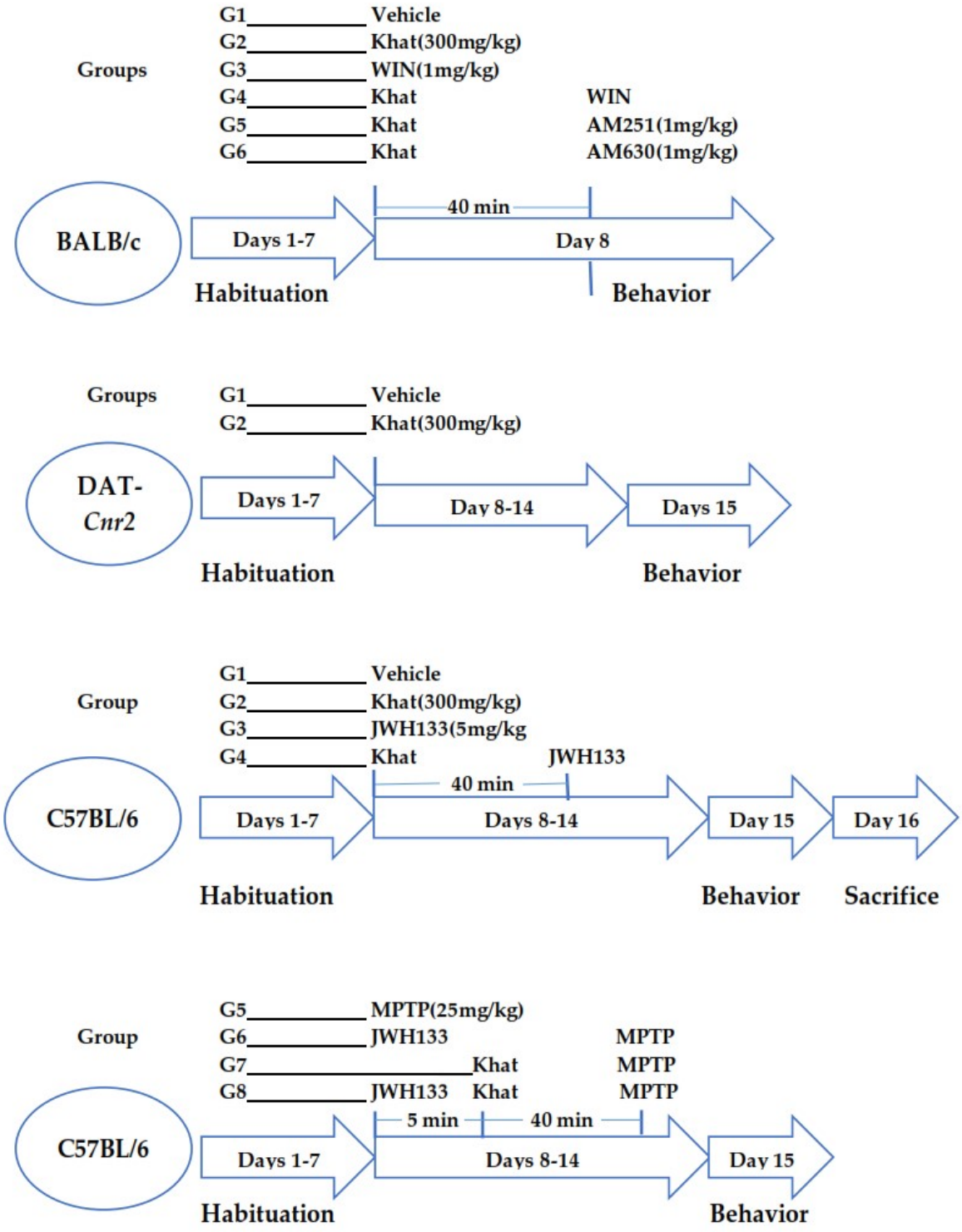

Figure 7. The schedule of drug treatments and behavioural tests.

3.5. MPTP-induced dopaminergic lesion in mice

Oxidative stress-induced mitochondrial dysfunction is hypothesized to be the main reason for the pathogenesis of PD. MPTPis commonly used to induce dopaminergic toxicity to produce neuropathologic abnormalities resembling the idiopathic PD in humans(Javitch et al., 1984). MPTP can cross the blood-brain barrier, and is converted into its active metabolite methyl-4-phenylpyridine (MPP+), which due to affinity with the dopamine transporter, is selectively transported to the mitochondria of the dopaminergic neurons. As a neurotoxic metabolite, MPP+ can block cell respiration and promote reactive oxygen species formation, thus inducing death of the dopaminergic neurons(Meng et al., 2017).

As in PD, MPTP causes greater loss of dopamine neurons in SNc than in VTA or retrorubral field. This specific and reproducible neurotoxic effect on the nigrostriatal system is the strength of this model. Although this model appears to be the best one for testing degeneration of the nigrostriatalpathway, some striking departures from PD need to be mentioned: these include the fact that 1. degeneration of dopaminergic neurons progress rapidly, i.e., days not years, 2. lesions are primarily if not exclusively dopaminergic, and 3. animals lack the typical PD proteinaceous inclusions called Lewy bodies (Blesa and Przedborski, 2014).

The neurotoxicity of MPTP is dependent on the activity of monoamine oxidase type B (MAO-B) enzyme, the enzyme that catalyzes the conversion of the MPTP protoxin to the dyhdropyrididinium intermediate, 1-methyl-4-phenyl-2,3-dihydropyridinium species (MPDP+), which is subsequently oxidized to the toxic MPP+. Thus, it has been proposed that differences in brain MAO-B levels could account for species and strain differences in sensitivity to MPTP. Rats are resistant to MPTP toxicity, and differences in MAO activity have been proposed as the

reason for their low susceptibility. The mouse strain most sensitive to MPTP, the C57BL/6 strain, is the only species in which MAO-B activity was greater in the brain than in the liver. Thus, the increased susceptibility of this mouse strain to MPTP may be due, in part, to the limited, systemic detoxification of MPTP by liver MAO-B. In this study, to investigate the role of khat in PD, a PD model in mouse was established by intraperitoneal injection of MPTP (25 mg/kg) once in a day for seven consecutive days to induce sub-acute parkinsonian symptoms in C57BL/6J mice. It is believed that sub-acute administration of MPTP causes up to 40% dopaminergic cell lose in the SNc and the peak time to greatest dopamine cell loss is achieved within 12 hours of MPTP administration(Meredith and Rademacher, 2011).

3.6. Behavioural experiments

3.6.1. Spontaneous locomotor activity test

Acute motor activity was assessed using a fully automated activity box (Linton Instrumentation, UK). Animals were placed gently in the activity box (46x25x15 cm) and frequency of activity was measured as indices of motor activity for 30 min. The parameter was recorded using software based on the breaking of each of the photo beam cells.

Sub-acute spontaneous locomotor activity was evaluated by using an infrared photobeam controlled open-field test chamber (MED Associates Inc., St. Albans, VT, USA).Mice were individually placed in the center of the box (43.2x43.2x30.5 cm) and allowed to freely explore the chamber for 10 min. The spontaneous locomotor activity was monitored using 16 evenly spaced infrared transmitters and receivers positioned around the periphery of the four sides of the chambers. The test boxes were connected to a computer and total distances travelled (ambulatory

distance traveled in centimeters) and stereotypic count (the rectilinear distance, in beams, traveled while inside the stereotypic box in the activity monitor) were obtained.

In both acute and sub-acute studies, the activity cages were frequently and thoroughly cleaned with a mild alcohol solution in order to avoid any possible cues. The locomotor measurement was performed under normal laboratory lighting in a ventilated and quiet room.

3.6.2. Y-maze

Short-term memory was assessed by spontaneous alternation behavior in the Y-maze task using the protocol described by Buyse et al., (2001). Experiments were carried out in a Y-maze constructed from wood and consisting of three identical alleys (40 x 15 cm with walls 30 cm high) diverging at a 120^0 angle one to the other on an equilateral triangular central area. Mice were placed at the end of one arm of the Y-maze and the sequence of arm entries were recorded during 5 min. The introduction arm was randomly assigned for each mouse. An arm visit was recorded when a mouse moved all four paws into the arm. An alternation was defined as consecutive entries into all three arms (e.g. 1,2,3 or 1,3,2). The number of maximum alternations was the total number of arm entries minus two and the percentage of alternations was calculated as the ratio of actual to maximum alternations multiplied by 100: (actual alternations/maximum alternations) x 100.

3.6.3. Elevated Plus Maze

The elevated plus maze was used to evaluate mice emotionality according to the protocol described elsewhere (Pellow et al., 1985). This is a wooden apparatus consisting of four arms (16 cm long, 5 cm wide). Two opposite arms are surrounded on three faces by 10 cm high walls (closed arms), while the two other arms are open (open arms). The maze is raised up to a height of 30 cm over the floor. The behavior was observed by an experimenter on a video placed outside the testing sound-attenuated room. The number of entries into open arms, the time spent in open arms (sec), and the total number of entries into both arms were evaluated (5 min test periods). The percentage of entries into open arms and the percentage of time spent in open arms were calculated as parameters for the anxiety related behavior.

3.7. Immunohistochemical staining for TH-immunoreactive neurons

Animals were anesthesized with ketamine/xylazine solution and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde (PFA). The brains were collected and postfixed overnight at 4 °C, cryoprotected with 30% sucrose in phosphate buffured saline (PBS) and then frozen and stored at -80 °C until analysed. Coronal sections (30 μm) containing the SNc and VTA were obtained. The slices were incubatedovernight at 4°C with polyclonal rabbit anti-TH antibody (Abcam, Cambridge, MA, USA). After washing, sections were incubated with goat polyclonal anti-rabbit secondary antibody, Alexa Flour® 488 (Abcam, Cambridge, MA, USA). The sections were mounted on slides and examined using confocal microscopy.Quantification of TH immunostaining was carried out on high-resolution digital microphotographs taken with a 20× objective and under the same conditions of light and brightness/contrast. Slides were used to measure the mean density of labeling in the selected area, using the analysis software ImageJ

(Wayne Rasband, NIH, Bethesda, MD, USA), which allows calibration that minimizes the influence of different backgrounds.

3.8. Quantitative reverse-transcription (qRT) PCR for mRNA quantification

qRT-PCR was used to estimate DAT mRNA expression in mouse midbrain. Two pairs of intron-spanning PCR primers with a Tm of 56-60℃ were designed and one of them was selected for qRT-PCR based on a single peak in melting curve, an amplification coefficient (AC) of "2" in a series of dilutions assay and/or a lower Ct value.

3.8.1. Sampling of VTA tissue from mice midbrain

Adult mice were sacrificed by rapid decapitation for brain collection. Midbrains were promptly dissected in metal mouse matrix (ZIVIC, PA, USA) from the brains, and the VTA was sliced out of the dissected coronal sections and transferred to pre-chilled tubes for RNA extraction.

3.8.2. RNA extraction

Total RNA of midbrain tissue or approximately 2 x 10^5 cells/well in 24-well plates was isolated by using 100 µl/10 mg tissue or 200 µl/well of TRIzol reagents (Ambion, MA, USA), following the manufacturer's protocol, and reconstituted in 20 µl RNase-free water. RNA concentration was estimated with NanoDrop Lit (Thermo Fisher Scientific). Approximately 10 µg RNA was extracted from every 10 mg tissue. RNA samples were stored at -80℃ till cDNA synthesis.

3.8.3. cDNA synthesis

One hundred ng RNA was reverse-transcribed into cDNA by using Verso cDNA synthesis kit (ThermoFisher Scientific) with oligo dT primers following the manufacturer's protocol. cDNA was diluted by 5 folds with DNase-free water prior to quantification by qRT-PCR or before being stored at -20℃.

3.8.4. qRT-PCR analysis of relative mRNA levels

cDNA samples were amplified in triplicates or quadruplicates by incubation in the Bio-Rad CFX Connect real-time system (Bio-rad, CA, USA). The amplification condition was 95˚C for 5 min, then for 49 cycles of 95˚C for 15 sec, 55˚C for 20 sec and 72˚C for 30 sec using SsoAdvanced Universal SYBR green supermix (Bio-rad, CA, USA). The final volume was 12.5 µl, containing 1 µl of cDNA and a final concentration of 0.5 µM for forward and reverse primers. Amplification coefficient (AC) was calculated from the Ct slope of the standard curve using the following formula: $AC=10^{-1/slope}$. Serial dilutions (1:2) of the starting template were prepared to generate eight points, and the Ct vs log cDNA concentration plot was constructed to calculate the Ct slope. This AC value was used in data analysis for relative mRNA levels, which were normalized with a reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.9. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Sigma Plot 12.0 and Statistical Package for Social Science SPSS 16.0 statistical programs were used. Statistical analyses were performed by student t-test and one-way analysis of variance (ANOVA). Post hoc comparisons of means were carried out with Tukey's test for multiple comparisons when appropriate. Values were considered statistically significant at $p<0.05$. T-test was used to analyze the sub-acute effect of khat on locomotor activity in C57BL/6J and DAT-Cnr2 mice. One-way ANOVA together with post hoc Tukey's test was used to see the effect of JWH133 on khat's locomotor activity, effect of khat on MPTP induced motor defficts, effect of khat on immunohistochemical staining for TH positive cell, effect of khat on the expression of DAT mRNA in C57Bl/6J mice upon sub-acute administration of khat. One-way ANOVA together with post hoc Tukey's test was also used to analyze the acute effect of khat on anxiety and working memory related behaviour in BALB/c albino mice.

4. Results

4.1. Acute effects

4.1.1. Effects working memory related behaviors

Working memory related behavioral alternation was investigated using Y-maze. The total alley visit [$F(5, 30) = 43.315$, $p < 0.001$] as well as percent alternation [$F(5, 30) = 10.251$, $p < 0.001$] were significantly associated with the treatment group. Post-hoc analysis revealed that both khat and WIN-55,212-2, when administered alone, failed to alter percent alternation compared to controls in the Y-maze. Combination of khat and WIN-55,212-2 significantly increased percent alternation when compared with controls ($p<0.001$),khat alone ($p<0.01$) or WIN-55,212-2 ($p<0.01$) alone. Compared to either the controls or khat treated mice, the AM251 and AM630 did not affect percent alternation when administered in combination with khat (Fig. 8).

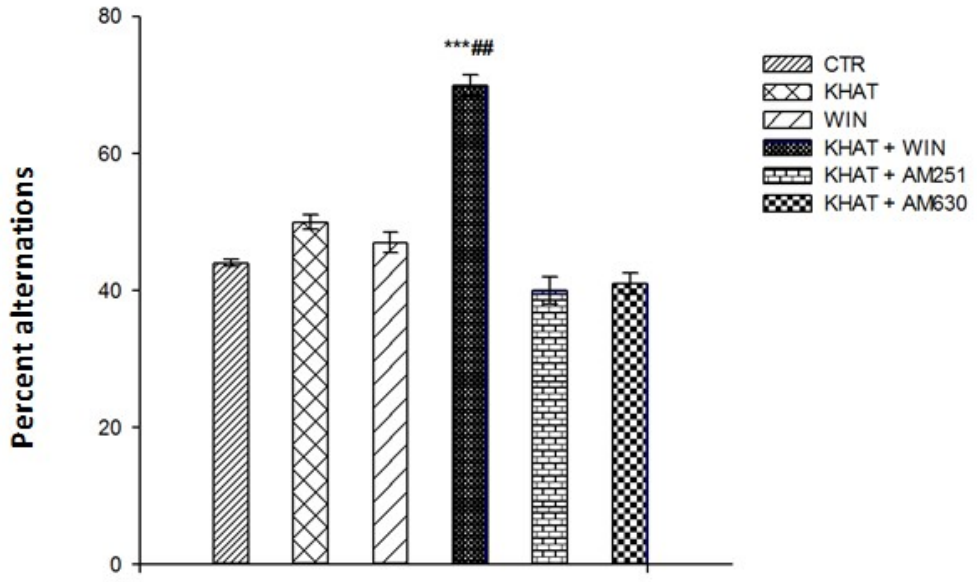

Figure 8. Acute effect of khat and cannabinoid ligands on percentage alternation in Y-maze in BALB/c albino mice. Values are mean ± SEM (n=6 in each group). Statistical analysis was performed by one way ANOVA followed by Tukey's multiple comparison test. $^{***}p < 0.001$ compared to CTR group; $^{\#\#}p < 0.01$ compared to KHAT group. CTR - control, KHAT - khat (300 mg/kg), WIN - non selective cannabinoid receptor agonist (1mg/kg), AM251 -CB1R antagonist(1 mg/kg), AM630-CB2R antagonist(1mg/kg).

4.1.2. Acute effects on anxiety like behavior

The elevated plus maze, with two open and two closed arms, was used to investigate anxiety like behaviour in mice. Percent arm entry and time spent in the open arm are the parameters recorded in this experiment to evaluate anxiety. The percentage of open arm entries [$F(5, 30) = 14.904$, $p<0.001$] and the percentage of time spent in the open arm [$F(5, 30) = 17.570$, $p<0.001$] were found to be significantly associated with the treatment group. Neither khat nor WIN-55,212-2 affected percent arm entry as well as percent time spent in open arms in the elevated plus maze when administered alone. Although khat tended to increase and WIN-55,212-2 to decrease percent arm entry, the diffrence failed to reach statistical significance (Fig. 9 and 10).

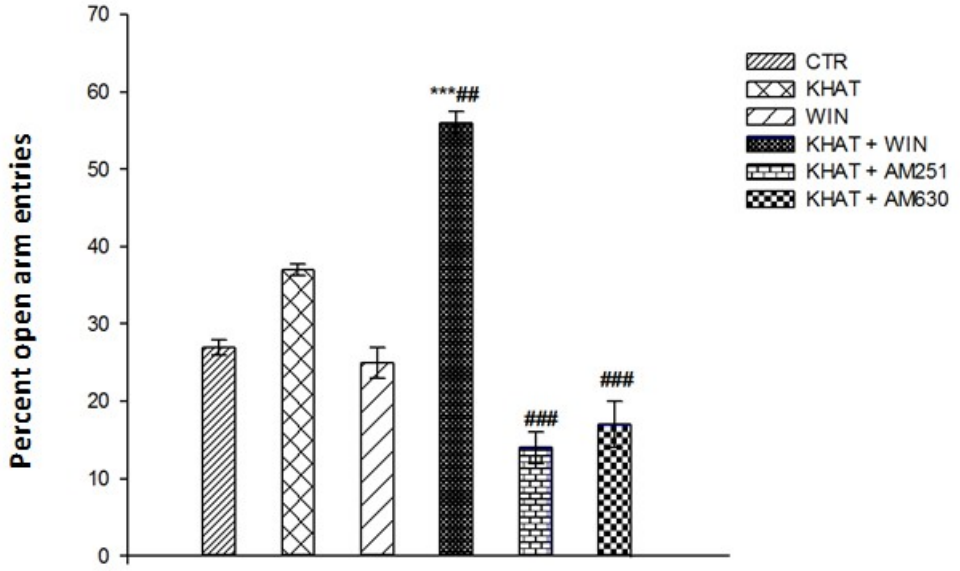

Figure 9.Acute effect of khat and cannabinoid ligands on percentage of open arm entries in elevated plus maze inBALB/c albino mice. Values are mean ± SEM (n=6 in each group). Statistical analysis was performed by one way ANOVA followed by Tukey's multiple comparison test. $^{***}p < 0.001$ compared to CTR group;$^{###}p < 0.001$, $^{##}p < 0.01$ compared to KHAT group. CTR - control, KHAT –khat(300 mg/kg), WIN - non selective cannabinoid receptor agonist (1mg/kg), AM251 - CB1R antagonist(1 mg/kg), AM630 - CB2R antagonist(1 mg/kg).

However, mice treated with a combination of khat and WIN-55,212-2 displayed a significant increase in open arm entry and time spent in the open arm compared to controls, khat and WIN-55,212-2 treated group. In stark contrast, the antagonists caused a significant decrease in both parameters compared to khat treated mice. Moreover, the values observed for both parameters with the antagonist-khat combination were significantly lower than that of khat-WIN-55,212-2 combination (Fig. 9 and 10).

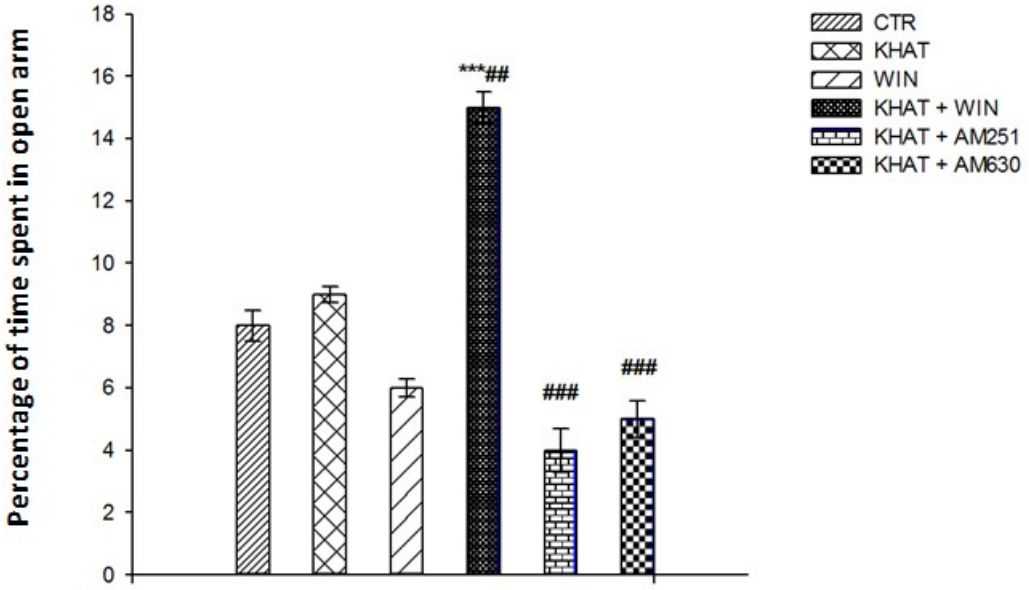

Figure10. Acute effect of khat and cannabinoid ligands on percentage of time spent in open arm in elevated plus maze in BALB/c albino mice. Values are mean ± SEM (n=6 in each group). Statistical analysis was performed by one way ANOVA followed by Tukey's multiple comparison test. $^{***}p < 0.001$ compared to CTR group; $^{###}p < 0.001$, $^{##}p < 0.01$ compared to KHAT group. CTR - control, KHAT - khat(300 mg/kg), WIN - non selective cannabinoid receptor agonist (1mg/kg), AM251 - CB1R antagonist(1 mg/kg), AM630 - CB2R antagonist(1 mg/kg).

4.1.3. Acute effects on locomotor activity

Acute motor activity in BALB/c albino mice was assessed using a fully automated activity box. Locomotor activity (Fig. 11) was significantly associated with treatment groups [$F(5,30)=29.052$, $p<0.001$]. During post-hoc analysis, treatment of mice with khat significantly increased ($p<0.001$) locomotion by 76.2% compared to controls. Treatment with WIN-55,212-2, did not, however, alter locomotion compared to control, but locomotion was significantly lower ($p<0.01$) than induced by khat. Interestingly, treatment with the combination of khat and WIN-55,212-2 produced locomotion that was significantly greater than the one produced by khatas well as the

vehicle. By contrast, treatment with khat and AM251 or AM630 significantly attenuated khat-induced hyper-locomotion by 31% ($p<0.001$) and 28.1% ($p<0.001$), respectively, and the value was brought down to the control level.

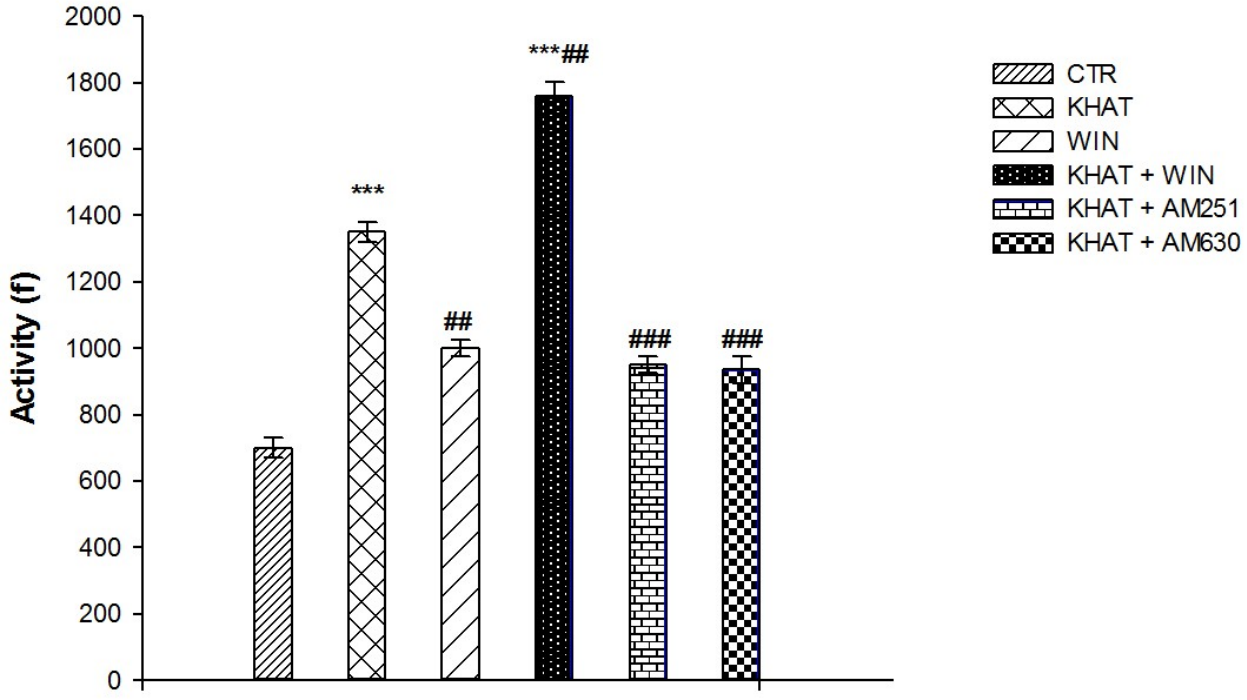

Figure 71. Acute effect of khat and cannabinoid ligands on locomotor activity in BALB/c albino mice. Values are mean ± SEM (n=6 in each group). Statistical analysis was performed by one way ANOVA followed by Tukey's multiple comparison test. $^{***}p < 0.001$ compared to CTR group; $^{###}p < 0.001$, $^{##}p < 0.01$ compared to KHAT group. CTR - control, KHAT - khat (300 mg/kg), WIN - non selective cannabinoid receptor agonist (1 mg/kg), AM251 - CB1R antagonist (1 mg/kg), AM630 - CB2R antagonist (1 mg/kg).

4.2. Sub-acute effects

4.2.1. Effects khat on locomotor activity in C57BL/6J WT mice

In sub-acute studies, distance travelled and stereotypic counts were evaluated to test mice motor behavior in the activity monitor apparatus. An independent *t*-test showed that, administration of khat at 300 mg/kg to the C57Bl/6J WT mice significantly ($p<0.001$) increased both the total distance travelled by 36.2% and the stereotypic count by 32.7% in the activity box compared to controls (Fig. 12 and 13).

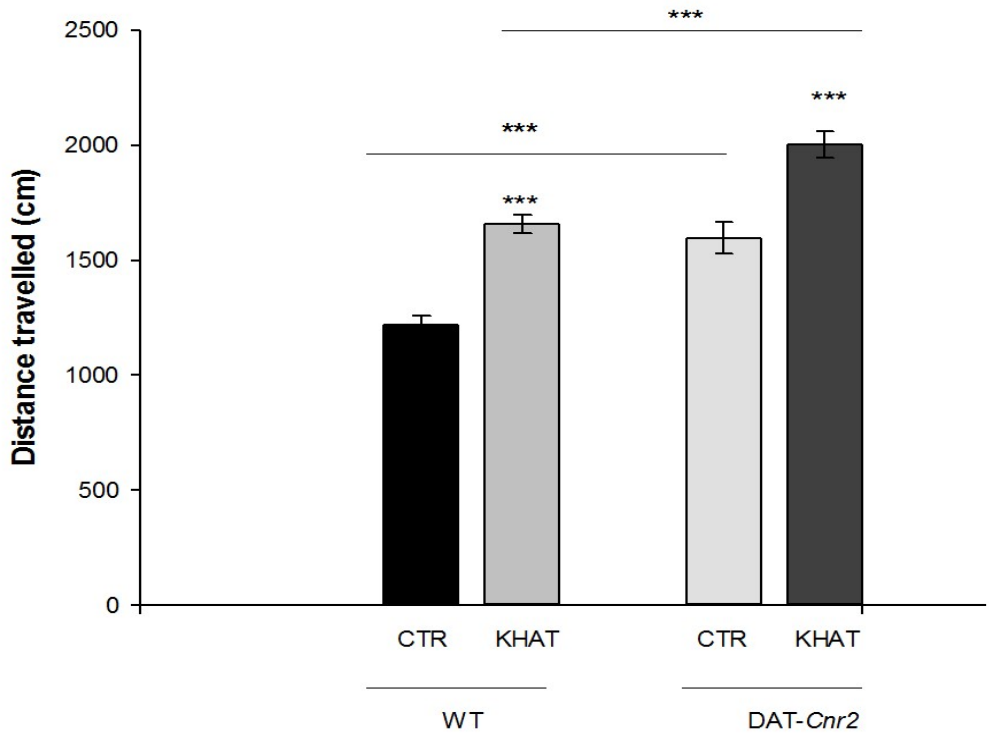

Figure 82. Sub-acute effect of khat on distance travelled (centimeters) in the activity monitor apparatus in C57BL/6J (WT) and DAT-*Cnr2*cKO mice. Values are mean ± SEM (n=6 in each group). Statistical analysis was done using student *t*-test. *** $p<0.001$.CTR - control, KHAT - khat (300 mg/kg). The vehicle and khat were administered to mice once daily for seven consecutive days.

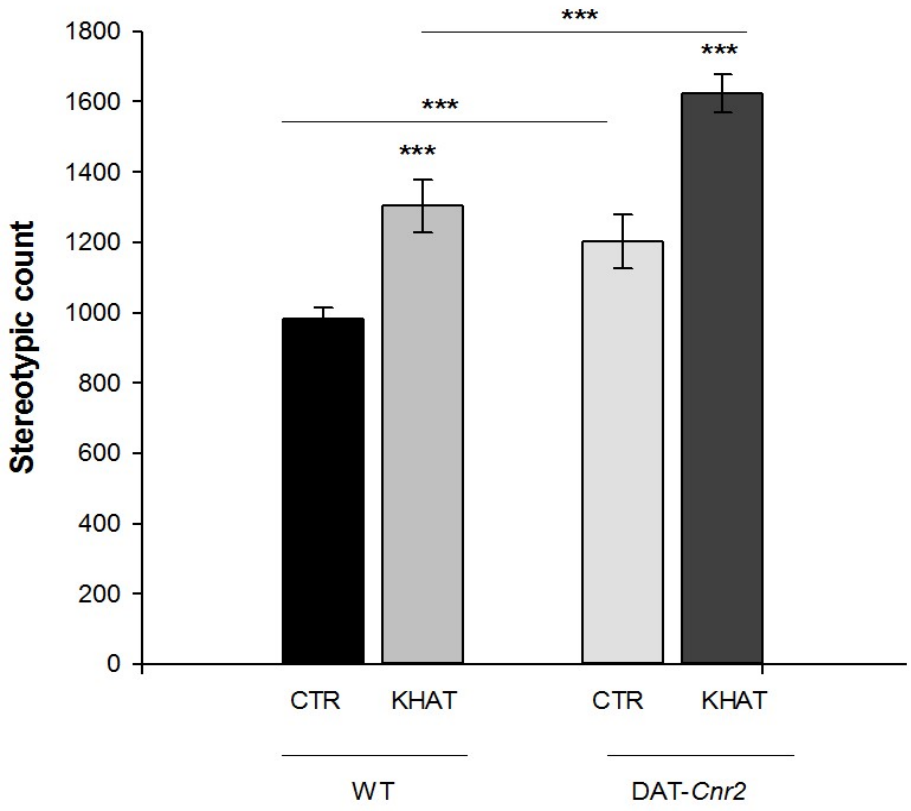

Figure 93. Sub-acute effect of khat on stereotypic count in the activity monitor apparatus in C57BL/6J (WT) and DAT-*Cnr2*cKO mice. Values are mean ± SEM (n=6 in each group). Statistical analysis was done using student *t*-test. $^{***}p<0.001$.CTR - control, KHAT - khat (300 mg/kg). The vehicle and khat were administered to mice once daily for seven consecutive days.

4.2.2. Effect of khat on locomotor activity in DAT-*Cnr2* mice

CB2Rs were previously thought to be predominantly expressed in immune cells and their involvement in cannabinoid-induced behaviors was largely unexplored. Recent studies showed that CB2Rs inhibits motor function and the deletion of CB2Rs in dopamine neurons induces enhanced motor function characterized by hyper-locomotion of the DAT-*Cnr2*cKO mice. The role of deletion of CB2Rs in dopamine neurons on the locomotor activity of khat was evaluated

by administering the extract to the WT and DAT-*Cnr2*cKO mice sub-acutely. The result from t-test depicts that deletion of CB2Rs in dopamine neurons per se significantly ($p < 0.001$) increased distance travelled in the activity box by 32% and stereotypic count by 28%. Administration of khat in a dose of 300 mg/kg significantly ($p < 0.001$) increased both the total distance travelled by 20.8% and stereotypic count by 34.9% in the DAT-*Cnr2* mice compared to the WT (Fig. 12 and 13).

4.2.3. Effect of co-administration of JWH133 with khat on the locomotor activity in C57BL/6J mice

The effect of co-administration of JWH133mice was evaluated using the activity monitor apparatus. One-way ANOVA showed there was significant association between distance travelled ($F(3,20) = 14.509$, $p< 0.001$) and treatment groups. Tukey's post hoc test showed that co-administration of JWH133 with khat significantly ($p< 0.05$) attenuated khat induced increase in distance travelled by 19.1% in the activity box. There is also a statistically significant difference ($F(3,20) = 13.285$, $p< 0.001$) in stereotypic count among the treatment groups. In a post hoc analysis, co-administration of JWH133 with khatsignificantly ($p< 0.01$) reduced the effect of khat alone by 25.5% on stereotypic count. On the other hand, JWH133 alone tended to reduce the distance travelled and stereotypic count compared to the control group, but the reduction was not statistically significant. In addition co-administration of JWH133 did not show apparent difference in distance travelled and stereotypic count compared to the control group (Fig. 14 and 15).

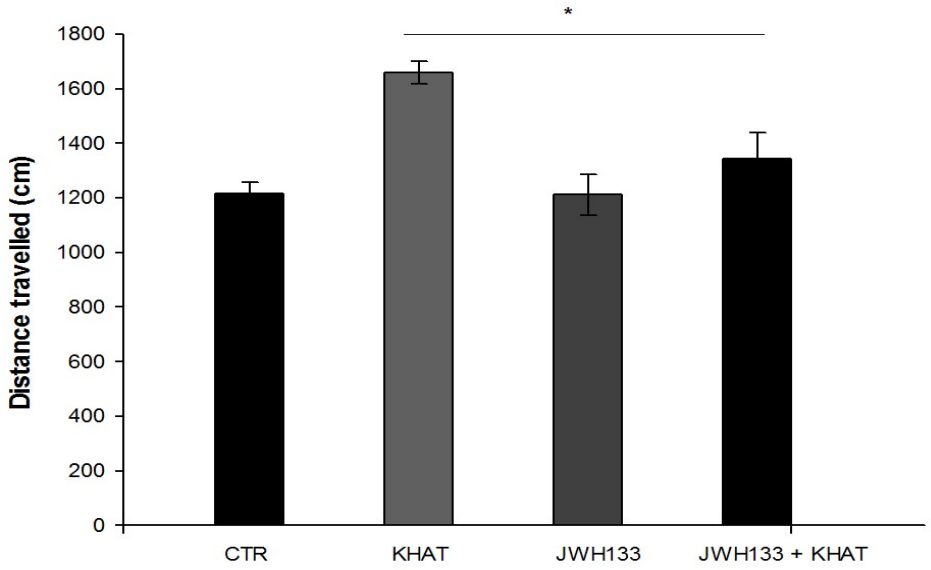

Figure 104. Effect of co-administration of the CB2R agonist JWH133 on khat induced increment in distance travelled (centimeters) in the activity box upon sub-acute administration in C57BL/6J mice. Values are mean ± SEM (n = 6 in each group). Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison test. *p< 0.05.CTR - control, KHAT - khat (300 mg/kg), JWH133 – CB2R agonist (5 mg/kg) and JWH133 + KHAT - khat(300 mg/kg) in combination with JWH133 (5 mg/kg). The vehicle, khat, and JWH133 were administered to mice once daily for seven consecutive days.

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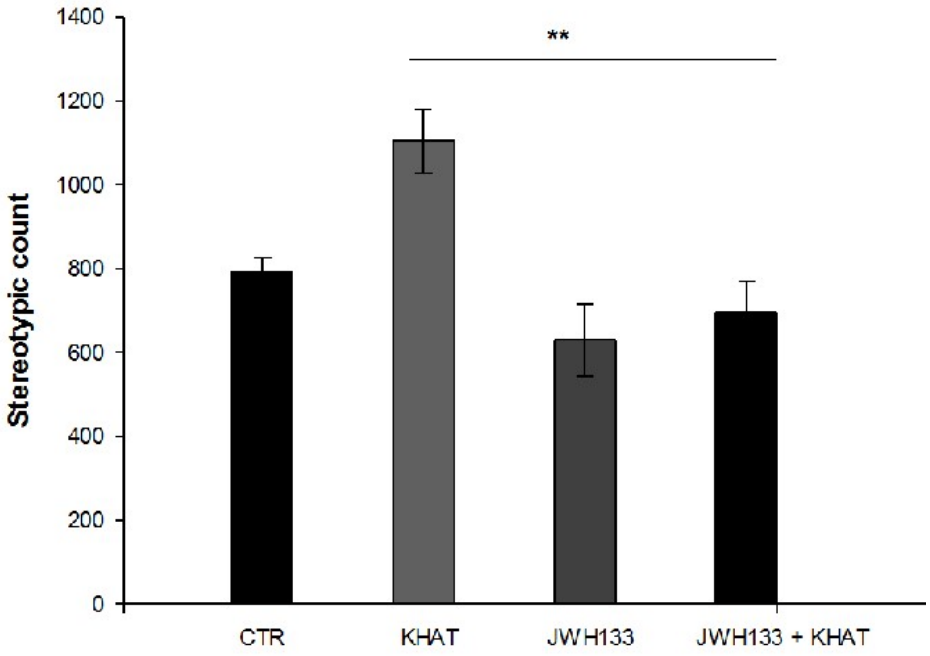

Figure 115. Effect of co-administration of the CB2R agonist JWH133 on khat induced increment in stereotypic count in the activity box upon sub-acute administration in C57BL/6J mice. Values are mean ± SEM (n = 6 in each group). Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison test. ***p*< 0.01. CTR - control, KHAT - khat (300 mg/kg), JWH133 – CB2R agonist (5 mg/kg) and JWH133 + KHAT - khat (300 mg/kg) in combination with JWH133 (5 mg/kg). The vehicle, khat, and JWH133 were administered to mice once daily for seven consecutive days.

4.2.4. Effect of khat on locomotor activity in MPTP-lesioned C57Bl/6J mice

The effect of khat on locomotor activity was evaluated in sub-acute MPTP lesioned mice. The locomotor activity was reduced in MPTP group compared to that of control group, confirming that the PD model was successfully established. Moreover, the locomotor activity of khat treated group was increased compared with that of MPTP group, which indicated that khat attenuated the motor deficits of PD mouse. One-way ANOVA showed that there is a statistically significant difference in distance travelled among the treatment groups ($F(4,25) = 54.029$, $p < 0.001$). Post hoc test showed that PD model mice treated with MPTP had a significant ($p < 0.001$) reduction in distance travelled by 52.9% compared to the control group, and this was significantly ($p < 0.01$) attenuated by 59.1% following treatment withkhat in PD mice (Fig. 16). There was also a statistically significant difference ($F(4,25) = 52.921$, $p < 0.001$) in stereotypic count among the treatment groups. Mice in the MPTP group had a reduced ($p < 0.001$) stereotypic count by 46.7% compared to those of the control group, which was reversed ($p < 0.01$) by 48.6% after khat treatment (Fig. 17).

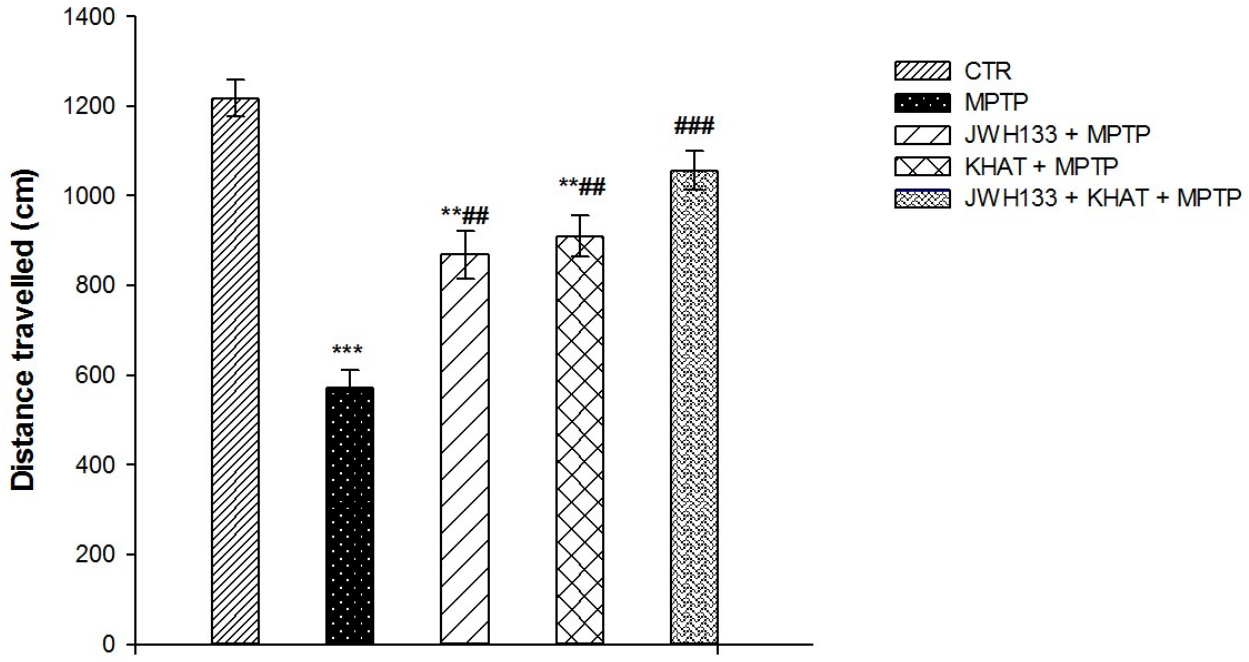

Figure 126.Effect of sub-acute administration of khat alone and in combination with JWH133 on distance travelled (centimeters) in MPTP mouse model. Values are mean ± SEM (n=6 in each group). Statistical analysis was done using One-wayANOVAfollowedbyTukey'smultiplecomparisontest.***p<0.001,**p<0.01comparedtoCTR group; $^{###}p$<0.001, $^{##}p$<0.01 compared to MPTP lesioned group. CTR - control, KHAT - khat (300 mg/kg), JWH133 – CB2R agonist (5 mg/kg), JWH133 + KHAT – combination of JWH133 (5 mg/kg) and khat(300 mg/kg), MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (25 mg/kg), JWH133 + MPTP – combination of JWH133 (5 mg/kg) and MPTP (25 mg/kg), KHAT + MPTP - combination of khat(300 mg/kg) and MPTP (25 mg/kg, JWH133 +KHAT + MPTP - combination of JWH133 (5 mg/kg), khat (300 mg/kg) and MPTP (25 mg/kg). The vehicle, JWH133, khat and MPTP were administered to mice once daily for seven consecutive days.

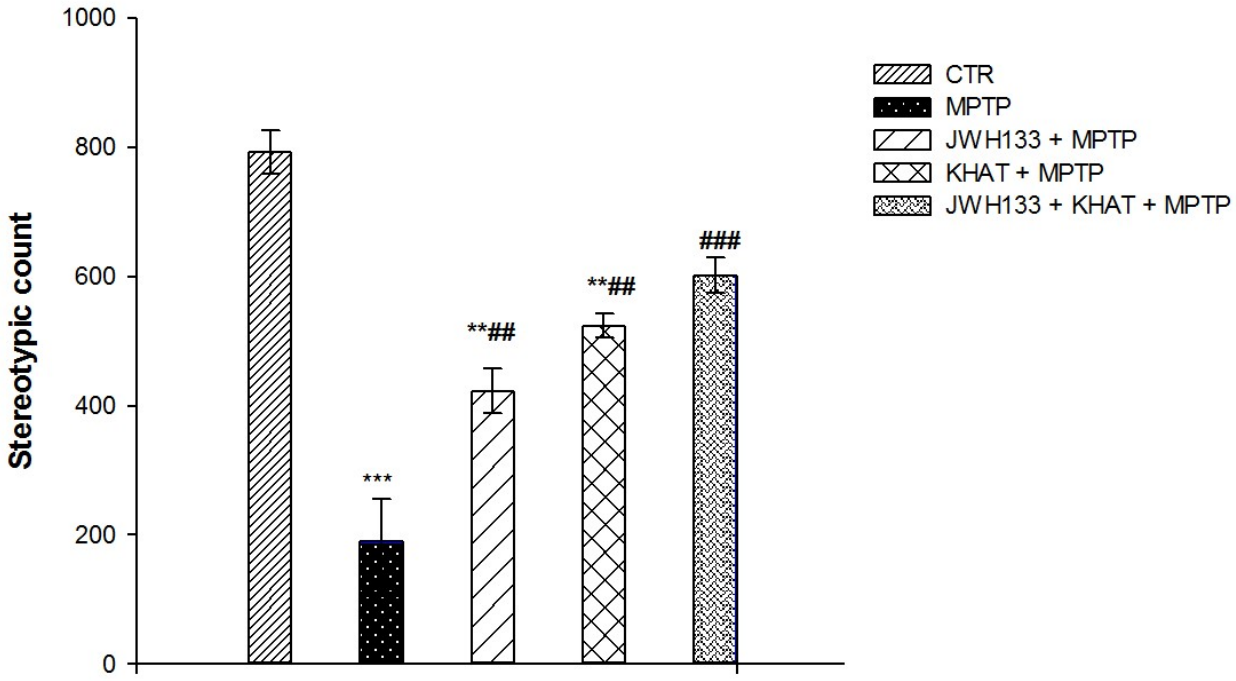

Figure 137.Effect of sub-acute administration of khat alone and in combination with JWH133 on stereotypic count in MPTP mouse model. Values are mean ± SEM (n=6 in each group). Statistical analysis was done using One-way ANOVA followed by Tukey's multiple comparison test. ***p<0.001, **p<0.01 compared to CTR group; $^{###}p$<0.001, $^{##}p$<0.01 compared to MPTP lesioned group. CTR - control, KHAT - khat (300 mg/kg), JWH133 – CB2R agonist (5 mg/kg), JWH133 + KHAT – combination of JWH133 (5 mg/kg) and khat (300 mg/kg), MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (25 mg/kg), JWH133 + MPTP – combination of JWH133 (5 mg/kg) and MPTP (25 mg/kg), KHAT + MPTP - combination of khat (300 mg/kg) and MPTP (25 mg/kg, JWH133 +KHAT + MPTP - combination of JWH133 (5 mg/kg), khat (300 mg/kg) and MPTP (25 mg/kg). The vehicle, JWH133, khat and MPTP were administered to mice once daily for seven consecutive days.

4.2.5. Effect of co-administration of JWH133 with khat on locomotor activity in MPTP-lesioned C57Bl/6J mice

The effect of administration of JWH133 alone and its co-administration on the locomotor activity of khat was also evaluated in sub-acute MPTP lesioned mice. One-way ANOVA showed that there is a statistically significant difference in distance travelled ($F(4,25) = 54.029$, $p<0.001$) and stereotypic count ($F(4,25)=52.921$, $p<0.001$) among the treatment groups. Administration of JWH133 alone significantly ($p < 0.01$) reversed MPTP induced reduction in total distance travelled by 36% and stereotypic count by 28%. JWH133 also significantly ($p<0.001$) increased khat induced increase in distance travelled by 15.9% (Fig. 16) and stereotypic count by 7.6% (Fig. 17) compared to MPTP lesioned mice in the activity box in post hoc test.

4.2.6. Immunohistochemical studies in C57Bl/6J mice

In order to determine the anatomical and structural integrity of dopaminergic neurons, brain sections were immunostained for TH immunoreactivity in the VTA and SNc of the C57BL/6J WT mice (Fig. 18A-D). The results of one-way ANOVA revealed that there is significant difference ($F(3,8) = 6.554$, $p < 0.01$) among groups in TH immunoreactivity in the VTA, but no apparent difference was detected in the SNc region. Tukey's test showed that mice treated with khat had significantly ($p < 0.05$) increased (12%) TH immunoreactive cells in the VTA region, compared to controls (Fig. 18E).

Administration of JWH133 alone didnot show a significant difference in TH immunoreactivity compared to both control and khat treated groups. Co-administration of JWH133 with khat (300 mg/kg) significantly ($p<0.05$) increased (18%) TH immunoreactive cells in the VTA region compared to controls. On the other hand, co-administration of JWH133 with khatdid not significantly alter THimmunoreactivity of khat in the VTA region compared to khat alone treated mice (Fig. 18E).

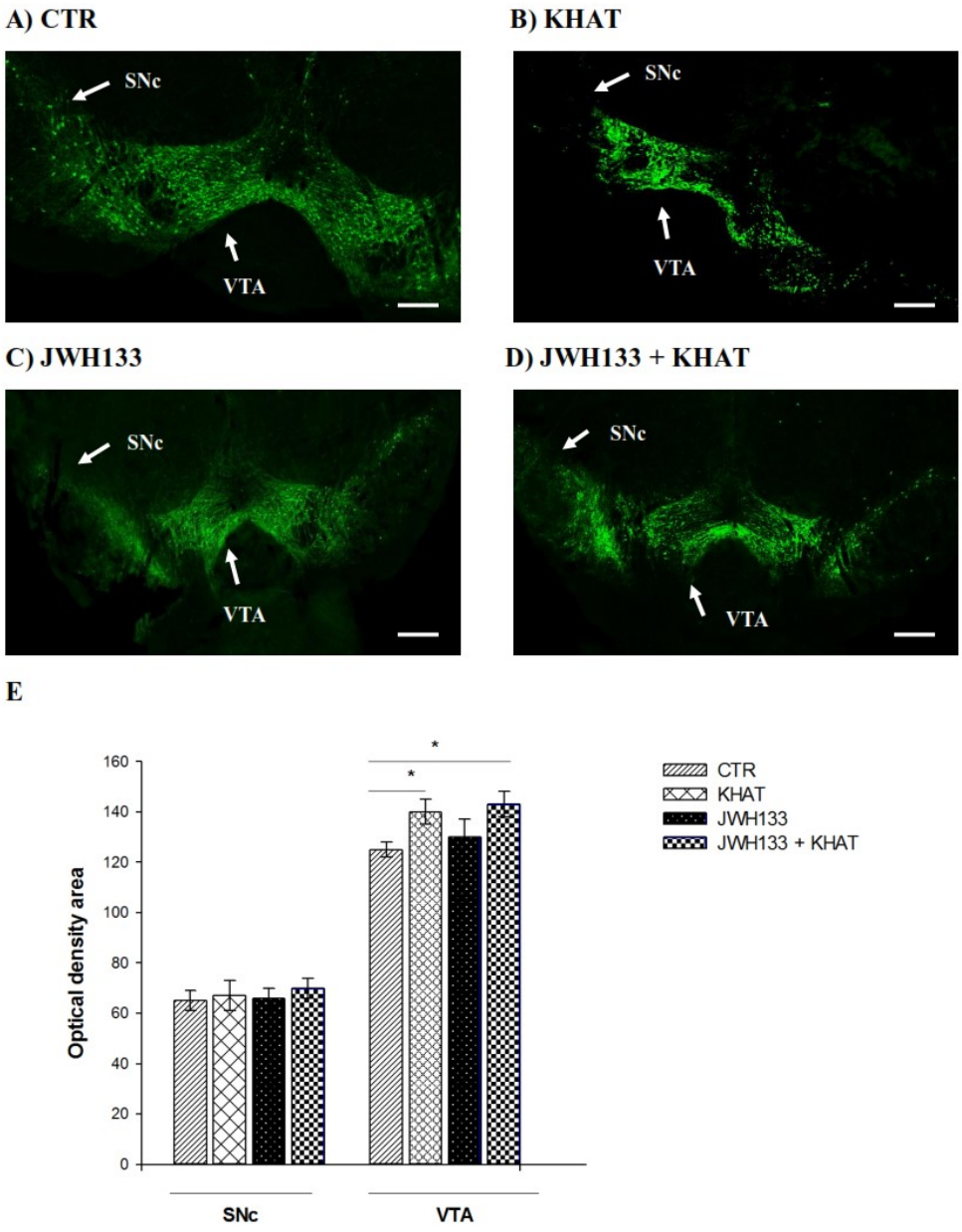

Figure 148.Effect of sub-acute administration of khat alone and in combination with JWH133 on immunohistochemical staining for TH-positive cells in C57BL/6J WT mice.(A-D) Representative photomicrographs of TH immunoreactive neurons in the substantianigra pars compacta (SNc) and ventral tegmental area (VTA) region. Scale bar, 100 µm. (E) The number TH positive cells (optical density area), and the data were expressed as mean ± SEM (n=6 in each group). Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison test. $^{*}p<0.05$. CTR - control, KHAT–khat (300 mg/kg), JWH133 - CB2R agonist (5 mg/kg), JWH133 + KHAT- combination of JWH133 (5 mg/kg) and khat (300 mg/kg). The vehicle, JWH133 and khatwere administered to mice once daily for seven consecutive days.

4.2.7. DAT mRNA gene expression in C57Bl/6J mice

The effect of khat administration on the expression of DAT mRNA was investigated using qRT-PCR technique. qRT-PCR was used to estimate DAT mRNA in the VTA region of C57BL/6J WT mice. The study demonstrated that khat administration increased the expression of DAT mRNA twofold compared to vehicle treated controls. To test for statistically significant difference, One-way ANOVA was performed. The result revealed that there is statistical difference in DAT mRNA expression among the groups ($F(3,8)=60.488$, $p<0.001$), and post hoc test showed that khat significantly ($p < 0.001$) increased expression of DAT mRNA compared to the control (Fig. 19).

Administration of JWH133 alone didn't show a significant difference in DAT mRNA expression compared to controls but JWH133 significantly ($p<0.001$) reduced expression of DAT mRNA compared to khat treated groups. Co-administration of JWH133 with khatsignificantly ($p<0.001$) increased expression of DAT mRNA compared to the control, however, there was no apparent difference detected in the expression of DAT mRNA in mice treated with a combination of JWH133 and khat compared to mice treated with khat alone (Fig. 19).

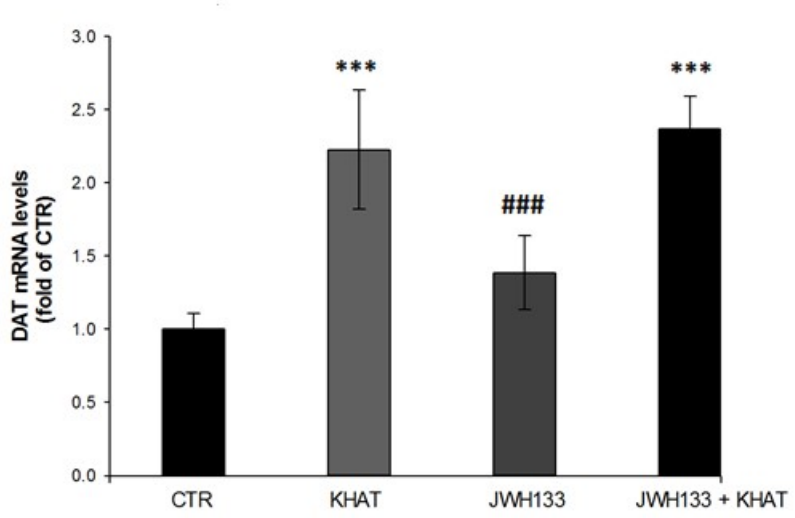

Figure 159.Effect of sub-acute administration of khat alone and in combination with JWH133 on DAT mRNA gene expression in WT mice. The quantification of dopamine transporter (DAT) mRNA levels and normalized by GAPDH mRNA in the VTA of WT mice after the administration of khat. Values are mean ± SEM (n=6 in each group). Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison test. ***$p < 0.001$ compared to CTR group; ###$p < 0.001$ compared to KHAT group. CTR – control, KHAT –khat (300 mg/kg), JWH133 - CB2R agonist (5 mg/kg),JWH133 + KHAT- combination of JWH133 (5 mg/kg) and khat (300 mg/kg). The vehicle, JWH133 and khat were administered to mice once daily for seven consecutive days.

5. Discussion

A large body of evidence exists in the literature, since its first description by Peter Forskal(Peters, 1952), about the adverse health aspects of khat, with a few mention of its pharmacology(Al-Habori, 2005; Al-Hebshi and Skaug, 2005; Cox and Rampes, 2003; Odenwald, 2007; Warfa et al., 2007). However, few studies have attempted to address the endogenous systems involved and the molecular mechanism for the observed effects of khat alone or its combination with other compounds. The aim of the present study was therefore to investigate the role of the ECS on the neurobehavioral effects of khat in WT and MPTP lesioned mice following concomitant administration of khat and cannabinoid receptor ligands. Furthermore, mice with cell type specific deletion of CB2Rs on dopamine neurons were used to probe the involvement of the ECS on the neurobehavioral effects of khat.

The present study showed that acute administration of khat had no effect on percentage alternations in the Y-maze test, a measure of working memory, which indicates that the extract didn't alter working memory in mice at the administered dose.In agreement with this, a study done to investigate the effect of acute exposure to crude khat on spatial learning and memory in mice using multiple T-maze test revealed that, khat did not produce any improvement in learning and memory compared to placebo at all doses used (Khan et al., 2018). In contrast to the present study, Kimani and Nyongesa (2008) reported that khat has differential effects on learning and memory in mice depending on dose, with low dose (40 mg/kg) having no effect on learning but impairing memory, whereas high dose (360 mg/kg) impairs learning but improves memory.The present study also showed that acute administration of WIN-55,212-2 had no effect on percentage alternations and hence, didn't alter working memory. However, previous works

showed that, administration of both synthetic and phytocannabinoids, including Δ^9-THC, WIN-55,212-2, and CP55,940, impair working memory and short-term memory through a CB1R-mediated mechanism in rats (Braida and Sala, 2000; Egashira et al., 2002). The contradiction between previous studies and the present study regarding the effect of cannabinoids on working memory related behaviour may emanate from variation in the doses administered, the behavioural paradigms (Morris water maze, Y-maze) used or the strain of animals used in the studies. But, unlike the individual compounds used alone in this study, the concurrent use of WIN-55,212-2 and khat increased behavior related to working memory, which points to the fact that there is an increased effect when these compounds are used together.

Previous studies done to evaluate the effect of khat on anxiety like behaviour demonstraetd that khat had mixed effects on the levels of anxiety depending on the parameter looked at and the doses used(Kimani et al., 2008). Studies on cannabinoids effect on anxiety revealed behaviour showed that, low doses of CB1R agonists elicit anxiolytic-like behaviour while higher doses produce anxiety-like behaviour(Rey et al., 2012; Braida et al., 2007; Onaivi et al., 1990). Here, the data demonstrated that the sole use of either khat or WIN-55,212-2 at the doses administered did not have anxiolytic-like effectsbut co-administration of these compounds exhibited anxiolytic-like effect in the elevated plus maze, as evidenced by a significant increase in percent open arm entry as well as duration. Further more, administration of the CB1R and CB2R antagonists significantly reduced khat's effect on percent open arm entry and duration, confirming the involvement of the ECS in modulating anxiety like behavioural effect of khat in this paradigm.

A possible explanation for the observed behavioral responses could be an interaction that occurs between cannabinoids and khat at the receptor/neurotransmitter system. Blockade of khat's behavioral responses by both CB1R and CB2R antagonists, AM251 and AM630, respectively suggests that there may be a common mechanism involved in mediating the effects of both khat and cannabinoids. Cannabinoid modulation of dopaminergic transmission is suggested by the ability of Δ^9-THC to affect motor and motivated behaviors in a manner similar to that produced by pharmacological manipulation of the nigrostriatal and mesocorticolimbic dopaminergic systems (Fitzgerald et al., 2012). Importantly, Δ^9-THC and other CB1R agonists activate dopaminergic transmission, like other abused drugs. In microdialysis studies, CB1R agonists increase dopamine levels in the nucleus accumbens (Chen et al., 1993, 1991, 1990). In agreement with this hypothesis, SR141716, a cannabinoid antagonist, reduces intravenous nicotine self-administration in rats, prevents nicotine-induced dopamine release in the shell of the nucleus accumbens and in the bed nucleus of the stria terminalis in freely moving rats, and antagonizes the amphetamine-like effects of nicotine (Cohen et al., 2002). Behavioural activity of khat is also associated with dopamine release (Banjaw and Schmidt, 2005; Kimani and Nyongesa, 2008). Taken together, these data collectively indicate that the dopaminergic system is involved in the interaction between khat and cannabinoids and therefore, thsese compounds might have a role in dopamine associated bahvioural dysfunctions in the CNS.

Our data also have shown that acute administration of crude khat significantly increased locomotor activity in WT mice, which is in line with previous studies(Geresu and Engidawork, 2010; Kalix, 1980). The stimulatory effect of khat is perceived as an increase in alertness and energy and relief from fatigue. Indeed, these effects have been reproduced in rats after oral administration of different concentrations of khat in which higher doses of the plant increased

motor activity (Hassan et al., 2007). The result of the study also revealed that acute administration of WIN-55,212-2 alone didn't increase locomotor activity. Synthetic, plant-derived and endogenous cannabinoids have powerful actions on motor activity in animals. In fact, these effects are bidirectional, depending on the dose. Large doses of cannabinoid reduced motor activity in a variety of behavioral tests and even produced strong catalepsy, whereas low doses stimulated motor activity as indicated by hyperlocomotion in WT animals(Chaperon and Thiébot, 1999; Fernández-Ruiz et al., 2002). In the present study, khat significantly increased locomotor activity but WIN55,212-2 failed to do so, owing to greater variation between individual values and small sample size that precluded statistical significance. It is of note that, co-administration of WIN55,212-2 with khat enhanced khat induced increase in locomotor activity, whereas, co-administration of AM251 and AM630 with khat reduced khat induced hypermotility, which reinforces the notion that the ECS modulateskhat's effect.

The acute effect of khat on locomotor activity in WT mice was also supported by sub-acute study. Like the acute study, sub-acute administration of khat also increased locomotor activity in WT mice which is in agreement with the notion that, repeated administration of psychostimulants in rodents results in a progressive and enduring augmentation of locomotor and stereotyped behaviors termed*behavioral sensitization*(Robinson and Berridge, 1993; Segal, 1975).

The involvement of the ECS in modulating behavioral effects of khat in the acute study was further sustained by the sub-acute study. Here, the role of CB2Rs in modulating sub-acute locomotor effect of khat was assessed by concomitant administration of khat with the CB2R agonist, JWH133, in WT mice and by administering khat alone in CB2R cKOmice. Sub-acute

administration of JWH133with khat in WT mice ablated khat-induced increase in locomotor activity. In the acute study, co-administration ofWIN-55,212-2 and khat significantly increased locomotor activity but co-administration of JWH133 with khat in the sub-acute study reduced khat's locomotor effect. The observed variation in response might be due to the low dose of WIN-55,212-2, since cannabinoids at lower dose increase locomotor activity (Chaperon and Thiébot, 1999; Fernández-Ruiz et al., 2002). The presence of functional cannabinoid CB2Rs in the brain has been controversial, and it is generally believed that the behavioral and psychotropic effects of cannabinoids are mediated by CB1Rs and CB2R ligands have no psychoactive effects. However, the purported lack of brain CB2Rs has been challenged by recent reports of CB2Rs on microglia (Fernández-Ruiz et al., 2002) and neuronal cells (Baek et al., 2008; Gong et al., 2006) in several brain regions including the anterior olfactory nucleus, cerebral cortex, cerebellum, hippocampus, striatum, and brainstem. Furthermore, activation of CB2Rs by 2-arachidonoylglycerol, JWH015, or JWH133 inhibits locomotion, morphine-6-glucuronide-induced emesis, and neuropathic pain, while stimulating neural progenitor proliferation and producing neuroprotective effects (Goncalves et al., 2008; Sagredo et al., 2009; Viscomi et al., 2009).

The role of CB2Rs in modulating the behavioral effects of khat was investigated by administering khat alone in CB2R cKOmice. It was found that, deletion of dopaminergic CB2R per se or administration of khat in CB2R cKOmice significantly increased locomotor activity.The observation that deletion of CB2Rs in dopamine neurons resulted with enhanced spontaneous motor activity, which is in agreement with previous studies (Canseco-Alba et al., 2019; Liu et al., 2017), reinforces the notion that CB2R mediates inhibition of spontaneous movement via modulation of the dopamine system, probably through reduction of neuronal

firing frequency (Den Boon et al., 2012). Further evidence for the inhibitory role of the CB2R comes from the observation that khat's effect on locomotion was attenuated when the receptor is activated but accentuated in the absence of the receptor. This finding is additional evidence that there is an interaction between khat and the ECS at the level of the dopaminergic system to modulate motor behavior that could probably have relevance to dopamine-related CNS disorders. The fact that khatinduces hyperlocomotor behavior in mice lacking CB2Rs in dopamine neurons provides evidence that brain CB2Rs may constitute a new therapeutic target for treatment of such disorders.

Besides the acute and sub-acute behavioral studies performed to investigate the involvement of the ECS in modulating the effect of khat, the interaction of the ECS with khat was also examined at the neuronal level using sub-acute studies. In this study, the effect of the ECS and khat on nigrostriataldopamine neurons degeneration induced by MPTP was also investigated by using MPTP lesioned mice. MPTP is a commonly used neurotoxin to induce a PD-like state in animals. As animals do not develop PD, the MPTP lesion model is one of several models that are used to investigate the underlying mechanisms of PD and to test novel compounds for their neuroprotective properties (Kopin and Markey, 1988; Nakamura and Vincent, 1986). The result of the present study showed that MPTP reduced locomotor activity in mice, and khat attenuated MPTP-induced motor deficits in mice. In addition, the result demonstrated that administration of JWH133 with khat enhanced the neuroprotective effect of khat as evidenced by increased locomotor activity in MPTP lesioned mice after co-administration of khat and JWH133. Previous findings have shown that activation of the CB2Rs rescued nigrostriataldopamine neurons from MPTP neurotoxicity (Chung et al., 2016; Ramírez et al., 2005; Shi et al., 2017; Walter et al., 2003)but the effect of co-administration of khat and JWH133 on locomotor activity in MPTP

lesioned mice had not been investigated. The result suggests that khat alone or in combination with the CB2R agonist might have a neuroprotective effect and it may have a significant role in reversing the motor deficits observed in PD patients.

In an effort to understand how khat affects the dopamine system, immunohistochemical staining for TH positive cells was performed. The data revealed that mice treated with khatshowed a significant increase in TH positive cells in the VTA compared to the controls. However, JWH133 failed to show a significant difference in TH immunoreactivity compared to controls when administered alone, which is in agreement with a previous study(Price et al., 2009). Co-administration of JWH133 with khat increased TH immunoreactive cells in the VTA region compared to controls but not compared to khat alone treated mice. The result also showed that, there was no seeming difference detected in TH positive cells in the SNc region of khat treated mice compared to controls. Although TH-immunoreactivity was not performed in MPTP-lesioned mice, the fact that increased immunoreactivity observed in VTA but not in SNc in normal mice suggests that khat may exert region specific effect during physiological and pathological conditions. Evidence for this assertion comes from the observation that though dopamine neurons in both regions share many physiological properties (Grace and Onn, 1989; Johnson and North, 1992; Lacey et al., 1989), they markedly differ in their sensitivity to addictive drugs such as nicotine (Keath et al., 2007).

Tyrosine hydroxylase is the rate-limiting enzyme of catecholamine biosynthesis (Daubner et al., 2011). The activity of the enzyme is regulated by two mechanisms; TH protein synthesis and phosphorylation. Catecholamines bind the active site of the enzyme and produce feedback inhibition but phosphorylation substantially decreases affinity of catecholamines thereby

relieving feedback inhibition and increasing enzyme activity (Gordon et al., 2008). Since cathinone, the active principle of khat, has similar mechanism of action (Al-Hebshi and Skaug, 2005)and related structure (Zelger et al., 1980)to amphetamine, it is worth expecting that khat has a similar dopamine releasing effect to amphetamine. In this context, the increased synaptic dopamine levels after khat are subject to enzymatic degradation rather than repacking into vesicles like the case of amphetamine (Siciliano et al., 2015),which ultimately results in increased dopamine turnover. The increase in TH expression after khat administration might be attributed to an enhanced dopamine turnover and consequently an enhanced expression of the enzyme required for its biosynthesis. Taken together, enhanced locomotor activity and increased expression of TH positive cells after khat administration may suggest that khat may have a significant therapeutic effect in PD.However there is a need for further studies to establish the molecular mechanisms involved in anti-PD potential of khat.

To produce furtherevidence for the role of khat and cannabinoids in dopaminergic system, the effect of khatalone and in combination with JWH133 was evaluated on the expression of DAT mRNA using qRT-PCR technique in WT mice. The result demonstrated that, khat administration increases the expression of DAT mRNA compared to vehicle treated controls. However, co-administration of khat with JWH133 failed to alter the expression of DAT mRNA compared to khat alone treated mice.

Previous studies on the effect of amphetamine on DAT expression showed that administration of amphetamine increases surface expression (Furman et al., 2009; Johnson et al., 2005a, 2005b)and mRNA level in the VTA and SNc(Dietz et al., 2005) of DAT.The relationship between DAT expression and amphetamine may be due to several factors. First, higher DAT

levels likely result in increased amphetamine transport and thus augmented intracellular amphetamine concentrations. In addition to increased cytoplasmic amphetamine levels, higher DAT levels may also allow for augmented DAT coupling to vesicular monoamine transporter-2 on synaptic vesicles, which together produce greatly augmented intravesicular amphetamine concentrations and thereby greater vesicular depletion of transmitter via amphetamine-induced disruption of vesicular pH gradients. Increased vesicular depletion results in augmented cytoplasmic dopamine levels, which can then be moved into the extracellular space via DAT-mediated reverse transport more readily due to augmented DAT levels (Siciliano et al., 2015; Sulzer and Rayport, 1990). Hence, the effect of khat on DAT mRNA expression in this study might be viewed similarly to the effect of amphetamine since the two compounds are presumed to have similar effect as mentioned above. Dopamine modulates physiological processes like locomotor activity, cognitive processes, reward and addiction. Dysfunction of the dopaminergic system is thought to be attributable to the development of multiple neurological disorders such as schizophrenia, PD, depression, attention deficit hyperactivity disorder and drug addiction (Zhu and Reith, 2008). This suggests that khat modulation of dopaminergic system could be exploited in studies of CNS disorders associated with dopamine dysfunction.

6. Strength and limitations of the study

6.1. Strength of the study

The strength of the present work is that, behavioral and molecular biology techniques were used to investigate the interaction between khat and the cannabinoid system in different strains of mice, which make the experimental design more rigorous and robust.

6.2. Limitations of the study

The major limitation of the present study is lack of sufficient amount of DAT-*Cnr2*cKO mice. In order to have a complete understanding of the interaction between khat and the cannabinoid system, TH immunostaining and DAT mRNA expression after administration of khat alone or in combination with the cannabinoid ligands should be done in DAT-*Cnr2* and MPTP lesioned C57BL/6 mice. The other limitation of the study isfailure to fractionate the active compound found in khat extract which is responsible for the observed effects. Isolating the active principle can help modify the structure and come up with a better therapeutic compound with minimal psychoactive effects.

7. Conclusions

In conclusion, work presented herein highlights the important modulation by the ECS of the neurobehavioral effect of khat depending on the paradigm used. The finding shows that activation of brain CBRs by WIN-55,212-2 enhances and inhibition by antagonists reducesthe effects of khat on locomotor activity, anxiolytic and working memory related behavior. This signifies the positive modulatory role of the ECS on behavioral effects of khat. On the other hand, co-administration of JWH133 inhibits khat's effect on locomotor activity and manipulation of CB2Rs augments khat induced hyperactivity, showing the negative modulatory role of the CB2Rs on locomotor activity of khat. In addition, this study also highlights the role of khat and the ECS on dopaminergic system. The data shows that khat attenuated MPTP-induced motor deficit and increased TH-positive cells and DAT mRNA expression, however, these neuroprotectiveeffects were not altered by concurrent administration with JWH133, suggesting that the CB2Rs selectively interact with khat-mediated neurobehavioral effects. This study provides a strong preclinical support for the therapeutic potential of khat and cannabionidsin the treatment of CNS disorders associated with dopamine dysregulation. Although khat is known to have several adverse effects that may limit the plant usefulness for therapeutic purposes, future research should address the culprit for most of the side effects as well as how to minimize intrinsic adverse effects associated with khat use.

8. Future directives

Whatever the mechanisms, the present findings, for the first time, reveal the interaction of the ECS and khat in mice. This work showed that, khat and cannabinoid based therapies may have the potential to be considered in treating disorders associated with aberrant dopamine function. Such an endeavor will require a better understanding of how khat, cannabinoids and manipulation of the CBRs influence behavior, especially over long-term treatment protocols. Investigations into cannabinoid control of dopamine neurotransmission have revealed important mechanisms by which neural circuits communicate through bidirectional synaptic signaling. A paucity of information remains, however, regarding the specific locale and conditions that drive eCB regulation of dopamine function. Previous studies done on khat also revealed significant behavioral effects of khat in animals and human beings, however, none of them mentioned the detailed molecular mechanisms involved in the observed behavioral effects. Further studiescan exploit thetherapeutic potential of khat and targeting the ECS in disorders associated with dopamine.

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