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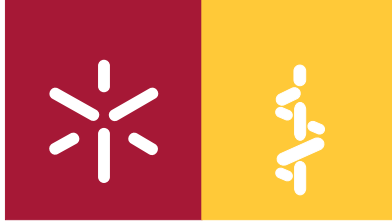
Ana Raquel Franky Gomes Carvalho

Modulation of limbic noradrenergic circuits by cannabinoids

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**Modulation of limbic noradrenergic
circuits by cannabinoids**

Tese de Doutoramento
Medicina - Medicina

Trabalho efectuado sob a orientação de
Doutora Elisabeth Van Bockstaele
Professora do Department of Neurosciences
Thomas Jefferson University, Philadelphia, PA – USA
Doutor Nuno Sousa
Professor da Escola de Ciências da Saúde
Universidade do Minho, Braga – Portugal

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DECLARAÇÃO

Nome: Ana Raquel Franky Gomes Carvalho

Endereço electrónico: arfranky@gmail.com

Telefone: +351 966390095

Número de Bilhete de Identidade: 11977651

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Orientadores:

Professora Elisabeth Van Bockstaele

Professor Doutor Nuno Sousa

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE A DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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Assinatura: _____

"É proibido não rir dos problemas
Não lutar pelo que se quer,
Abandonar tudo por medo,
Não transformar sonhos em realidade."

Pablo Neruda

"A place for everything, everything in its place."
Benjamin Franklin

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To all my family.

To all my friends.

Abstract

The endocannabinoid system has been implicated in the regulation of several physiological functions. The widespread distribution of the endocannabinoid system in the central nervous system (CNS) accounts for many effects attributed to cannabinoids. Importantly, cannabinoids have been shown to modulate mood, cognition and memory. There is growing evidence suggesting that cannabinoids can interact with the noradrenergic system. Noradrenergic transmission in the CNS has also been implicated in the regulation of mood, cognition and memory. In the present work, the hypothesis that cannabinoids can impact noradrenergic transmission in the limbic system was examined. Firstly, localization of the cannabinoid receptor type 1 (CB1r) was performed in the nucleus accumbens (Acb) and in the nucleus of the solitary tract (NTS), using immunohistochemical techniques, to clarify the anatomical substrates underlying potential interactions. It was shown that CB1r is present in noradrenergic neurons of the NTS. In addition, CB1r was found in the Acb but rarely in noradrenergic terminals. Furthermore, the effects of cannabinoid administration on adrenergic receptor (AR) expression in the Acb were studied. Western blot analysis of accumbal tissue revealed that exogenous administration of the synthetic cannabinoid WIN 55,212-2 decreases α2A- and β1-AR expression. Finally, the importance of norepinephrine (NE) in cannabinoid-induced behaviors was tested. Using the place conditioning paradigm and the elevated zero maze (EZM), the effects of cannabinoids on aversion and anxiety, respectively, were tested following depletion or blockade of noradrenergic transmission in the Acb or in the bed nucleus of the stria terminalis (BNST). Using an immunotoxin approach, NE depletion restricted to the Acb, but not BNST, blocked the expression of aversion to WIN 55,212-2. Depletion of NE had no effect on WIN 55,212-2-induced anxiety. Moreover, the fact that blockade of β1-AR in the Acb prevents WIN 55,212-2-induced aversion suggests that noradrenergic transmission via β1-AR is critical for eliciting this behavior.

In conclusion, the present work provides new evidence supporting the idea that cannabinoids can impact noradrenergic transmission in the limbic system. In addition, cannabinoid-induced aversion is dependent on intact noradrenergic transmission in the Acb. Taken together, the studies provide herein clarify the anatomical and neurochemical substrates for cannabinoid actions in the CNS.

Resumo

O sistema endocanabinóide tem sido implicado na regulação de várias funções fisiológicas. A dispersa distribuição do sistema endocanabinóide no sistema nervoso central (SNC) explica os muitos efeitos atribuídos aos canabinóides. De realçar que tem sido demonstrado que os canabinóides modelam o humor, cognição e memória. Existe uma crescente evidência sugerindo uma interacção entre o sistema endocanabinóide e o sistema noradrenérgico. Por seu lado, transmissão noradrenérgica no SNC tem sido implicada na regulação do humor, cognição e memória. No presente trabalho, a hipótese de que os canabinóides podem afectar a transmissão noradrenérgica no sistema límbico foi examinada. Inicialmente, a localização do receptor dos canabinóides tipo 1 (CB1r) no núcleo accumbens (Acb) e no núcleo do tracto solitário (NTS) foi efectuada utilizando técnicas de imunohistoquímica, de forma a clarificar os substratos anatómicos subjacente a potenciais interacções. Foi demonstrado que CB1r está presente em neurónios noradrenérgicos do NTS. Para além disso, CB1r foi encontrado no Acb mas raramente em terminais noradrenérgicos. Adicionalmente, os efeitos da administração de canabinóides na expressão de receptores adrenérgicos no Acb foram estudados. Análise por western blot de tecido do Acb revelou que administração exógenea do canabinóide sintético WIN 55,212-2 diminui a expressão dos receptores adrenérgicos α2A e β1. Finalmente, a importância da noradrenalina (NA) nos comportamentos induzidos pelos canabinóides foi testada. Utilizando o paradigma de "place conditioning" e o teste "elevated zero maze" (EZM), os efeitos dos canabinóides na aversão e anxiedade foram testados após depleção ou bloqueio da transmissão noradrenérgica no Acb ou no núcleo da estria terminalis (BNST). Utilizando uma imunotoxina, a depleção restrita de NA no Acb, mas não no BNST, bloqueou a aversão ao WIN 55,212-2. Enquanto que depleção de NA não teve nenhum efeito na anxiedade provocada por WIN 55,212-2. Mais, o facto de o bloqueio do receptor adrenérgico β1 no Acb prevenir a aversão induzida por WIN 55,212-2 sugere que a transmissão noradrenérgica via este receptor é fundamental para a expressão deste comportamento. Em conclusão, o presente trabalho fornece nova evidência suportando a ideia de que os canabinóides podem afectar a transmissão noradrenérgica no sistema límbico. Mais, a aversão induzida por canabinóides é dependente da transmissão noradrenérgica no Acb. Em conjunto, os estudos apresentados neste trabalho esclarecem os substratos anatómicos e neuroquímicos das acções dos canabinóides no SNC.

Contents

Abbreviations

2-AG - 2-arachidonoylglycerol

Δ9-THC - (−)-trans-Δ9-tetrahydrocannabinol

Acb - Nucleus accumbens

AR - Adrenergic receptor

BNST - Bed nucleus of the stria terminalis

CB1r - Cannabinoid receptor type 1

CB2r - Cannabinoid receptor type 2

CNS - Central nervous system

CPA - Conditioned place aversion

CPP - Conditioned place preference

CTA - Conditioned taste aversion

DAGL - Diacylglycerol lipase

DSE - Depolarization-induced suppression of excitation

DSI - Depolarization-induced suppression of inhibition

EMT - Endocannabinoid membrane transporter

EPM - Elevated plus maze

ERK - Extracellular signal-regulated kinase

EZM - Elevated zero maze

FAAH - Fatty acid amide hydrolase

FAK - Focal adhesion kinase

GPCR - G-protein coupled receptor

LC - Locus coeruleus

LC-NE - Locus coeruleus noradrenergic

LTD - Long-term depression

LTP - Long-term potentiation

MAGL - Monoacylglycerol lipase

mGLuR - Metabotropic glutamate receptor

NAPE-PLD - *N*-acylphosphatidylethanolamine-hydrolizing phospholipase D

NAT - *N*-acyltransferase

NE - Norepinephrine

NET - Norepinephrine transporter

NTS - Nucleus of the solitary tract

PE - Phosphatidylethanolamine

PFC - Prefrontal cortex

PLA1 - Phospholipase A1

PLC - Phospholipase C

PTSD - Posttraumatic stress disorder

TH - Tyrosine hydroxylase

VTA - Ventral tegmental area

Introduction

1.1 The endocannabinoid system

Marijuana (Cannabis sativa) has been used medicinally and recreationally for thousands of years (Crippa *et al.*, 2010). Descriptions of its effects include ability to alter perception and judgment, increase euphoria and appetite, decrease nausea and impairment of motor coordination. Since the identification of its main psychoactive component, (−)-trans-Δ9-tetrahydrocannabinol (Δ9-THC) (Gaoni & Mechoulam, 1964), numerous related compounds have been synthesized or isolated, and together they form a class of drugs called the cannabinoids.

The endocannabinoid system is constituted by its endogenous ligands, enzymes for synthesis and degradation and its receptors (Figure 1). The endocannabinoid system is widely distributed in the brain tissue of several vertebrates. The discovery of the cannabinoid receptors, type 1 (CB1r) in 1990 (Matsuda *et al.*, 1990) and type 2 (CB2r) in 1993 (Munro *et al.*, 1993), together with the identification of the endogenous ligands, anandamide (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), led to an increased interest in this system.

1.1.1 The synthesis and degradation of endocannabinoids

The first endocannabinoid to be identified was *N*-arachidonoylethanolamide (anandamide) in isolates derived from the pig brain (Devane *et al.*, 1992). Three years later, a second endocannabinoid (2-AG) was identified by two independent laboratories (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). Despite their similar chemical structures, anandamide and 2-AG possess distinct biosynthesis pathways. The existence of different enzymatic routes for their synthesis suggests that, under certain circumstances, these two endocannabinoids might operate independently of each other (Stella & Piomelli, 2001). In addition, anandamide and 2-AG show different binding efficacies to both CB1r and CB2r as 2-AG has been shown to have higher affinity than anandamide (Kano *et al.*, 2009). In fact, 2-AG acts as a full agonist while anandamide is seen as a partial agonist (Burkey *et al.,* 1997). Interestingly, both ligands show higher affinity to CB1r than CB2r. Nonetheless, the production of both endocannabinoids seems to be dependent on cell activation and increases in intracellular Ca^{2+} (Piomelli, 2003).

Biosynthesis of anandamide

Anandamide can be synthesized from enzymatic condensation of free arachidonic acid and ethanolamine by the enzyme fatty acid amide hydrolase (FAAH) (Deutsch & Chin, 1993). However, this pathway does not seem to be the main route for anandamide synthesis as it requires very high concentrations of arachidonic acid and ethanolamine (Devane & Axelrod, 1994; Kruszka & Gross, 1994; Ueda *et al.*, 1995; Sugiura *et al.*, 1996). In fact, FAAH is seen as the main enzyme responsible for hydrolysis of anandamide (Deutsch & Chin, 1993; Piomelli, 2003; Gulyas *et al.*, 2004). At least one other pathway for the synthesis of anandamide is known and it is thought to be the main source of anandamide. This pathway is composed of two enzymatic reactions (Cadas *et al.*, 1997), the transfer of an arachidonate group from phospholipids to phosphatidylethanolamine (PE) by the enzyme *N*-acyltransferase (NAT), yielding the formation of *N*-arachidonoyl phosphatidylethanolamine. Secondly, *N*-arachidonoyl phosphatidylethanolamine is hydrolyzed to anandamide and phosphatidic acid by *N*-acylphosphatidylethanolamine-hydrolizing phospholipase D (NAPE-PLD). NAT is Ca^{2+}-dependent and it is thought to be the rate-limiting step in the anandamide production (Cadas *et al.*, 1996). Ca^{2+} also stimulates NAPE-PLD along with Mg^{2+} (Okamoto *et al.*, 2004).

Biosynthesis of 2-arachidonoylglycerol

Several pathways have been described for the synthesis of 2-A. At present, the main pathway for the synthesis of 2-AG involves the enzymes phospholipase C (PLC) and diacylglycerol lipase (DAGL). First, membrane phospholipids containing arachidonic acid, like phosphatidylinositol, are hydrolyzed to diacylglycerol by PLC. Subsequently, diacylglycerol is hydrolyzed to 2-AG by DAGL. Other pathways for production of 2-AG include sequential reactions by phospholipase A1 (PLA1) and lysoPI-specific PLC (Ueda *et al.*, 1993; Sugiura *et al.*, 1995), conversion from 2-arachidonoyl lysophosphatidic acid to 2-AG by phosphatase (Nakane *et al.*, 2002) and formation from 2-arachidonoyl phosphatidic acid through 1-acyl-2-arachidonoylglycerol (Bisogno *et al.*, 1999; Carrier *et al.*, 2004).

Degradation of endocannabinoids

Anandamide and 2-AG possess distinct degradation pathways. Anandamide is hydrolyzed by FAAH. FAAH is located predominantly postsynaptic and juxtaposed to axon terminals containing

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CB1r (Egertova *et al.*, 2003; Gulyas *et al.*, 2004). On the other hand, 2-AG is hydrolyzed by monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002; Dinh *et al.*, 2004) which is believed to be present mostly in axon terminals (Dinh *et al.*, 2002).

1.1.2 The cannabinoid receptors

CB1r and CB2r are the two major cannabinoid receptors. Despite the fact both are G-protein coupled receptors (GPCR), mainly coupled to Gi (inhibitory) protein, they share only 44% amino acid sequence identity (Munro *et al.*, 1993) and show a very distinct distribution. CB1r is highly expressed in the central nervous system (CNS); however, its distribution is not homogeneous, with highest densities observed in the cerebral cortex, hippocampus, basal ganglia and cerebellum (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991). CB2r is found mainly in immune cells (Munro *et al.*, 1993; Galiegue *et al.*, 1995). However, in the last years, CB2r has been identified in the CNS albeit in lower levels than CB1r (Gong *et al.*, 2006; Onaivi, 2006). Interestingly, in the CNS, CB2r is reported to be distributed in neuronal somata and dendrites, but not in axon terminals like CB1r.

CB1r
CB1r was first cloned in 1990 by Matsuda and colleagues (Matsuda *et al.*, 1990). CB1r is known to be primarily coupled to the Gi family of G proteins. As a result, activation of CB1r mediates inhibition of adenylyl cyclase leading to a decrease of intracellular cAMP (Howlett *et al.*, 1986; Pertwee, 1997; Demuth & Molleman, 2006). However, coupling of CB1r to Gs and Gq proteins has been suggested (Glass & Felder, 1997; Maneuf & Brotchie, 1997; Lauckner *et al.*, 2005). In addition to its effects on adenylyl cyclase activity, activation of CB1r activates A-type (Hampson *et al.*, 1995) and inwardly rectifying K^+ channels (Mackie *et al.*, 1995), inhibits N- and P/Q-type Ca^{2+} channels (Twitchell *et al.*, 1997) and D- and M-type K^+ channels (Mu *et al.*, 1999; Schweitzer, 2000). CB1r activation has also been shown to activate focal adhesion kinase (FAK) (Derkinderen *et al.*, 1996), mitogen-activated protein kinase (Bouaboula *et al.*, 1995) and extracellular signal-regulated kinase (ERK) (Derkinderen *et al.*, 2003).

CB2r

CB2r was identified in 1993 (Munro *et al.*, 1993) in macrophages. Since its identification, CB2r was seen as the peripheral target for cannabinoids, with actions mainly in the immune system. As a regulator of the peripheral immune system it was expected that CB2r could also be a modulator of the central immune system and, in fact, CB2r was later identified in microglia (Nunez *et al.*, 2004; Ashton *et al.*, 2006). Additionally, CB2r has also been identified in neurons of the cerebellum and hippocampus (Gong *et al.*, 2006; Onaivi, 2006).

CB2r are GPCR, coupled to Gi proteins. Contrary to CB1r that is able to signal through Gs, CB2r is unable to couple to Gs (Glass & Felder, 1997; Maneuf & Brotchie, 1997). In addition, CB2r are also unable to regulate ion channels (Felder *et al.*, 1995).

<u>Endocannabinoid role in synaptic system</u>

As mentioned earlier, CB1r is located mainly to axon terminals and presynaptic sites. This localization is consistent with the findings that cannabinoids mediate suppression of neurotransmitter release by retrograde signaling (Llano *et al.*, 1991; Pitler & Alger, 1992; Wilson & Nicoll, 2001), a phenomenon known as depolarization-induced suppression of inhibition (DSI) or excitation (DSE), placing the endocannabinoid system as a synaptic modulatory system. In this signaling pathway, postsynaptic cells are depolarized leading to an increase in endocannabinoid production. Endocannabinoids, mainly 2-AG, are released to act on presynaptic receptors. Activation of presynaptic receptors will decrease the likelihood of release of glutamate or GABA from the terminal (Wilson & Nicoll, 2002; Piomelli, 2003). Because DSI is absent in CB1r knockout mice, CB1r is seen as the presynaptic target of endocannabinoids (Varma *et al.*, 2001; Wilson *et al.*, 2001). In addition to this fast, "on demand" mechanism to modulate synaptic transmission, endocannabinoids have also been implicated in synaptic plasticity, namely, by affecting long-term depression (LTD) and long-term potentiation (LTP) (Sullivan, 2000). Modulation of LTD/LTP by endocannabinoids requires, as in DSI/DSE, the release of endocannabinoids by the postsynaptic cell in response to a Ca^{2+} rise and/or activation of group I metabotropic glutamate receptors (mGluR-I) on the postsynaptic cell, to act on presynaptic CB1r (Chevaleyre *et al.*, 2006). The difference between DSI/DSE and LTD/LPT resides in the amount, nature and duration of endocannabinoid release.

Cannabinoids are also able to induce long lasting changes in neuronal morphology. After chronic treatment with CB1r agonists, changes in dendritic morphology were observed in areas like prefrontal cortex (PFC), nucleus accumbens (Acb) and hippocampus (Kolb *et al.*, 2006;

Tagliaferro *et al.*, 2006). In summary, cannabinoids can have a long-term impact in the CNS, but the functional implications of such actions are still unclear.

1.1.3 The endocannabinoid system as a potential therapeutic target

Due to its wide distribution, the endocannabinoid system has a great range of potential therapeutic applications. From management of nausea and vomiting to neuroprotection or
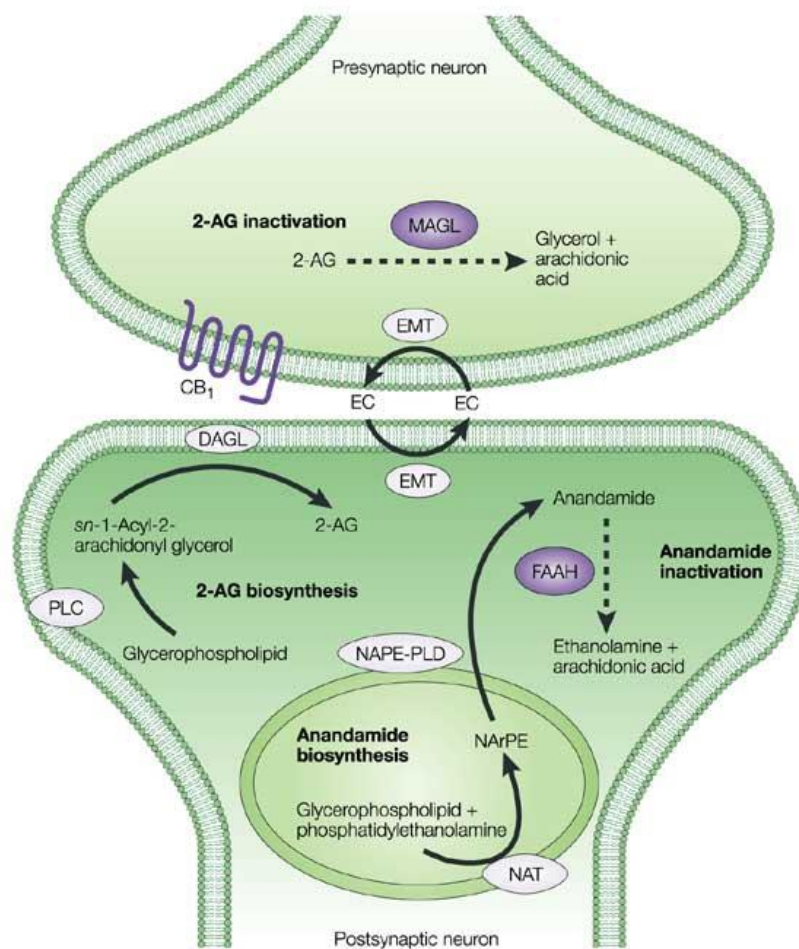
Figure 1. The endocannabinoid system. The enzymes for 2-AG biosynthesis, PLC and DAGL seem to be mostly localized in postsynaptic neurons. MAGL, responsible for inactivation of 2-AG is localized in presynaptic neurons, while FAAH, for degradation of anandamide, is localized in postsynaptic neurons. The localization of anandamide biosynthetic enzymes NAT and NAPE-PLD is not yet known, but thought to be postsynaptic. CB1r is found mainly presynaptic in accordance with the retrograde signaling hypothesis proposed for the endocannabinoid system. Lastly, the not yet identified endocannabinoid membrane transporter (EMT) seems to facilitate both endocannabinoid release and re-uptake, and might be localized on both pre- and postsynaptic neurons. With permission from Di Marzo *et al.*, 2004

antitumoral activity (Köfalvi, 2008), several are the fields where cannabinoid modulation could display therapeutic actions. However, with the systemic use of cannabinoid agonists/antagonists, some side effects have been reported which can preclude the use of such agents in a more broad number of patients. For instance, the synthetic Δ9-THC, dronabinol, is indicated to stimulate appetite in patients with AIDS suffering from anorexia with weight lost. Dronabinol is also indicated to treat nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional antiemetic treatments. Yet, the side effects of dronabinol, especially on the nervous system where it can exacerbate underlying mental illness such as mania, depression or schizophrenia (Food and Drug Administration, 2004), may be limiting the number of patients that could benefit from the drug. Additionally, the CB1r antagonist, rimonabant, was in clinical trials for the treatment of obesity, diabetes mellitus and cardiometabolic risk factors (Steinberg & Cannon, 2007). However, due to reservations about its safety, especially in patients with history of psychiatric disorders, clinical trials were suspended and the drug has been removed from the market (Sanofi-Aventis, 2008).

1.2 The noradrenergic system

The noradrenergic system, together with the serotoninergic, cholinergic and dopaminergic systems, is typically viewed as a neuromodulatory system. In contrast to glutamatergic or GABAergic neurons that have a widespread distribution, neuromodulatory neurons are confined to specific nuclei in the brain. Nevertheless, neuromodulatory neurons have widespread projections (Sara, 2009). The noradrenergic system, in particular, has the cell bodies grouped in nuclei in the brainstem, namely the locus coeruleus (LC) and the nucleus of the solitary tract (NTS) (Foote *et al.*, 1983; Weinshenker & Schroeder, 2007; Itoi & Sugimoto, 2010). While the LC is a homogenous nucleus in which most cells are noradrenergic (Foote *et al.*, 1983), the NTS contains several neurotransmitters (Barraco *et al.*, 1992). The noradrenergic neurons of the NTS are distributed throughout the caudal NTS (subpostremal and commissural NTS) (Barraco *et al.*, 1992). The LC, located within the dorsal wall of the rostral pons, in the lateral floor of the fourth ventricle, is the largest noradrenergic nucleus in the brain (Foote *et al.*, 1983) and is the sole source of norepinephrine (NE) in the forebrain (Sara, 2009). The LC is seen as the "arousal" center, important for regulation of sleep and vigilance, and activation of the LC is important for

selective attention (Southwick *et al.*, 1999; Sara, 2009). On the other hand, the NTS works as relay station for sensory signals arising from the viscera. The NTS integrates visceral information with other regulatory information coming from the brainstem, diencephalon and forebrain (Barraco *et al.*, 1992; Itoi & Sugimoto, 2010). The NTS is known to send efferents to autonomic centers in the brainstem but also to send ascending efferents to higher levels of the neuroaxis (Barraco *et al.*, 1992).

NE can interact with three families of adrenergic receptors (ARs): α1, α2 and β(1-3) receptors. α1 receptors are coupled to Gq proteins, hence activating phospholipase C and phosphotidyl inositol intracellular pathway, resulting in activation of protein kinase C and release of intracellular calcium (Duman & Nestler, 1995). α2-ARs, found pre- and postsynaptically (MacDonald *et al.*, 1997), are coupled to Gi proteins which can lead to a decrease in intracellular cAMP (Duman & Nestler, 1995). Presynaptic localized α2-ARs work as autoreceptors, since activation of these receptors will decrease intracellular cAMP and Ca^{2+}, inhibiting the release of neurotransmitters. β-ARs are coupled to Gs proteins, activating adenylyl cyclase and increasing intracellular cAMP (Duman & Nestler, 1995).

1.2.1 The noradrenergic system and mental health

Abnormalities of the noradrenergic system have been implied in some of the features of psychiatric disorders, such as schizophrenia, anxiety, depression and posttraumatic stress disorder (PTSD) (Friedman *et al.*, 1999; Southwick *et al.*, 1999; Nutt, 2002; Nutt, 2006; Itoi & Sugimoto, 2010). Several studies have revealed alterations in the levels of adrenergic receptor expression in depressed suicide victims. α2-AR density was found to be increased in brains of depressed suicide victims (Meana *et al.*, 1992; De Paermentier *et al.*, 1997; Callado *et al.*, 1998), while β1-AR density was reported to be decreased (De Paermentier *et al.*, 1990). These changes were not found throughout the brain suggesting that specific areas of the brain may be involved in the pathophysiology of the mood disorders. Moreover, pharmacological depletion of monoamines (e.g. reserpine) produces depressive-like behaviors in animal models, suggesting a role for monoamines (including NE) in pathophysiology of depression. Additionally, most antidepressants drugs in the market act by increasing the levels of synaptic monoamines. Hence, low levels of NE seem to account to the expression of depressive symptoms. It has been reported

an up-regulation of the rate-limiting enzyme in the synthesis of NE, tyrosine hydroxylase (TH) in the LC of depressed patients, which can be seen as a response to the low levels of NE observed in depression (Zhu *et al.*, 1999).

1.3 The nucleus accumbens and mental health

1.3.1 The limbic system

The limbic system is often synonymous of emotional brain. Initially, the limbic system was defined anatomically and included the cingulated cortex, the hippocampus, the thalamus, and the hypothalamus (MACLEAN, 1954). Later, this definition was questioned and it was suggested that the limbic system would not be defined only by anatomical localization to the limbic lobe and its connections but through functional connections which influence in emotional behavior (LeDoux, 1996; Berridge, 2003; Franks, 2006). Thus, areas like the amygdala, PFC and Acb are now recognized to be part of the limbic system (Berridge, 2003).

The study of the limbic system as the system controlling emotions is of great interest. More than understanding how the human brain processes emotions, it allows us to understand how the brain is disrupted in the disease processes such as in the case of mood disorders. While most research in the depression field has focused on hippocampus and PFC, great interesting on several subcortical structures such as the Acb, amygdala and hypothalamus, implicated in reward, fear and motivation, is emerging (Nestler & Carlezon, 2006). The role of the hippocampus in memory and spatial learning along with the PFC functions in working memory, attention and impulse control are consistent with some cognitive deficits seen in patients with depression and other mood disorders. Nonetheless, these areas do not seem to account for the diversity of symptoms found in these patients (Nestler & Carlezon, 2006).

1.3.2 The nucleus accumbens

Although the Acb is mostly seen as part of the reward/pleasure system, it was not initially considered part of the traditional limbic circuit (Berridge, 2003). Its role in emotion regulation is, however, indisputably striking. Due to its connectivity with the amygdala, the hippocampus, PFC
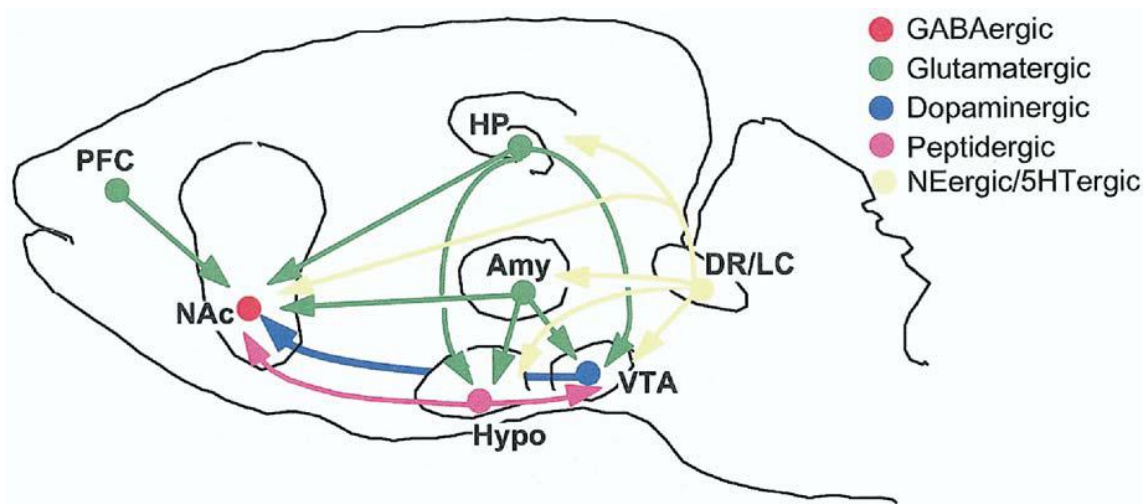
Figure 2. The neural circuitry of mood. The figure shows a highly simplified summary of a series of neural circuits in the brain that are believed to contribute to the regulation of mood. Amy, amygdale; DR, dorsal raphe; HP, hippocampus; Hypo, hypothalamus; LC, locus coeruleus; NAc, nucleus accumbens; NE, norepinephrine; PFC, prefrontal cortex; VTA, ventral tegmental area. With permission from Nestler & Carlezon, 2006.

and the ventral tegmental area (VTA), the Acb has been proposed to work as the ''limbic-motor interface'' (Mogenson *et al.*, 1980; Bonelli *et al.*, 2006; Meredith *et al.*, 2008) (Figure 2). Glutamatergic amygdalar projections transmit information about affective/emotional memory while glutamatergic afferents from the hippocampus and PFC convey contextual features from the environment. In addition, the Acb receives dopaminergic afferents from VTA which encode for the reward properties of behavior. As a "limbic-motor interface" Acb is positioned to modulate behavior and, in fact, Acb activity has been found to be disrupted in animal models of depression (Newton *et al.*, 2002; Shirayama & Chaki, 2006).

Anatomically, the Acb is part of the ventral striatum and is composed of a central "core" subregion and a peripheral and medially situated "shell" subregion. The core subregion works as a modulator of generic motor responses, while the shell seems to integrate emotional and motivational valences into a motor response (Maldonado-Irizarry & Kelley, 1994). The Acb is constituted by GABAergic medium spiny neurons (90%) and cholinergic interneurons (10%) (Meredith, 1999). Connectivity between the two subregions has been described (van Dongen *et al.*, 2005; van Dongen *et al.*, 2008) suggesting that, although the two subregions seem to have different roles in behavior, they have the ability to modulate each other.

1.4 The interplay between the endocannabinoid and noradrenergic systems

Manipulation of the cannabinoid system exerts effects on mood and cognition that have some similarities with manipulations of the noradrenergic system. Briefly, increasing cannabinoid tone has been shown to improve mood like increasing noradrenergic tone with antidepressants. Moreover, overactivation of the cannabinoid system can cause mania (Henquet *et al.*, 2006), a side effect that has been reported by patients using antidepressants (Peet, 1994; Bond *et al.*, 2008; Tondo *et al.*, 2010). Taken together, the effects of cannabinoid and noradrenergic manipulation on CNS suggest that the two systems may interact or share some signaling pathways. Consistent with this, a study performed with human subjects revealed that administration of the β-AR blocker, propranolol, before consumption of marijuana prevented the cardiovascular effects of marijuana and prevented the learning impairment produced by marijuana (Sulkowski *et al.*, 1977). In line with this, early anatomical studies using radioautography, have identified moderate CB1r binding and CB1r mRNA in the principal noradrenergic nuclei, the LC and NTS (Herkenham *et al.*, 1991; Mailleux & Vanderhaeghen, 1992; Matsuda *et al.*, 1993; Derbenev *et al.*, 2004; Jelsing *et al.*, 2008). Characterization of CB1r distribution in the LC showed that CB1r is localized to somato-dendritic profiles as well as to axon terminals and neurochemical characterization of LC neurons showed that some of the CB1r-positive neurons are noradrenergic (Scavone *et al.*, 2010). The existence of CB1r in the LC and NTS prompts the hypothesis that cannabinoids may modulate noradrenergic activity. In fact, administration of cannabinoid-like agents has been shown to increase Fos expression in LC noradrenergic (LC-NE) neurons (Patel & Hillard, 2003; Oropeza *et al.*, 2005) and in NTS neurons (Jelsing *et al.*, 2009). Moreover, cannabinoid-like agents are also able to modulate LC and NTS firing (Himmi *et al.*, 1996; Himmi *et al.*, 1998; Mendiguren & Pineda, 2004; Mendiguren & Pineda, 2006; Muntoni *et al.*, 2006) suggesting that CB1r in the LC and NTS are functional. The anatomical and functional studies reveal a potential mechanism by which cannabinoids exert their effects on mood and cognition. The ability of cannabinoids to modulate LC and NTS activity can impact noradrenergic transmission in critical regions for regulation of mood and cognition. In fact, cannabinoids have been shown to increase NE release in the PFC (Oropeza *et al.*, 2005).

1.5 Aims of the study

Drugs targeting the endocannabinoid system are being explored to ameliorate, or even treat, several pathological processes. However, some safety issues persist, namely psychiatric side effects, demanding a better understanding of the mechanisms of these side effects. There is evidence suggesting that the endocannabinoid system can modulate noradrenergic transmission in the brain. As the noradrenergic system play a role in some symptoms of several psychiatric disorders, identifying how, where and when the endocannabinoid system is modulating the noradrenergic system becomes very pertinent. The clinical applications of such knowledge can be, at least, applied in two distinct perspectives. On one hand, it may allow us to understand and prevent some side effects of modulators of the endocannabinoid system and, on the other hand, to use modulators of the endocannabinoid system to revert impairments/disruption of the noradrenergic system.

In summary, this thesis aims to:

- Characterize the localization of CB1r in the Acb with respect to noradrenergic afferents
- Understand the implications of cannabinoid administration in adrenergic receptor expression in the Acb
- Investigate the role of NE in cannabinoid-induced behaviors

1.6 References

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Experimental work

Chapter 2.1

Carvalho AF, Mackie K and Van Bockstaele EJ

Cannabinoid modulation of limbic forebrain noradrenergic circuitry

Cannabinoid modulation of limbic forebrain noradrenergic circuitry

Ana F. Carvalho,[1,2] Kenneth Mackie[3] and Elisabeth J. Van Bockstaele[1]

[1]Neurosurgery, Farber Institute for Neurosciences, Thomas Jefferson University, 900 Walnut Street, Suite 417, Philadelphia, PA 19107, USA
[2]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[3]Psychological and Brain Sciences, Indiana University, Bloomington, IN, USA

Abstract

Both the endocannabinoid and noradrenergic systems have been implicated in neuropsychiatric disorders. Importantly, low levels of norepinephrine are seen in patients with depression, and antagonism of the cannabinoid receptor type 1 (CB1R) is able to induce depressive symptoms in rodents and humans. Whether the interaction between the two systems is important for the regulation of these behaviors is not known. In the present study, adult male Sprague–Dawley rats were acutely or chronically administered the CB1R synthetic agonist WIN 55,212-2, and α2A and β1 adrenergic receptors (AR) were quantified by Western blot. These AR have been shown to be altered in a number of psychiatric disorders and following antidepressant treatment. CB1R agonist treatment induced a differential decrease in α2A- and β1-ARs in the nucleus accumbens (Acb). Moreover, to assess long-lasting changes induced by CB1R activation, some of the chronically treated rats were killed 7 days following the last injection. This revealed a persistent effect on α2A-AR levels. Furthermore, the localization of CB1R with respect to noradrenergic profiles was assessed in the Acb and in the nucleus of the solitary tract (NTS). Our results show a significant topographic distribution of CB1R and dopamine beta hydroxylase immunoreactivities (ir) in the Acb, with higher co-localization observed in the NTS. In the Acb, CB1R-ir was found in terminals forming either symmetric or asymmetric synapses. These results suggest that cannabinoids may modulate noradrenergic signaling in the Acb, directly by acting on noradrenergic neurons in the NTS or indirectly by modulating inhibitory and excitatory input in the Acb.

Introduction

The cannabinoid receptor type 1 (CB1R) can be found in several areas of the brain such as the frontal cortex, basal ganglia, hippocampus, amygdala and brainstem (Mackie, 2005), and it has been implicated in the regulation of learning and memory as well as in depression, anxiety and pain. CB1R activation is known to inhibit GABA and glutamate release in several brain regions, including the hippocampus, dorsal striatum, cerebellum and nucleus accumbens (Acb; Hoffman & Lupica, 2000; Daniel & Crepel, 2001; Gerdeman & Lovinger, 2001; Robbe et al., 2001). In addition to the effects of cannabinoids on GABA and glutamate transmission, growing evidence points to a significant role for monoamines in cannabinoid-induced behaviors. Previous studies have shown important interactions between the cannabinoid and noradrenergic systems (Oropeza et al., 2005, 2007; Page et al., 2007; Fox et al., 2009; Jelsing et al., 2009). Systemic administration of the synthetic cannabinoid agonist WIN 55,212-2 was shown to increase the release of norepinephrine (NE) in the prefrontal cortex (PFC) (Oropeza et al., 2005). In addition,

WIN 55,212-2 increased c-fos expression in the locus coeruleus (LC) and in the nucleus of the solitary tract (NTS; Oropeza et al., 2005; Jelsing et al., 2009). Efferents of the LC and the NTS account for most of the noradrenergic projections to the forebrain. The noradrenergic input to cortical and limbic structures is important for brain arousal, memory and mood (Aston-Jones et al., 1991; Heninger et al., 1996). Dysregulation of this system plays a role in the pathophysiology of depression (Heninger et al., 1996; Anand & Charney, 2000; Nutt, 2002). Noradrenergic deficiency and dysfunction of adrenergic receptors (AR) may be present in some patients with depression and may be important for the response to antidepressants (Anand & Charney, 2000). Consistent with this, various studies show an increase in α2-AR density in brains of depressed suicide victims (Meana et al., 1992; De Paermentier et al., 1997; Callado et al., 1998) while β1-AR density is decreased (De Paermentier et al., 1990). With regard to antidepressant treatment, the levels of α2- and β1-ARs have been shown to decrease (De Paermentier et al., 1991, 1997) in areas such as temporal cortex, amygdala and thalamus of antidepressant-treated suicides. Moreover, chronic administration of WIN 55,212-2 has been shown to desensitize α2-ARs in noradrenergic-enriched areas (Moranta et al., 2009). Elucidating the effects of cannabinoid administration on the expression of these ARs may contribute to

Correspondence: A. F. Carvalho, [1]Neurosurgery, as above.
E-mail: arfranky@gmail.com

identifying the mechanism by which cannabinoids are involved in mood-related disorders (Hill & Gorzalka, 2005a; Witkin *et al.*, 2005; Leweke & Koethe, 2008).

In the present study, we studied the impact of a cannabinoid agonist on limbic forebrain noradrenergic circuitry using biochemical and neuroanatomical approaches. The limbic region analyzed, the Acb, is a brain region involved in the integration of motivation-related information, with important implications for drug addiction and mood disorders (Di Chiara, 2002; Shirayama & Chaki, 2006). Understanding how cannabinoids may impact noradrenergic input to the Acb may provide important information regarding the effects of CB1R compounds on drug-induced behaviors.

Materials and methods

Subjects

Adult male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 220–250 g were housed two or three per cage in a controlled environment (12-h light schedule, temperature at 20°C). Food and water were provided *ad libitum*. The care and use of animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University and were conducted in accordance with the NIH *Guide for the care and use of laboratory animals*. All efforts were made to reduce the number of animals used.

Antibody characterization and specificity

A list with the characterization of all the primary antibodies used can be found in Table 1. An affinity-purified polyclonal antibody directed against the CB1R was used; it was generated against a fusion protein containing the last 15 amino acids of the C-terminal of the rat CB1R fused to glutathione *S*-transferase. The specificity of this CB1R antibody has been determined in somatosensory cortex of mice lacking CB1R by Bodor *et al.* (2005). In addition to the aforementioned study, additional controls were conducted here. For example, immunoperoxidase detection of the CB1R antibody was conducted in tissue sections obtained from the forebrain of mice deficient in the CB1R (provided by Kenneth Mackie) and compared to that of similar sections obtained from wild-type mice. In these experiments, peroxidase detection for CB1R was absent in knockout tissue but present in wild-type samples (Fig. 1). In addition, specificity controls involved controlling for the secondary antibody by processing tissue that lacked primary antibody incubation. In such experiments, run in parallel, peroxidase immunoreactivity or immunogold–silver particles were not detected in tissue sections from which the primary antibody had been

omitted (Supporting information, Fig. S1). To evaluate possible cross-reactivity of secondary antibodies with the primary antisera in the dual labeling experiments, some sections were processed for dual labeling with omission of one of the primary antisera.

The monoclonal antibody against dopamine beta hydroxylase (DBH) was raised against purified bovine DBH. The specificity of the DBH antibody has also been demonstrated previously in our laboratory (Oropeza *et al.*, 2007). More specifically, preabsorption with the respective antigen (Alpha Diagnostics, San Antonio, TX, USA) resulted in an absence of immunolabeling in tissue sections from the frontal cortex.

The monoclonal (clone CL-300) antibody directed against calbindin was generated using purified calbindin-D from chicken gut. This antibody revealed the same distribution in the Acb as described by others (Voorn *et al.*, 1989; Jongen-Relo *et al.*, 1994).

The monoclonal antibody direct against the NE transporter (NET) was generated using a peptide (amino acids 05–17) of the mouse and rat NET coupled to keyhole limpet hemocyanin by the addition of a C-terminal cysteine. To test the specificity of the NET antibody, preabsorption of the antibody with the blocking peptide (1 μg/mL; MabTechnologies, Stone Mountain, GA, USA) resulted in the absence of immunolabeling in rat tissues containing the Acb (supporting Fig. S2).

The polyclonal antibody against the α2A-AR was developed against a synthetic peptide (Arg-Ile-Tyr-Gln-Ile-Ala-Lys-Arg-Arg-Thr-Arg-Val-Pro-Pro-Ser-Arg-Arg-Gly) derived from amino acids 218–235 of human, mouse, rat and pig α2A-AR. The polyclonal antibody against the β1-AR was raised against a synthetic peptide (His-Gly-Asp-Arg-Pro-Arg-Ala-Ser-Gly-Cys-Leu-Ala-Arg-Ala-Gly) derived from amino acids 394–408 of mouse and rat β1-AR. The specificity of α2A- and β1-AR antibodies was determined by preabsorption of the antibodies with the respective blocking peptide (10 μg/mL; Sigma-Aldrich, St Louis, MO, USA); this resulted in the absence of labeling in the blots loaded with whole-brain protein samples (supporting Fig. S3).

The monoclonal antibody against microtubule-associated protein (MAP2) was raised in mouse against rat brain MAP. The specificity of the MAP2 antibody has been described (Teng *et al.*, 2001), who found no detectable band in Western blots from brain extracts of MAP2-deficient mice.

Drug administration and Western blot analysis

WIN 55,212-2 (Sigma-Aldrich) was dissolved in 5% dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ, USA) in 0.9% saline and injected intraperitoneally in a volume of 1 mL/kg body weight. A dose–response study was performed in which animals

TABLE 1. Characterization of the primary antibodies

Antigen	Immunogen	Manufacturer	Host, mono/polyclonal	Catalog no.	Dilution
CB1R	Last 15 aa of the C-terminal of the rat CB1R	Dr K. Mackie*	Rabbit polyclonal	–	1:500 1:1000
DBH	Purified bovine DBH	Chemicon, Millipore	Mouse monoclonal	MAB308	1:1000
Calbindin D-28	Purified calbindin D-28 from chicken gut	Abcam	Mouse monoclonal	ab9481	1:300
NET	Peptide, aa 5–17 of mouse and rat NET	MabTechnologies	Mouse monoclonal	NET05-1	1:1000
α2A-AR	Synthetic peptide, aa 218–235 of human, mouse, rat and pig	Sigma-Aldrich	Rabbit polyclonal	A-271	1:500
β1-AR	Synthetic peptide, aa 394–408 of mouse and rat	Sigma-Aldrich	Rabbit polyclonal	A-272	1:1000
MAP2	Rat brain MAP	Abcam	Mouse monoclonal	ab11267	1:1000

*Synthesized in the laboratory of Dr K. Mackie; Chemicon, Millipore, Bedford, MA, USA; Abcam, Cambridge, MA, USA; MabTechnologies, Stone Mountain, GA, USA; Sigma-Aldrich, St Louis, MO, USA.
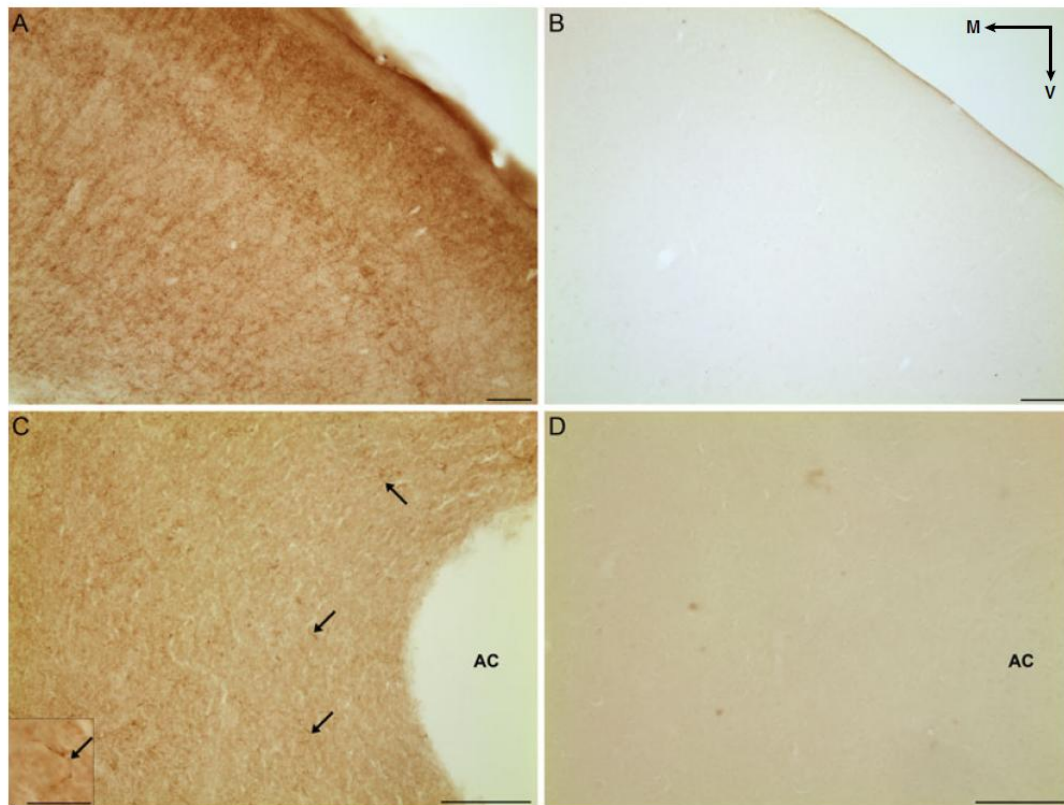
FIG. 1. Specificity of CB1R primary antibody. (A and C) Brightfield photomicrographs showing immunoperoxidase labeling for CB1R in a cross-section of (A) the frontal cortex (FC) and (C) the Acb (arrows) of a wild-type mouse brain. (B and D) Immunohistochemistry for CB1R in equivalent cross-sections of a CB1R-knockout mouse reveals an absence of immunolabeling in (B) the FC and (D) the Acb. Inset in C shows higher magnification of CB1R-labelled fiber indicated in C. AC, anterior commissure; M, medial; V, ventral. Scale bar, 100 μm.

received an acute injection of WIN 55,212-2 at 0.3, 1.0, 3.0 or 7.0 mg/kg ($n = 20$) or vehicle (5% DMSO in saline, $n = 5$). Another set of animals was divided into three treatment groups (acute, chronic and chronic + abstinence). In the acute group, animals received one injection of 3.0 mg/kg WIN 55,212-2 ($n = 8$) or vehicle ($n = 6$). The chronic group received a daily injection of WIN 55,212-2 ($n = 8$) or vehicle ($n = 6$) for 7 days. Animals in the dose–response study and in the acute and chronic groups were killed 40–45 min after the last injection. The chronic + abstinence group received repeated injections (7 days) of WIN 55,212-2 ($n = 8$) or vehicle ($n = 6$) and were killed 7 days after the last injection. Experimental animals were anesthetized with isoflurane (Isoflurane, USP; Webster Veterinary, Sterling, MA, USA) and decapitated. Brains were removed and a coronal section containing the whole extension of the Acb (from ∼0.7 to 2.7 mm anterior to bregma) was cut. The area punched was located medially to the anterior commissure and ventrally to the lateral ventricle, and included the shell and medial core of the Acb (as shown in Fig. 2). Part of the cerebellum was also collected from a coronal section from ∼10.50 to 12 mm posterior to bregma. Proteins were extracted in radio immunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein quantification was performed using the bicinchoninic acid reagent. Protein samples were loaded at equal concentrations and run on a 4–12% Tris-glycine gel (Invitrogen, Carlsbad, CA, USA). Gels were then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA) at 25 V for 2 h. Membranes were probed for rabbit anti-α2A-AR (1:500; Sigma-Aldrich), rabbit anti-β1-AR (1:1000; Sigma-Aldrich) or mouse anti-NET (1:5000; MabTechnologies) using the Western Breeze Chemilluminescent Kit (Invitrogen). In order to control for protein loading, each blot was stripped using Restore Stripping Buffer (Pierce, Rockford, IL, USA) and re-probed for β-actin (1:5000; Sigma-Aldrich).

Light microscopy and immunofluorescence

Seven naïve animals were used for light and immunofluorescence microscopy. Animals were deeply anesthetized with sodium pento-barbital (60 mg/kg), administered intraperitoneally, and transcardially perfused with 50 mL of heparinized saline followed by 400 mL of 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, brains were removed and postfixed in the same fixative. Coronal sections throughout the Acb and the NTS were cut at 40 μm using a Vibratome
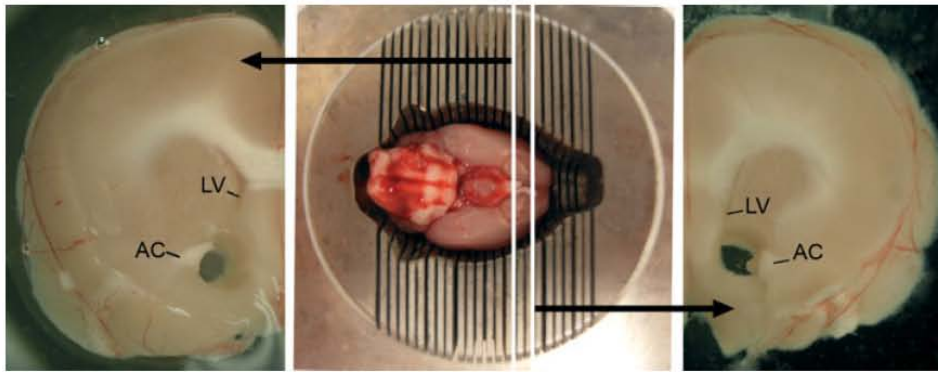
FIG. 2. Representative photomicrographs of the region of the Acb excised for protein analysis. A coronal section, ranging from just rostral to the optic chiasm to 2 mm anterior, was obtained in order to include the entire rostrocaudal extent of the Acb. Bilateral punches of the Acb were performed using a trephine medially to the anterior commissure (AC) and ventrally to the lateral ventricle (LV).

(Technical Product International, St Louis, MO, USA) and collected into 0.1 M PB. Every sixth section of the Acb was processed for immunohistochemical visualization of calbindin, CB1R or DBH immunoreactivities. Free-floating sections were treated with 1% sodium borohydride in 0.1 M PB for 30 min. They were then rinsed with 0.1 M PB and later washed in 0.1 M Tris saline buffer (TS; pH 7.6). The sections were blocked in 0.5% bovine serum albumin (BSA) in 0.1 M TS for 30 min and then washed for 5 min, twice. Sections were incubated overnight at room temperature with a mouse antibody for calbindin (1:300; Abcam, Cambridge, MA, USA), a rabbit antibody directed against CB1R (1:500) or a mouse monoclonal antibody recognizing DBH (1:1000; Chemicon, Millipore) in 0.1% BSA with 0.25% Triton-X 100 in 0.1 M TS. The sections were then washed in 0.1 M TS, three times for 10 min. Then, sections were incubated in a secondary biotin-conjugated donkey antirabbit or donkey antimouse IgG (1:400; Jackson ImmunoResearch, West Grove, PA, USA) in 0.1% BSA with 0.25% Triton-X 100 in 0.1 M TS for 30 min at room temperature. Then sections were washed in 0.1 M TS, three times for 10 min. Sections were incubated in an avidin–biotin complex solution (VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) in 0.1 M TS for 30 min and then washed. CB1R and DBH immunoreactivity was visualized with a red reaction by incubating the tissue sections in a red peroxidase substrate (VECTOR NovaRED substrate kit; Vector Laboratories) for 5 min, while the calbindin immunoreactivity was visualized with a blue reaction by incubating the sections in a blue peroxidase substrate (VECTOR SG substrate kit) for 10 min. The reaction was stopped by rinsing the sections in distilled water and then the sections were washed in 0.1 M TS. For dual immunofluorescence, every sixth section of Acb and NTS was processed as described above except that tissues were incubated overnight in a cocktail with rabbit anti-CB1R (1:500 for Acb sections, 1:5000 for NTS sections) and mouse anti-DBH antibodies or rabbit anti-CB1R and mouse anti-MAP2 (1:1000; Abcam) in 0.1% BSA+2% TritonX- 100 in 0.1 M TS. Tissue sections were then incubated in a secondary antibody solution containing fluorescein isothiocyanate donkey antimouse IgG (1:200; Jackson ImmunoResearch) and tetramethyl rhodamine isothiocyanate antirabbit IgG (1:400; Jacskon ImmunoResearch) in 0.1% BSA+2% Triton-X 100 in 0.1 M TS, for 2 h at room temperature. Sections were then washed in 0.1 M PB. Both dual- and single-labeled sections were mounted onto gelatinized glass slides from a 0.05 M PB solution. The slides were dehydrated through a graded series of alcohols and cleared

in xylene before being coverslipped with Permount (light microscopy; Fisher Scientific, Pittsburgh, PA, USA) or DPX (immunofluorescence; Sigma-Aldrich) mounting mediums.

Electron microscopy

Although DBH was an adequate marker for noradrenergic terminals using light and fluorescence microscopy because it was possible to increase penetration with detergents in thicker tissue sections, this vesicular-bound enzyme was more difficult to consistently detect using electron microscopy with low concentrations of permeabilization agents. Therefore, without using detergents, NET was used as a marker to detect noradrenergic axon terminals and did not compromise the ultrastructural preservation of the neuropil. NET and CB1R were visualized in sections through the Acb obtained from naïve rats ($n = 7$) that were perfused with 50 mL of heparinized saline followed by 100 mL 3.8% acrolein (Electron Microscopy Sciences) and 400 mL of 2% formaldehyde (Electron Microscopy Sciences) in 0.1 M PB. Sections were processed following the protocol described for light microscopy except that Triton-X 100 was not added to the solution for antibody incubation. The sections were incubated overnight, at room temperature, in a primary antibody solution containing rabbit anti-CB1R (1:500) and mouse anti-NET (1:1000) with 0.1%BSA in 0.1 M TS. The NET antibody was visualized using immunoperoxidase detection by incubating sections in biotinylated donkey antimouse IgG (1:400; Jackson ImmunoResearch Laboratories) followed by avidin–biotin complex (Vector Laboratories). The sections were then reacted with 22 mg of 3-3′ diaminobenzidine (DAB; Sigma-Aldrich) containing 0.05% hydrogen peroxide for 15 min. For immunogold detection of CB1R, sections were then incubated in ultrasmall gold-conjugated goat antirabbit IgG (1:50; Electron Microscopy Sciences) with 0.8% BSA in 0.01 M PBS containing 0.1% fish gelatin (Amerhsam Corp., Amerhsam, UK) for 2 h. Sections were rinsed in the same buffer and then rinsed in 0.01 M PBS and incubated in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.01 M PBS for 10 min, followed by washes in 0.01 M PBS and 0.2 M sodium citrate buffer (pH 7.4). A silver enhancement kit (Amersham Corp.) was used for silver intensification of the gold particles. Following intensification, tissue was rinsed in 0.1 M PB and incubated in 2% osmium tetroxide in 0.1 M PB for 1 h, washed in 0.1 M PB, dehydrated and flat-embedded in Epon 812 (Electron Microscopy Sciences). The reverse labeling was performed in which

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CB1R was visualized by immunoperoxidase detection by using biotinylated donkey antirabbit (1 : 400; Jackson ImmunoResearch Laboratories) and immunogold detection of NET was visualized by ultrasmall gold-conjugated goat antimouse IgG (1 : 50; Electron Microscopy Sciences). Thin sections of 74 nm in thickness from the mid-ventral shell of the Acb were cut using diamond knife and collected on copper mesh grids.

Controls

For Western blot analysis, in order to minimize differences in the areas excised for protein extraction, the same investigator conducted the tissue dissection. To minimize protein loading errors, all gels run were loaded by the same person.

For immunohistochemical experiments, to control for specificity of the secondary antibodies, controls in which the primary antisera was omitted were run in parallel. Sections processed in the absence of primary antibody did not exhibit immunoreactivity. To evaluate cross-reactivity of labeling of the primary antiserum by secondary antisera, some sections were processed for dual labeling with omission of one of the primary antisera. To assure that DBH and NET stained the same profiles, dual immunofluorescence for DBH and NET was performed in tissue sections containing the Acb as described before (supporting Fig. S4).

Data analysis

Western blot

Blots were scanned into a PC computer and band intensities were quantified using Kodak Molecular Imaging Software (Version 4.5; Carestream Health Inc., Rochester, NY, USA). Intensities of bands for the adrenergic receptor proteins were normalized to that of β-actin in the same sample. Average intensity of bands for acute control tissues was arbitrarily set at 1. Statistical analysis was performed using SPSS 16.0 Graduate Student Version. Statistical analysis of data from the dose–response study was conducted using a one-way ANOVA followed by *post hoc* Bonferroni (with significance set at $P < 0.05$). For the analysis of the effects of acute, chronic and abstinence, a 2×3 ANOVA on the interaction between drug treatment (vehicle and drug) and treatment duration (acute, chronic and abstinence) was conducted. When a significant interaction was observed between the two factors, simple effects tests were conducted and a Bonferronni correction was applied. The results are expressed normalized to vehicle group values and SEM values are given.

Light microscopy

Slides with single-labeled sections were visualized using a Leica DMRBE microscope (Wetzlar, Germany), and images were acquired using SPOT Advanced software (Diagnostics Instruments, Inc., Sterling Heights, MI, USA). Figures were then assembled and adjusted for brightness and contrast in Adobe Photoshop CS2. Schematics showing the distribution of CB1R and DBH immunoreactivity are represented on coronal diagrams (from 2.7 to 1.0 mm anterior to bregma) from the rat brain atlas of Paxinos & Watson (1997) by direct visualization of slides using a light microscope. Schematics were subsequently assembled in Adobe Photoshop CS2.

Dual immunofluorescence

For immunofluorescence, sections were visualized using a confocal microscope (Zeiss LSM 510 Meta; Carl Zeiss Inc., Thornwood, NY,

USA). Z-stacks from areas with dual labeling were collected and analyzed; single optical planes were analyzed individually for distribution and co-localization of the two markers throughout the thickness of the section. The data presented represent projections of six to nine single optical planes except for dual fluorescence in the NTS and CB1R and MAP2 pictures, for which a single plane from the z-stack is shown. Digital images were obtained and imported using the LSM 5 image browser. Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop CS2.

Electron microscopy

For ultrastructural analysis, at least 15 grids containing four to eight thin sections (74 nm of thickness) each were collected from at least three plastic-embedded sections of the mid-ventral shell of the Acb from each animal. Thin sections were viewed using a Morgagni 268 digital electron microscope (FEI Company, Hillsboro, OR, USA), initially at low magnification to ensure that background labeling in the neuropil, deemed spurious, was not commonly encountered, then at higher magnification to verify adequate cellular morphology. For quantification, electron micrographs from thin sections of three animals that showed optimal preservation of ultrastructural morphology were taken at different magnifications, usually at 11 000× and then at 14 000× to 22 000× for better resolution of the structures analyzed. Figures presented were assembled and adjusted for brightness and contrast in Adobe Photoshop CS2. Selective gold–silver-labeled profiles were identified by the presence of at least two gold particles within a cellular compartment. The criterion of a minimum of two gold particles as indicative of positive immunolabeling is based on the fact that one gold particle could occasionally be found in profiles known to lack CB1R or NET immunoreactivity, such as myelin and blood vessels. Immunoperoxidase labeling was regarded as positive when the electron-dense precipitate in individual profiles was considerably greater than that seen in other morphologically similar profiles in the neuropil. The cellular elements were identified based on the description of Peters *et al.* (1991). Somata contained a nucleus, Golgi apparatus and smooth endoplasmic reticulum. Proximal dendrites contained endoplasmic reticulum, were postsynaptic to axon terminals and were > 0.7 μm in diameter. A terminal was considered to form a synapse if it showed a junctional complex, a restricted zone of parallel membranes with slight enlargement of the intercellular space and/or associated with postsynaptic thickening. Asymmetric synapses were identified by thick postsynaptic densities (Gray's type I); in contrast, symmetric synapses had thin densities (Gray's type II) both pre- and postsynaptically. The term 'undefined' synaptic contact was used to denote parallel membrane association of an axon terminal plasma membrane juxtaposed to that of a dendrite or soma which lacked recognizable membrane specializations in the plane of section analyzed, and with no intervening glial processes. The term 'apposition' is also used to denote close parallel membrane associations of axon terminals with other axon terminals and/or dendrites which lacked recognizable specializations but were otherwise not separated by glial processes.

Results

WIN 55,212-2 altered the expression of adrenergic receptors in the Acb

The influence of a cannabinoid agonist on adrenergic receptor expression in the Acb was assessed by Western blot analysis of protein extracts that were obtained from the Acb of animals that received either an acute systemic injection of WIN 55,212-2, repeated

systemic injections of WIN 55,212-2 or repeated systemic injections of WIN 55,212-2 followed by a period of abstinence. The region targeted for tissue dissection included the area medial to the anterior commissure (shell and medial core). As reported by others (Rudoy & Van Bockstaele, 2007), protein extracts indicative of α2A-AR could be identified at ~45 kDa while proteins indicative of β1-AR migrated to ~65 kDa.

Several studies have reported that CB1R agonists have biphasic effects on behavior according to the dose used, with lower doses stimulating locomotion and higher doses inhibiting it (Rodriguez de Fonseca *et al.*, 1998; Drews *et al.*, 2005). Cannabinoids have also been shown to have anxiolytic and anxiogenic effects on animals (Witkin *et al.*, 2005). For the dose–response study, a one-way ANOVA demonstrated a significant difference among treatment groups in β1-AR protein expression ($P = 0.02$; $F_{3,12} = 10.833$) and *post hoc* comparison tests revealed that acute administration of WIN 55,212-2 induced a decrease in the expression of β1-AR at concentrations of 1.0 and 3.0 mg/kg ($P < 0.05$) when comparing to vehicle-treated animals
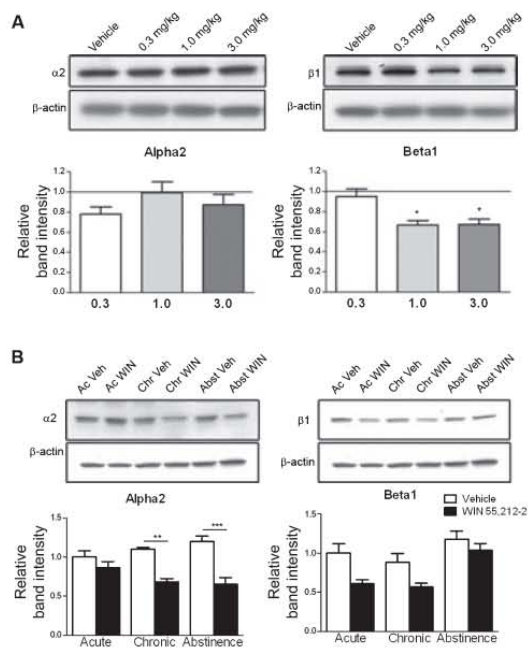

FIG. 3. Western blot for α2A and β1 adrenergic receptors (α2A- and β1-AR) from the Acb following WIN 55,212-2 treatment. Bands shown are representative of one sample from one animal of each group. (A) Dose–response study showing that acute administration of WIN 55,212-2 decreased the levels of β1-AR in the Acb at 1.0 and 3.0 mg/kg (*$P < 0.05$). None of the doses used had an effect on the levels of α2A-AR. (B) Western blot for α2A- and β1-AR in protein extracts from the Acb of rats administered WIN 55,212-2 (3.0 mg/kg) or vehicle, acutely (one injection) or chronically (7 days) and killed 40–45 min or 7 days (Chr+Abst group) after the last injection. α2A-AR expression was not altered by acute treatment with WIN 55,212-2. However, after chronic treatment there was a significant (**$P < 0.01$) decrease in the expression of α2A-AR and this decrease persisted in the absence of the drug for 7 days (Chr+Abst group; ***$P < 0.001$). Two-way ANOVA shows that β1-AR expression was significantly reduced after treatment with WIN 55,212-2 when compared to vehicle-treated animals. Data are presented as mean (+SEM) of change in band intensity normalized to values for vehicle-treated animals, with acute vehicle-treated animals set at 1.

(Fig. 3A). Conversely, one-way ANOVA revealed no significant difference in α2A-AR protein expression ($P = 0.271$; $F_{3,14} = 1.49$), demonstrating that none of the concentrations used had an effect on α2A-AR protein levels in the Acb with an acute injection (Fig. 3A). To investigate the effects of repeated administration of WIN 55,212-2, the 3.0 mg/kg concentration was used as it has also been shown that this concentration, but not 1.0 mg/kg, increases extracellular NE in the PFC (Oropeza *et al.*, 2005). In addition, 3.0 mg/kg has been shown to induce c-fos expression in the NTS (Jelsing *et al.*, 2009). Worthy of note, the high dose used (7.0 mg/kg) had very pronounced sedative effect on the animals, making this dose unsuitable for future studies.

To assess the effects of repeated administration of WIN 55,212-2 in α2A-AR and β1-AR protein expression a two-way ANOVA on the interaction between drug treatment (vehicle and drug) and treatment duration (acute, chronic and abstinence) was conducted (Fig. 3B). With respect to the effects in α2A-AR expression, the analysis revealed a significant interaction between the two factors ($P = 0.03$, $F_{2,26} = 4.103$). Therefore, a simple effects tests comparing vehicle- and drug-treated animals were conducted for the acute, chronic and abstinence conditions. A Bonferronni correction was applied. No differences in α2A-AR expression were observed with an acute injection of WIN 55,212-2 (3.0 mg/kg). However, the mean difference observed in the chronic condition (mean difference 0.42) was significant ($P < 0.01$; $t(5) = 7.09$). Similar effects were observed for the abstinence condition [mean difference 0.54; $P < 0.001$; $t(11) = 4.87$]. This shows that repeated treatment with WIN 55,212-2 (3.0 mg/kg) for 7 days (chronic group) significantly decrease the expression of α2A-AR and that this effect persisted over time as α2A-AR expression levels remained below control levels in the abstinence group. With respect to the effects in β1-AR protein expression, the analysis revealed a significant effect of drug treatment ($P < 0.001$; $F_{1,32} = 15.32$) and treatment duration ($P < 0.001$; $F_{2,32} = 10.67$). The effect of treatment condition suggested that subjects given WIN 55,212-2 showed a significantly decrease in β1-AR expression comparing to vehicle-treated animals. However, no interaction between the two factors was found ($P = 0.353$; $F_{2,21} = 1.076$).

Two-way ANOVA revealed no significant interaction between α2A-AR ($P = 0.668$; $F_{2,16} = 0.414$) and β1-AR ($P = 0.29$; $F_{2,18} = 1.327$) protein expression in samples from the cerebellum, an area rich in CB1R and noradrenergic input (data not shown). No significant effect was observed with respect to NET expression after treatment with WIN 55,212-2 (3.0 mg/kg; $P = 0.466$; $F_{1,18} = 0.555$; supporting Fig. S5).

Topographic distribution of CB1R in Acb core and shell subregions

The Acb extends for ~2.2 mm in the ventral striatum and is composed of a central 'core' and a peripheral and medially situated 'shell' subregion (Zahm, 1999; van Dongen *et al.*, 2008). To adequately distinguish the neuroanatomical boundaries of the core and shell subregions within the Acb, calbindin immunoreactivity was used as a marker to define these two subregions in adjacent coronal sections. As previously reported (Jongen-Relo *et al.*, 1994; Tan *et al.*, 1999), calbindin immunoreactivity was more prominent in the core and the overlying striatum where it often appeared in cell bodies (Fig. 4B). Our data are consistent with these reports, as calbindin immunoreactivity appeared prominently in the Acb core where peroxidase-labeled cell bodies could be identified immediately adjacent to the anterior commissure and approaching the lateral ventricle dorsally. The
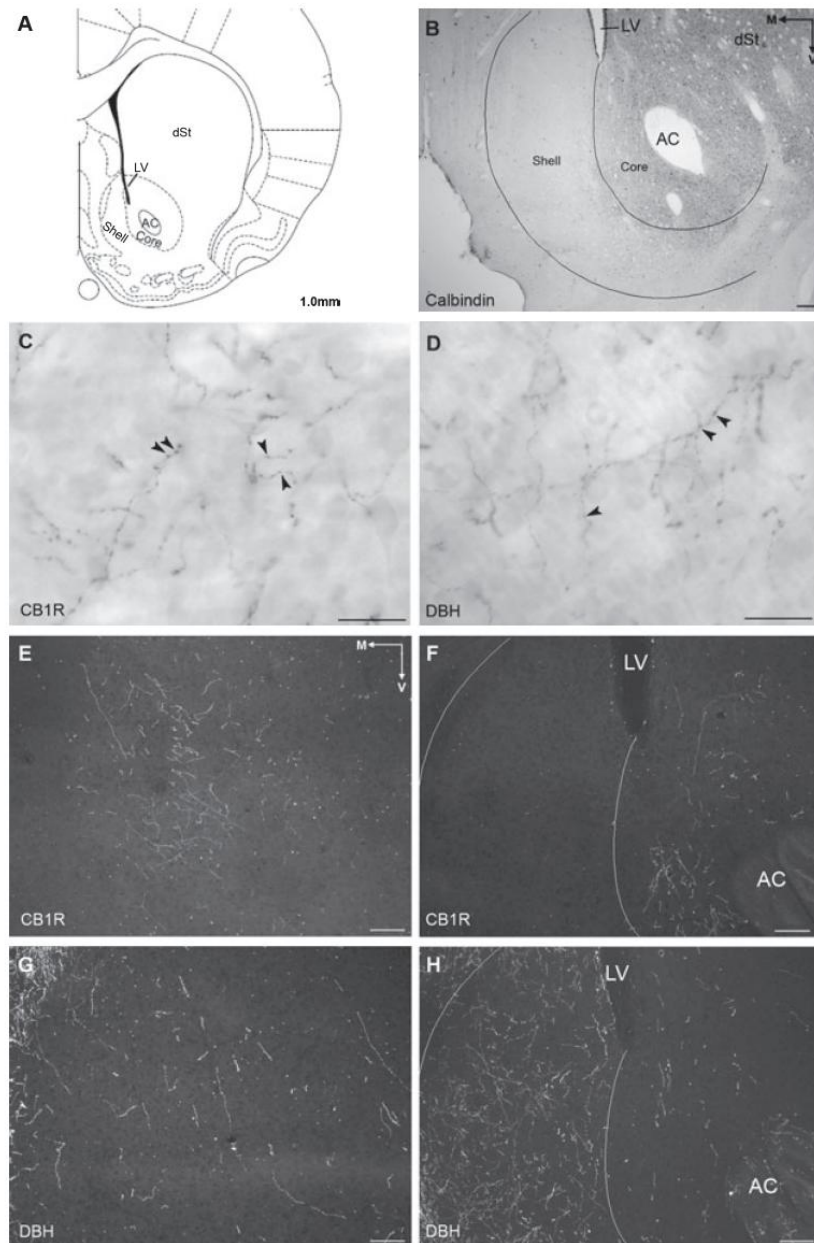
36

FIG. 4. (A) Diagram of a coronal section of rat forebrain adapted from the rat brain atlas of Paxinos & Watson (1997) showing subregions of the Acb in the ventral striatum. The core subregion of the Acb surrounds the anterior commissure (AC) whereas the shell subregion is situated medial and ventral to the lateral ventricle (LV). (B) Brightfield photomicrograph of calbindin immunoreactivity in a coronal section of rat brain at an equivalent level to that shown in panel (A). Calbindin immunoreactivity is more intense in the core and dorsal striatum (dSt), with almost no labeling in the shell. (C and D) Brightfield photomicrographs, from the shell of the Acb at ∼1.7 mm anterior to bregma, of CB1R and DBH-ir, respectively. High magnification of CB1R and DBH-ir shows tortuous and beaded (arrowheads) processes. (E and F) Darkfield photomicrographs of CB1R immunoreactivity at two different levels of the Acb. CB1R-ir is seen (E) in the shell at mid-levels, ∼0.7 mm anterior to bregma and (F) in the core at more caudal levels (1.0 mm anterior to bregma). (F) At this level, CB1R immunoreactivity is almost completely absent from the dorsal shell. (G and H) Darkfield photomicrographs showing DBH immunoreactivity at the same level as CB1R immunoreactivity in E and F, respectively. (G) Some DBH immunoreactivity is seen at mid-levels of the shell and (H) intense DBH immunoreactivity is seen in the shell at caudal levels. M, medial; V, ventral. Scale bar, 100 μm (B and E–H), 25 μm (C and D).

distribution of calbindin immunoreactivity, along with the anterior commissure and lateral ventricle, were used as references to identify the level of Acb and its subregions when analyzing the distribution of CB1R and DBH (Fig. 4A and B). The rostrocaudal segment of the Acb was systematically categorized into three levels for the purpose of the analysis: rostral (from 2.7 to 1.7 mm anterior to bregma), middle (from 1.7 to 1.0 mm anterior to bregma) and caudal (from 1.0 to 0.6 mm anterior to bregma), coordinates according to the rat brain atlas of Paxinos & Watson (1997).

Localization of CB1R in the Acb was consistent with previous reports (Robbe *et al.*, 2001; Pickel *et al.*, 2004; Kearn *et al.*, 2005). Immunoperoxidase and immunofluorescence labeling of CB1R was identified in long, beaded processes (Figs 4C, and 5A and B) consistent with axonal profiles and punctate deposits that were more consistent with a postsynaptic distribution (Fig. 5A). For simplicity, only CB1R processes are represented on the schematic illustrations (Fig. 6). CB1R-immunoreactive (-ir) processes were found throughout the rostrocaudal extent of the Acb but with a differential distribution within the shell and core subregions. CB1R-ir shifted from dorsal to ventral aspects of the shell with caudal progression through the Acb. However, in the caudal third division of the Acb, CB1R-ir was more prominent within the Acb core subregion (up to +1.0 mm from bregma), with little immunoreactivity in the shell (Fig. 4F). Clusters of CB1R-ir processes were particularly evident in the mid-ventral shell and in the core at caudal levels (Figs 4E and F, and 5B). CB1R labeling was seen in long processes running either medially or ventrally. Although not depicted on the schematic illustrations, CB1R was also seen in profiles consistent with somatodendritic structures (Fig. 5A) as reported by others (Pickel *et al.*, 2004; Kearn *et al.*, 2005; Villares, 2007). To confirm the somatodendritic localization of CB1R in this region, dual immunofluorescence of CB1R and the somato-dendritic marker MAP2 showed double labeling, indicating that CB1R was also present postsynaptically (Fig. 5E).

At the ultrastructral level, using the immunogold–silver detection method, CB1R was identified both pre- (Fig. 7A) and postsynaptically (Fig. 7B) in cellular profiles. Of 342 CB1R-ir cellular profiles examined, 55% (189/342) were found in axon terminals and 45% (153/342) in dendrites. Pickel *et al.* (2004) reported similar values: 59% in terminals and 41% in dendrites. Similar values were obtained when CB1R was visualized using immunoperoxidase detection. Immunocytochemical labeling for CB1R-ir was identified along the plasma membrane of axon terminals as well as within the axoplasm (Fig. 7). Axon terminals that exhibited CB1R-ir were unmyelinated and contained synaptic vesicles that were heterogeneous in nature. CB1R-ir dendrites contained mitochondria and endoplasmic reticulum and were postsynaptic, mainly to unlabeled terminals. Of the axon terminals exhibiting CB1R-ir, synaptic specializations were charac-terized as symmetric or asymmetric. Semiquantitative analysis showed that, out of 189 profiles counted, 17% (32/189) formed symmetric synapses while 20% (37/189) formed asymmetric synapses (Fig. 7A). The remaining profiles did not form sufficiently clearly recognizable synaptic specializations in the plane of section analyzed to be accurately classified.

Topographic distribution of DBH in Acb core and shell subregions

Although distributed throughout the entire rostrocaudal extent of the Acb, DBH-ir fibers also showed a topographic distribution (Fig. 4 and 6). DBH-ir was found in both the shell and core of the Acb except at more caudal levels (+1.0 to +0.7 mm from bregma), where DBH-ir

was found mainly in the shell (Fig. 4H). This level corresponded to the area of the Acb with the highest density of DBH-ir, where abundant, beaded and tortuous DBH-ir fibers were seen. The density of DBH-ir fibers decreased towards more rostral levels, with few fibers being detected at the most rostral level (2.7 mm anterior to bregma). Regions of high overlap between DBH-ir and CB1R-ir included the ventromedial shell at mid-levels of the Acb (Fig. 6, panels 1.7 and 1.6 mm).

Ultrastructural analysis of noradrenergic terminals was also assessed by electron microscopy. NET was used as a marker to detect noradrenergic axon terminals and did not compromise the ultrastruc-tural preservation of the neuropil. In order to assure that NET labeled the same profiles as DBH, dual immunofluorescence was performed; co-localization of the two markers in the same profiles occurred (supporting Fig. S4). At the ultrastructural level, NET was detected only in axon terminals. The peroxidase reaction resulted in a diffuse labeling within the terminals, with more intense labeling adjacent to the plasma membrane (arrows in Fig. 7), while immunogold–silver particles were found mainly in the cytoplasm, as reported by Miner *et al.* (2003) in the PFC. Detection of NET with immunogold–silver particles allowed better characterization of the synaptic specialization of these axon terminals in 31% of the terminals analyzed (47/153). NET was found to form mainly symmetric synapses (31 of 47; 66%), in accordance with studies in the PFC (Miner *et al.*, 2003), while asymmetric synapses were found in 34% (16/47).

Immunofluorescence microscopy showed that CB1R and DBH overlapped in both core and shell subregions of the Acb

Dual immunofluorescence for CB1R and DBH was conducted in the same section of tissue to determine whether noradrenergic afferents exhibit CB1R immunoreactivity. Both CB1R- and DBH immuno-reactivity were found in beaded and tortuous processes (Fig. 5). The beaded morphology was more evident within the DBH-ir fibers (Fig. 5B and C). The distribution of CB1R- and DBH immunoreac-tivity in the Acb was in concordance with the data obtained from single labeling described above. Although CB1R immunoreactivity was often found in areas containing noradrenergic fibers, rarely were noradrenergic fibers positive for CB1R. However, in these areas of overlap, CB1R- and DBH immunoreactivity appeared to converge on common structures as the processes appeared to delineate cell bodies of neurons in the Acb (double arrows in Fig. 5B), suggesting that noradrenergic fibers and fibers containing CB1R may be converging on common neurons.

Noradrenergic afferents to the shell of the Acb showed a low frequency of co-existence with CB1R

The dual immunofluorescence data suggested multiple sites of interaction between CB1R and noradrenergic afferents that could only be fully resolved using ultrastructural analysis. The selection of the mid-ventral shell of the Acb for EM analysis was based on the light-microscopic data showing significant overlap in this area (Fig. 6). NET was detected using immunoperoxidase while CB1R was localized using immunogold–silver deposits (Fig. 7). In the area sampled, CB1R and noradrenergic terminals were found to physically interact in two ways. Some noradrenergic terminals were found to have CB1R and some were found to be apposed to unlabeled profiles containing CB1R. More specifically, 7.7% (9/113) of all NET-ir axon terminals contained CB1R immunogold–silver particles, while 4.8% (9/189) of all CB1R-containing axon terminals were found in NET-ir
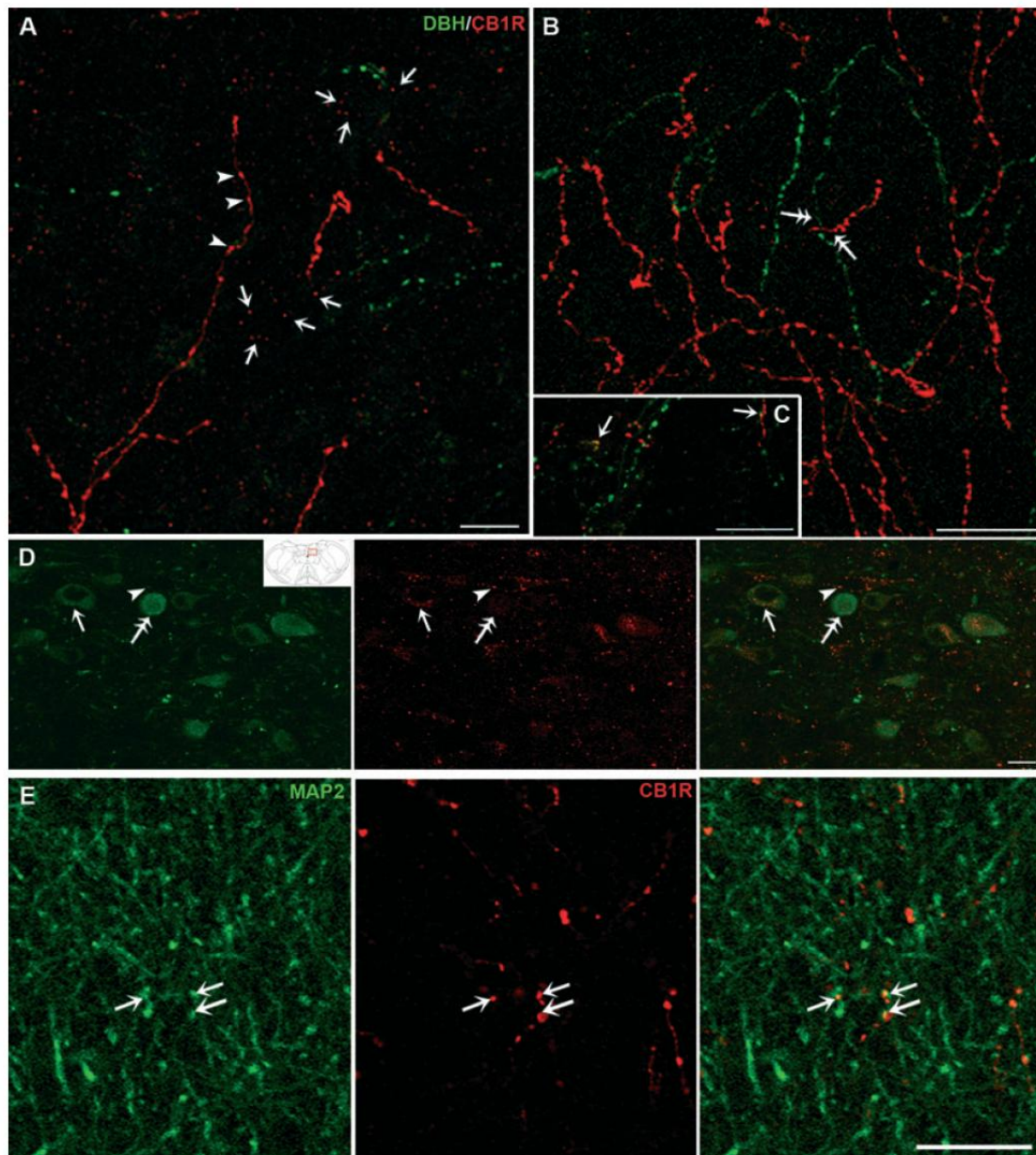
Fig. 5. Confocal fluorescence photomicrographs showing dual-labeling for CB1R and DBH in coronal sections of (A–C) the Acb and (D) the NTS, and (E) CB1R and MAP2 in the Acb. CB1R was detected using a rhodamine isothiocyanate (red)-conjugated secondary antisera and DBH and MAP2 were detected using a fluorescein isothiocyanate (green)-conjugated secondary antisera. Inset is a schematic diagram adapted from the rat brain atlas of Paxinos & Watson (1997) showing the level (14.08 mm posterior to bregma) at which the photomicrograph was taken. (A–C) CB1R and DBH immunoreactivity are frequently seen in the same field throughout the Acb. Both immunoreactivities show beaded processes resembling axonal structures (arrowheads in A) and punctate labeling consistent with postsynaptic profiles (arrows in A). Some co-localization of the two markers (arrows in C) can be seen. In addition, independently labeled fibers appear to converge on common structures (double arrows in B). (D) CB1R immunoreactivity is associated with DBH-labeled neurons (arrow) as well as unlabeled neurons (arrowhead) in the NTS. Some of the DBH-labeled neurons lack CB1 immunoreactivity (double arrows). (E) Localization of CB1R in somatodendritic profiles labeled with MAP2 (arrows). Scale bars, 20 μm.
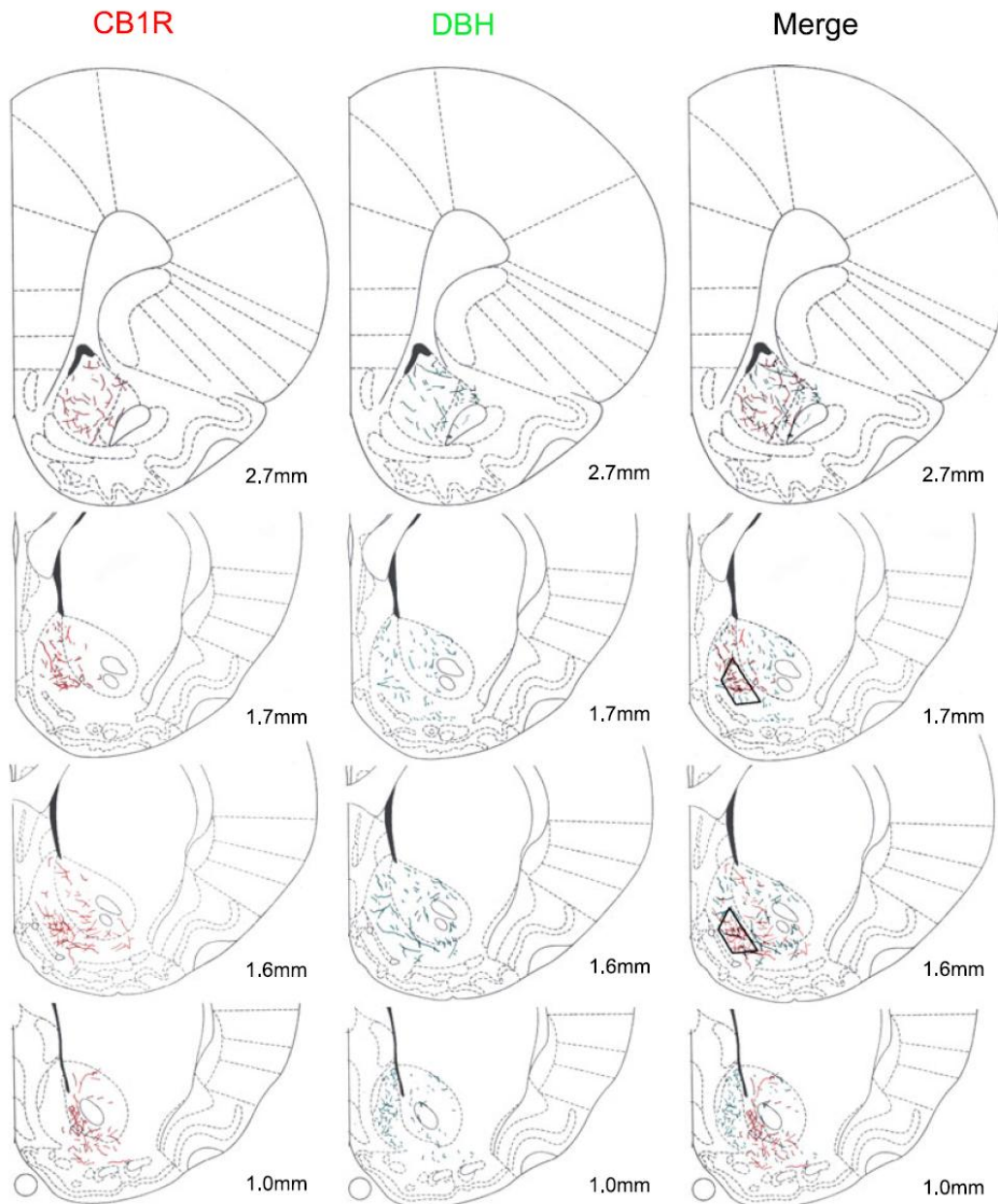
FIG. 6. Distribution of CB1R and DBH immunoreactivities along the extent of the Acb shown in schematics adapted from the rat brain atlas of Paxinos & Watson (1997). Distances shown represent location anterior to bregma. CB1R immunoreactivity is depicted in schematics on the left while DBH immunoreactivity is shown in schematics in the middle column. CB1R immunoreactivity was found diffusely in the core and in the shell subregions in the rostral third area of the Acb. At mid-levels (1.7–1.0 mm), CB1R was found mainly in the shell and there was a rostrocaudal shift in CB1R immunoreactivity from the dorsal shell to the ventral shell. In the caudal third of the Acb (up to 1.0 mm), CB1R immunoreactivity was found almost exclusively in the core. DBH immunoreactivity was found diffusely in the shell and core subregions, with increased density as the nucleus progressed caudally. In the caudal third of the Acb (up to 1.0 mm), DBH immunoreactivity was very intense in the shell and less so in the core. Trapezoids in the right column indicate the region sampled for ultrastructural analysis of CB1R and DBH distribution.

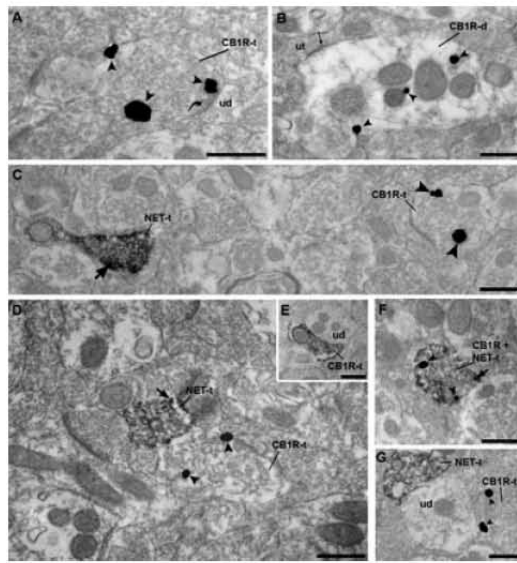FIG. 7. Electron photomicrographs from the Acb shell subregion showing immunogold–silver labeling (arrowheads) for CB1R and immunoperoxidase labeling for NET. Irrespective of whether the immunolabeling was in (A) an axon terminal or (B) a dendrite, immunogold–silver particles for CB1R could be detected within the cytoplasm as well as associated with the plasma membrane. (A) Example of immunogold–silver labeling for CB1R in an axon terminal (CB1R-t) forming an asymmetric, excitatory-type synapse (curved arrow) with an unlabeled dendrite (ud). (B) CB1R immunolabeling is present in a dendrite (CB1R-d) that receives a symmetric, inhibitory-type synapse (thin arrow) from an unlabeled terminal (ut). (C) Dual localization of NET and CB1R using immunoperoxidase detection for NET and gold–silver labeling for CB1R. NET can be identified in an axon terminal by the presence of a diffuse peroxidase precipitate with intense immunoreactivity along the plasma membrane (thick black arrow). The immunoperoxidase-labeled NET axon terminal (NET-t) is found in the same field as a CB1R-t that is labeled with gold–silver particles (arrowheads). (D) A NET-t is apposed to a CB1R-t labeled with immunogold–silver. (E) Example of reverse labeling using immunoperoxidase for CB1R which is apposed to an unlabeled dendrite (ud). (F) An example of an axon terminal that exhibits labeling for both CB1R and NET (CB1R + NET-t). (G) A NET-t exhibiting immunoperoxidase labeling and a CB1R-t converging onto the same unlabeled dendrite (ud). Scale bars, 500 nm.

axon terminals (Fig. 7F). With respect to the apposed labeling, 6.2% (7/113) of all NET-ir axon terminals were apposed to profiles containing CB1R immunogold–silver particles (Fig. 7D). Conversely, 1.6% of all CB1R-containing axon terminals were apposed to NET-ir axon terminals, while 2.6% of all CB1R-containing dendrites were apposed to NET-ir axon terminals. In regions of apposition, no synaptic specialization of the CB1R-containing terminals was recognizable in the cross-section analyzed.

CB1R were located in noradrenergic neurons in the NTS

As the cannabinoid agonist was administered systemically, the effects of WIN 55,212-2 on the expression of AR in the Acb could also be due to its actions on CB1R located in noradrenergic nuclei projecting to the Acb, i.e. the NTS. To assess this, dual immunofluorescence for CB1R and DBH was performed in tissue sections containing the NTS. DBH immunoreactivity was found diffusely in cell bodies as well as in processes resembling dendrites (Fig. 5D). CB1R immunoreactivity

exhibited a punctate distribution and co-localized in the cytoplasm of noradrenergic neurons (positive for DBH) as well as non-noradrenergic neurons (lacking DBH-ir). Some of the DBH-labeled neurons lacked CB1R-ir.

Discussion

The present study demonstrates that systemic administration of a cannabinoid agonist alters the expression of ARs in a key limbic forebrain region related to motivated behaviors. Light and ultrastructural microscopy studies indicate several potential cellular sites for interaction between the two systems that include co-existence in common axon terminals, serial modulation by convergence of separately labeled axon terminals on common postsynaptic targets and indirect effects on noradrenergic brainstem perikarya that provide afferent input to the Acb.

Methodological considerations

The present study analyzed the expression of AR in the Acb following treatment with WIN 55,212-2 or vehicle. The Acb can be divided into core and shell subregions. At more rostral levels, the two regions can be easily microdissected but at more caudal levels the core subregion completely surrounds the anterior commissure while the shell subregion surrounds it ventrally. To avoid dissecting the anterior commissure we oriented our micropunches to target the Acb medial to the anterior commissure, leaving out the core that sits lateral to it and part of the ventrolateral shell from the dissection. As micropunches of the Acb were used for the quantification of the ARs, the exact area where these changes occurred (shell vs. core subregions, rostral vs. caudal) cannot be established. Also, whether the changes observed are due to a decrease in both pre- and postsynaptic receptors cannot be defined.

A potential limitation known to be associated with the pre-embedding immunolabeling technique is penetration of immunoreagents in thick Vibratome sections (Chan *et al.*, 1990). To circumvent this possibility, analysis of ultrathin sections was carried out exclusively on sections near the tissue–plastic interface where penetration is maximal. Limitations associated with the specificity of immunogold labeling were overcome by quantifying only the profiles containing two or more immunogold–silver particles. This may lead to an underestimation of actual cellular relationships. However, this approach minimized the reporting of potential spurious gold labeling.

AR changes in the Acb following CB1R agonist treatment

To our knowledge, we are the first to report a change in adrenergic receptor expression in the Acb following exposure to systemic administration of the synthetic cannabinoid agonist WIN 55,212-2. Our results demonstrate a decrease in $\beta1$- and $\alpha2A$-AR protein expression in the Acb following acute and/or chronic exposure. A decrease in protein expression levels may be related to downregulation of the receptor as adrenergic receptors, which belong to the G protein-coupled receptor (GPCR) superfamily, are known to desensitize, internalize and downregulate their expression following binding of an agonist (Heck & Bylund, 1997; Dunigan *et al.*, 2002). Because desensitization does not seem to depend on protein degradation (as removal of agonist rapidly restores receptor function; Hein & Kobilka, 1995), no differences in total receptor protein would be expected during desensitization. In contrast, downregulation of

GPCRs can be defined as a loss of total cellular binding activity or decrease in receptor density (Barturen & Garcia-Sevilla, 1992; Hein & Kobilka, 1995; Heck & Bylund, 1997). Mechanisms for downregulation may include protein degradation, destabilization of the receptor mRNA or repression of gene transcription.

We have previously reported that acute and chronic systemic administration of WIN 55,212-2 is capable of increasing NE release in the PFC with concomitant activation of c-fos activation in brainstem noradrenergic neurons (Oropeza *et al.*, 2005; Page *et al.*, 2007). In addition, others have shown that WIN 55,212-2 (3.0 mg/kg) is able to induce c-fos expression in the NTS (Jelsing *et al.*, 2009). It is tempting to speculate that the downregulation of AR in the Acb following WIN 55,212-2 may occur due to an increase in NE release in the Acb. The fact that NET expression in the Acb is not affected by WIN 55,212-2 administration suggests that the reuptake of NE by this transporter remains constant although binding tests should be performed to confirm this. We have recently described a decrease in β1-AR levels in the PFC after chronic treatment with WIN 55,212-2, with no changes in the levels of α2A-AR (Reyes *et al.*, 2009). The distinct effect of WIN 55,212-2 on the levels of ARs in the PFC and Acb may account for the anatomical and functional differences between the two areas. Anatomically, the PFC receives its noradrenergic input solely from the LC while the Acb is innervated mainly by the NTS (Delfs *et al.*, 1998; Olson *et al.*, 2006). The present study, therefore, by assessing noradrenergic afferents to the Acb, provides information regarding the interaction of the cannabinoid system with limbic-forebrain projections originating specifically from the NTS. Also, the subcellular localization of the AR in the Acb is not known but they are found to be both pre- and postsynaptic in other brain regions (MacDonald *et al.*, 1997; Ramos & Arnsten, 2007; Wang *et al.*, 2007). The localization of AR with CB1R is being analyzed and, based on previous studies showing preferential presynaptic localization of α2-AR (Flugge *et al.*, 2004), we hypothesize that axon terminals in the Acb expressing α2-AR will be apposed to terminals containing CB1R. Considering our localization of CB1R in dendrites and the known association of β1-AR receptors with the postsynaptic density protein in other brain regions (Strader *et al.*, 1983; Aoki *et al.*, 1987; Hu *et al.*, 2000), we also anticipate a potential co-localization of β1-AR and CB1R postsynaptically. It has been proposed that activation of CB1R can sequester G proteins, making them unavailable for other GPCRs such as α2-AR and somatostatin receptors (Vasquez & Lewis, 1999). Whether disruption of this GPCR signaling can ultimately lead to their downregulation has not been addressed yet. Activation of CB1R is also known to lead to changes in membrane potential and to alter the levels of intracellular cAMP (Demuth & Molleman, 2006). cAMP can initiate intracellular pathways that can lead to inhibition of AR synthesis or to destabilization of AR mRNA, contributing to downregulation of the receptor (Kirigiti *et al.*, 2001; Dunigan *et al.*, 2002). In addition, stimulation of CB1R activates protein kinases that could participate in the regulation of gene expression (Piomelli, 2003).

CB1R and DBH were topographically distributed within the Acb

Our data are in agreement with others' with regard to the presence of noradrenergic terminals and CB1R immunoreactivity in the Acb (Berridge *et al.*, 1997; Delfs *et al.*, 1998; Tsou *et al.*, 1998; Robbe *et al.*, 2001). However, the present study adds a detailed analysis of the distribution of CB1R immunoreactivity not provided in these studies. Robbe *et al.* (2001) identified CB1R immunoreactivity in

large, poorly branched fibers exhibiting intensely immunostained varicosities that were localized mostly in the core subregion of the Acb. We report the same type of immunostaining for CB1R but we provide new data showing that CB1R is also found in the shell subregion. Our analysis shows that CB1R immunoreactivity is not uniform throughout the Acb. CB1R immunoreactivity is mainly found in the core in the caudal third of the Acb and is found in the remaining two-thirds of the nucleus in the shell subregion. Hence, CB1R immunoreactivity seems to be more abundant in the shell. Careful analysis by light microscopy of CB1R immunoreactivity in both the core and the shell subregions did not reveal major differences in the immunostaining pattern between the subregions, suggesting that CB1R may function similarly in both the shell and core. The distribution of DBH-ir in the Acb presented in this study is in agreement with previous studies (Berridge *et al.*, 1997; Delfs *et al.*, 1998). More specifically, DBH-ir was reported to be more evident in the shell at caudal levels but it was also found at more rostral levels, both in the shell and in the core.

In summary, our mapping of CB1R and DBH immunoreactivity in the Acb shows an interesting topographic distribution of the two markers. CB1R and DBH were shown to have an uneven distribution throughout the nucleus. This fact may be relevant for the anatomic and functional heterogeneity proposed for the Acb (Zahm, 1999). Anatomical and behavioral studies support a rostro-caudal gradient for appetitive vs. aversive behaviors (Reynolds & Berridge, 2001, 2002, 2003). These studies suggest that the rostral shell is important for appetitive/hedonic behaviors whereas the caudal shell is important for aversive/fear behaviors, and that GABAergic and glutamatergic transmission (through GABA$_A$ and AMPA receptors) is involved. Modulation of GABAergic and glutamatergic transmission in the intermediate shell produces combined positive and negative motivational effects. Whether the overlapping region of DBH and CB1R immunoreactivities described in the present study correlates with these behaviors cannot be established. However, our ultrastructural analysis of the middle third of the shell subregion localized CB1R to terminals forming symmetric (inhibitory) and asymmetric (excitatory) synapses, suggesting that activation of CB1R can modulate inhibitory and excitatory input in the Acb and therefore modulate behavior. In addition, previous studies have shown that cannabinoids are able to inhibit glutamate and GABA transmission in the Acb (Hoffman & Lupica, 2001; Manzoni & Bockaert, 2001; Robbe *et al.*, 2001; Hoffman *et al.*, 2003), mainly through a presynaptic mechanism. Future studies should also address whether the presence of noradrenergic fibers in this specific region is important for the modulation of the abovementioned behaviors.

Subcellular localization of CB1R in the Acb

The CB1R subcellular distribution in the shell of the Acb analyzed in the present study by electron microscopy is agreement with previous studies but also shows some differences (Pickel *et al.*, 2004; Matyas *et al.*, 2006). Discrepancies in anatomical studies may arise from multiple factors. For example, the region of the Acb analyzed may differ from study to study. As shown in the present study, the distribution of both CB1R and noradrenergic fibers varies considerably throughout the nucleus and sampling differences between laboratories may lead to different results. In the present study, the area selected for ultrastructural analysis was restricted to the mid-ventral shell due to the higher incidence of overlap between CB1R and DBH immunoreactivity observed by light and fluorescence

microscopy. In addition, different criteria were used to quantify profiles that exhibited CB1R immunoreactivity. In the present study, only profiles containing two or more gold particles were included in the semi-quantitative analysis whereas other groups (Pickel *et al.*, 2004) considered single immunogold–silver profiles as indicative of positive labeling for CB1R. Finally, another difference relates to the type of synapses formed by terminals containing CB1R. Matyas *et al.* (2006) showed that all single-labeled CB1R-containing axon terminals and dual-labeled CB1R and GABA axon terminals formed exclusively symmetric synapses. On the other hand, Pickel *et al.* (2004) reported that 42% of the CB1R-labeled axon terminals formed asymmetric synapses while only 7% formed symmetric synapses. In the present study, a similar number of terminals formed symmetric and asymmetric synapses, although > 60% of the profiles exhibited synaptic specifications that could not be unequivocally established in the plane of section analyzed. Nevertheless, as mentioned before, cannabinoids have been found to affect both glutamate and GABA transmission in the Acb (Manzoni & Bockaert, 2001; Robbe *et al.*, 2001). The localization of CB1R in terminals forming symmetric and asymmetric synapses in the present study is consistent with this.

As reported by Pickel *et al.* (2004), the present study shows immunolabeling for CB1R in somatodendritc profiles in the Acb. This is an interesting finding as cannabinoid actions are thought to be mainly presynaptic. However, there is evidence for self-inhibition of cortical interneurons by cannabinoids in an autocrine manner, whereby cannabinoids are synthesized postsynaptically and activate nearby CB1R (Piomelli, 2003; Bacci *et al.*, 2004). Moreover, the fact that fatty acid amide hydrolase (one of the enzymes responsible for degradation of endocannabinoids) is located mainly in cell bodies and dendrites (Egertova *et al.*, 2003; Piomelli, 2003) may suggest that cannabinoids might be able to act postsynaptically. Nevertheless, whether CB1R located postsynaptically in the Acb are functional and activating intracellular pathways was not investigated in the present study and warrants further investigation.

Anatomical data show interaction between CB1R and DBH

Our anatomical data show multiple sites for interaction between the cannabinoid and noradrenergic systems in the Acb and the NTS (Fig. 8). CB1R was found in noradrenergic terminals, in unlabeled terminals apposed to noradrenergic terminals and in dendrites in the Acb as well as in noradrenergic and non-noradrenergic neurons of the NTS (Fig. 8, panel 1). Based on our anatomical data, we proposed four potential mechanisms by which WIN 55,212-2 is modulating AR expression. WIN 55,212-2 may be modulating the levels of AR directly by activating CB1R present in profiles that express AR (dendrites or axon terminals; Fig. 8, panel 2). WIN 55,212-2 can also act on CB1R present in noradrenergic terminals (Fig. 8, panel 2) modulating the release of NE. Continued agonist activation of AR by NE can lead to receptor downregulation (Hein & Kobilka, 1995; Heck & Bylund, 1997). A third intra-accumbal mechanism may account for modulation by WIN 55,212-2 of AR. The majority of CB1R was found in unlabeled profiles. The nature of these profiles is unknown, but activation of CB1R by WIN 55,212-2 may contribute to modulation of these profiles' transmission with consequent effects on noradrenergic terminals and profiles containing AR (Fig. 8, panel 3). Ultimately, WIN 55,212-2 may be acting on CB1R present in the NTS, increasing the noradrenergic input to the Acb (Fig. 8, panel 4). In fact, WIN 55,212-2 administration has been shown to induce c-fos activation in the NTS (Jelsing *et al.*, 2009). However, whether this
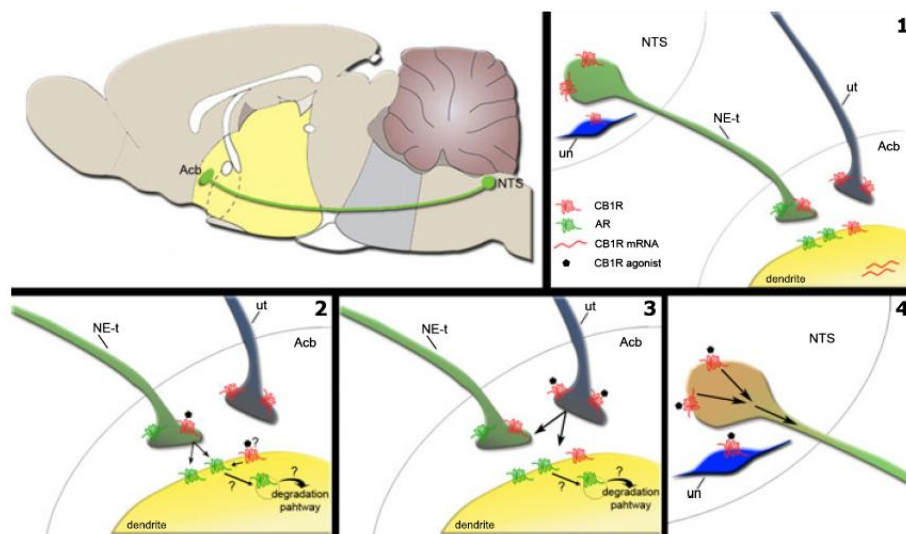

FIG. 8. Possible sites for modulation of noradrenergic transmission in the Acb by cannabinoids. (Top left panel). Schematic of a saggital rat brain showing the noradrenergic input to the Acb arising from the NTS. (1) The present study shows that, in the Acb, CB1R is found in noradrenergic terminals (NE-t), unlabeled terminals (ut) and in dendrites. CB1R is also present in somatodendritic profiles of noradrenergic neurons and unlabeled neurons (un) in the NTS. Adrenergic receptors (AR) can be found pre- and postsynaptically (MacDonald *et al.*, 1997; Ramos & Arnsten, 2007; Wang *et al.*, 2007). CB1R mRNA has been shown to be present in the Acb (Hohmann & Herkenham, 2000; Hurley *et al.*, 2003). We hypothesize that cannabinoids may modulate noradrenergic transmission in the shell of the Acb as follows. (2) Directly, through activation of CB1R present on noradrenergic terminals or dendrites. Whether dendritic CB1Rs are functional requires further studies. Nevertheless, if functionally active these receptors could influence adrenergic receptor expression. (3) Indirectly, through activation of CB1R in terminals apposed to noradrenergic terminals. (4) Indirectly, through activation of CB1R in the NTS neurons that send projections to the Acb.

neuronal activation increases NE release in the Acb remains to be elucidated.

Functional implications

Convergent studies in the literature suggest that cannabinoids may play a role in several neuropsychiatric disorders (Maldonado *et al.*, 2006; Leweke & Koethe, 2008; Moreira & Lutz, 2008) such as depression or schizophrenia. Interestingly, the CB1R antagonist rimonabant was withdrawn due to an unacceptably high incidence of neuropsychiatric side effects (Nissen *et al.*, 2008; Sanofi-Aventis), while CB1R agonists have been shown to alleviate depressive-like behaviors in animal models (Gobbi *et al.*, 2005; Hill & Gorzalka, 2005b). Moreover, Gobbi *et al.* (2005) showed that increased levels of anandamide evoked an increase in noradrenergic neuron activity in the LC. This is supported by previous work from our laboratory, showing that administration of a synthetic cannabinoid is able to activate the LC with increased levels of NE in the PFC (Oropeza *et al.*, 2005; Page *et al.*, 2007). The present study adds to these data, as the decrease in α2A-AR expression may account for the assumed increase in NE in the Acb, as α2A-AR seem to function as autoreceptors by inhibiting NE release from the presynaptic terminal (Kable *et al.*, 2000). In fact, local administration of α2-AR agonists in the Acb has been shown to reduce the efflux of NE measured by microdialysis, while administration of antagonists of α2-AR increased the release of NE (Aono *et al.*, 2007). Moreover, downregulation of β1-AR can be seen as a mechanism which is adaptive to an increase in synaptic NE. Although activation of α2-AR can decrease dopamine release in other brain regions such as the PFC and hippocampus (Guiard *et al.*, 2008; Jentsch *et al.*, 2008) this does not seem to be the case in the Acb. Ihalainen and colleagues have shown that administration of an α2-AR agonist would decrease dopamine in the Acb when administered systemically but not when it was locally administered (Ihalainen & Tanila, 2004). This supports the idea that α2-AR may be localized mainly in noradrenergic terminals in the Acb. Therefore, the impact of chronic WIN 55,212-2 on α2-AR levels in the Acb seems to be selective for noradrenergic terminals. As NE is an important target for the treatment of depression (Heninger *et al.*, 1996; Nutt, 2002), it is tempting to speculate that cannabinoids may impact mood- and motivation-related behaviors by activating limbic forebrain noradrenergic circuits.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Specificity of the secondary antibody.

Fig. S2. Specificity of NET primary antibody.

Fig. S3. Specificity of β1-AR and α2A-AR antibodies.

Fig. S4. Confocal fluorescence photomicrographs showing dual-labeling for NET and DBH in coronal sections of the Acb. NET and DBH are co-localized to the same profiles.

Fig. S5. Results of western blot analysis for NET in the Acb showing that treatment with WIN 55,212-2 (3.0 mg/kg) does not affect NET expression.

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Abbreviations

Acb, nucleus accumbens; AR, adrenergic receptor; BSA, bovine serum albumin; CB1R, cannabinoid receptor type 1; DBH, dopamine beta hydroxylase; GPCR, G protein-coupled receptor; -ir, -immunoreactive; LC, locus coeruleus; MAP2, microtubule associated protein 2; NE, norepinephrine; NET, norepinephrine transporter; NTS, nucleus of the solitary tract; PB, phosphate buffer; PFC, prefrontal cortex; TS, Tris saline buffer.

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Carvalho AF, Reyes AS, Sterling RC, Unterwald E and Van Bockstaele EJ

Contribution of limbic norepinephrine to cannabinoid-induced aversion

Contribution of limbic norepinephrine to cannabinoid-induced aversion

Ana Franky Carvalho · Arith-Ruth S. Reyes ·
Robert C. Sterling · Ellen Unterwald ·
Elisabeth J. Van Bockstaele

Abstract

Rationale The cannabinoid system has risen to the forefront in the development of novel treatments for a number of pathophysiological processes. However, significant side effects have been observed in clinical trials raising concerns regarding the potential clinical utility of cannabinoid-based agents. Understanding the neural circuits and neurochemical substrates impacted by cannabinoids will provide a better means of gaging their actions within the central nervous system that may contribute to the expression of unwanted side effects.

Objectives In the present study, we investigated whether norepinephrine (NE) in the limbic forebrain is a critical determinant of cannabinoid receptor agonist-induced aversion and anxiety in rats.

Methods An immunotoxin lesion approach was combined with behavioral analysis using a place conditioning paradigm and the elevated zero maze.

Results Our results show that the non-selective CB1/CB2 receptor agonist, WIN 55,212-2, produced a significant place aversion in rats. Further, NE in the nucleus accumbens was critical for WIN 55,212-2-induced aversion but did not affect anxiety-like behaviors. Depletion of NE from the bed nucleus of the stria terminalis was ineffective in altering WIN 55,212-2-induced aversion and anxiety.

Conclusions These results indicate that limbic, specifically accumbal, NE is required for cannabinoid-induced aversion but is not essential to cannabinoid-induced anxiety.

Keywords Anxiety · Conditioned place aversion · Nucleus of the solitary tract · Saporin · Mood disorders

A. F. Carvalho (✉) · A.-R. S. Reyes · E. J. Van Bockstaele
Neuroscience, Farber Institute for Neurosciences,
Thomas Jefferson University,
Philadelphia, PA 19107, USA
e-mail: arfranky@gmail.com

A. F. Carvalho
Life and Health Sciences Research Institute (ICVS),
School of Health Sciences, University of Minho,
Braga, Portugal

R. C. Sterling
Department of Psychiatry and Human Behavior,
Thomas Jefferson University,
Philadelphia, PA, USA

E. Unterwald
Department of Pharmacology and Center for Substance Abuse
Research, Temple University School of Medicine,
Philadelphia, PA, USA

Abbreviations

Acb	Nucleus accumbens
ANOVARM	Repeated measures ANOVA
BNST	Bed nucleus of stria terminalis
BSA	Bovine serum albumin
CB1r/CB2r	Cannabinoid receptor type1/Cannabinoid receptor type2
CeA	Central nucleus of amygdala
CNS	Central nervous system
DBH	Dopamine beta hydroxylase
DSAP	Saporin conjugated with antibody against DBH
EZM	Elevated zero maze
Ir	Immunoreactivity
KOR	Kappa opioid receptor
NE	Norepinephrine
NTS	Nucleus of the solitary tract
PB	Phosphate buffer
PFC	Prefrontal cortex
ROI	Region of interest

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SAP   Saporin
TS   Tris saline buffer

Introduction

The endocannabinoid system has been implicated in a variety of physiological functions due to abundant expression of its receptors and endogenous ligands in the central nervous system (CNS; Herkenham et al. 1991; Mackie 2005; Mackie 2008) as well as in adipose tissue, gastrointestinal tract, skeletal muscle, heart, and the reproductive system (for review, Pacher et al. 2006). The endocannabinoid system controls emotional reactivity, motivated behaviors, and energy homeostasis. In the brain, the cannabinoid receptor type 1 (CB1r) is the most abundant while the cannabinoid receptor type 2 (CB2r) is found mainly in cells of the immune and hematopoietic systems (Piomelli 2003). The diverse localization of the endocannabinoid system underscores its importance as a potential target in the treatment of a variety of disorders. However, when targeting the endocannabinoid system, a high number of unwanted side effects occur, as evidenced by increased incidence of anxiety and depression in obese patients treated with the CB1r antagonist, rimonabant (Steinberg and Cannon 2007). Cannabinoid agonists have also been shown to induce anxiety and dysphoria (Reilly et al. 1998; Williamson and Evans 2000). Hence, identifying neurochemical targets of cannabinoids is essential. Some studies have suggested that the dysphoric/aversive effects seen upon cannabinoid administration are due to its anxiogenic properties (McGregor et al. 1996; Ghozland et al. 2002). However, conclusive evidence is lacking to support this hypothesis.

The present study explored the role of limbic norepinephrine (NE) in cannabinoid-induced aversion and anxiety. NE is involved in cognition and attention (Aston-Jones et al. 1991) as well as in the pathophysiology of mood disorders (Heninger et al. 1996; Anand and Charney 2000). Previous studies have shown an interaction between the cannabinoid system and the NE system in areas such as the prefrontal cortex (PFC) (Oropeza et al. 2005, 2007; Page et al. 2007), nucleus accumbens (Acb; Carvalho et al. 2010), locus coeruleus (Oropeza et al. 2005; Scavone et al. 2010) and the nucleus of the solitary tract (NTS) (Jelsing et al. 2009; Carvalho et al. 2010). Limbic regions such as the Acb and bed nucleus of the stria terminalis (BNST) have been implicated in aversive and anxiety-like behaviors (Davis 1998; Aston-Jones et al. 1999; Ventura et al. 2007; Carlezon and Thomas 2009). In the present study, we investigated the role of NE in the Acb and BNST in cannabinoid-induced aversion and anxiety. For this purpose, an immunotoxin lesion approach was used to target NE fibers in the Acb and BNST and behavioral tests were performed on rats after administration of a CB1r/CB2r agonist, WIN 55,212-2.

Methods

Subjects

Sixty four male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) weighing 220–250 g were housed two or three per cage in a controlled environment (12-h light schedule, temperature at 20°C). Food and water were provided ad libitum. The care and use of animals were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and were conducted in accordance with the NIH Guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Surgery

Rats were anesthetized with an intraperitoneal (i.p.) injection of a saline solution containing a cocktail of Ketamine HCl (100 mg/kg; Phoenix Pharmaceutical, Inc. St. Joseph, MO, USA) and Xyla-Ject (2 mg/kg; Phoenix Pharmaceutical, Inc.) and subsequently placed in a stereotaxic surgical frame (Stoelting Corp., Wood Dale, IL, USA). The anesthesia was maintained by administration of isoflurane (Webster Veterinary Supply, Inc., Sterling, MA, USA) through a nose cone. Animals received bilateral injections of saporin conjugated with an antibody against dopamine-beta-hydroxylase (DSAP, Chemicon International, Inc., Temecula, CA, USA; 0.21 μg/μl in phosphate buffer (PB), pH 7.4) or control solution with non-conjugated saporin (SAP, Advanced Targeting Systems, San Diego, CA, USA, 0.0441 μg/μl in PB) into the Acb (n=32, 250 nl bilaterally; AP, 1.7 mm rostral to bregma; ML, ±0.8 mm; DV, −7.0 mm) or the BNST (n=32, 300 nl bilaterally; AP, 0.4 mm caudal to bregma; ML, ±4.0 mm; DV, −7.4 mm, with an angle of 19.6°), according to Rat Brain Atlas of Paxinos and Watson (1997) coordinates. The dose of DSAP and SAP used was based on previously published studies (Ritter et al. 2001; Ritter et al. 2003). The volume of DSAP and SAP injected at each site was determined from pilot experiments in our laboratory using a similar protocol. Previous immunohistochemical studies indicated that a period of 2 weeks was sufficient for transport of the immunotoxin and degeneration of the affected neurons (Wrenn et al. 1996; Ritter et al. 2003). Therefore, animals were given 15–18 days before the start of the behavioral tests described below.

Drug preparation and administration

WIN 55,212-2 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 5% dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ, USA) in 0.9% saline and injected i.p. (3.0 mg/kg) in a volume of 1 ml/kg body weight. Vehicle injections consisted of 5% DMSO in 0.9% saline.

Place conditioning

An unbiased place conditioning procedure was used so that the side of the apparatus used to conditioned animals was counterbalanced in all the groups. The paradigm consisted of three phases: pre-test, conditioning, and test. On pre-test day (day 1), animals were placed in the apparatus and allowed to freely explore both sides of the apparatus for 20 min. The time spent in each side was recorded by an investigator and animals with preference for one side higher than 200 s were removed from the study (eight animals of a total of 64). During the conditioning phase (days 2–6), the rats were injected twice daily. In the morning, animals were injected with vehicle and confined to one side of the apparatus for 45 min. In the afternoon, animals were injected with WIN 55,212-2 (3.0 mg/kg) and confined to the opposite side for 45 min. Control groups of animals received vehicle in both sessions. On the test day (day 7), animals were placed in the apparatus and allowed to explored both sides for 20 min. The test trial was recorded on camera and time spent in each side was measured by an investigator. No injection was given to the animals on the test day.

Spatial reference memory test

Verifying that the lesion of noradrenergic input to the Acb and BNST did not alter spatial memory performance, animals were tested in the spatial reference memory test (Morris 1984). Animals were tested 4 days following place conditioning. WIN55,212-2 was not injected at any point during the test period. This control experiment was included to verify that spatial memory was intact in animals with a selective depletion of norepinephrine in the Acb and BNST. The test was conducted in a circular black tank (1.8 m diameter) filled to a depth of 31 cm with water at 22°C and placed in a dimly lit room with extrinsic clues. The hidden platform remained at a fixed spatial location for the entire acquisition period. The acquisition phase consisted of four daily trials (inter-trial interval of 30–45 min.) over 4 days. Each trial started with the animals being placed into the water, facing the wall of the maze, at one of four starting points: N, E, S, and W. Four different starting positions were randomly used in each training block. A trial

was considered complete when the rat escaped onto the platform; when this escape failed to occur within 120 s, the animal was gently guided to the platform and an escape latency of 120 s was recorded for that trial. Rats were allowed to spend 10 s on the escape platform before being returned to home cage. Time needed to reach the platform (escape latency), length of the path described (distance swam) and swim velocity were recorded using HVS Image 2020 Plus tracking system (Version 9/05, HVS Image, Buckingham, UK).

The probe trial was assessed after the last trial of the acquisition period, removing the platform from the pool. Animals were released on the side opposite to where the platform was for a single trial of 60 s, during which the percent time spent in each quadrant was measured. For analysis, the time spent in the target quadrant was compared with the average time spent in the remaining three quadrants.

Elevated zero maze

The elevated zero maze (EZM) is a modification of the elevated plus maze that is also a reliable and sensitive model of anxiety-like behavior in rodents (Shepherd et al. 1994). The EZM consists of a black ABS plastic annular platform (~120 cm diameter) elevated ~70 cm above the ground. It is divided into four equal quadrants which are ~20 cm wide: two opened and two closed. The two opened quadrants are opposite each other and are surrounded by a 1 cm "lip". The two closed quadrants are enclosed by walls (~27 cm high) on both the inner and outer edges of the platform. Testing was conducted the day after the spatial reference memory test in a dimly lit room with a constant illumination on the opened arms of the maze. Vehicle and WIN 55,212-2 were injected i.p. 30–35 min prior to the start of the test. At the start of the 10 min testing session, each rat was placed on the same opened arm facing the center of the maze. The maze was cleaned with 65% ethanol and dried after each testing session. Time spent in the closed arm and total number of entries was used as the output measure for this maze.

Locomotor activity

After the EZM, locomotor activity was assessed in a subset of animals from each treatment group to determine whether treatment influenced locomotor activity. Animals were placed in a home cage-like environment within the Home Cage Video Tracking System (Med Associates, St. Albans, VT, USA) which includes a sound-attenuating cubicle, video tracking interface, and Activity Monitor 5 software (Med Associates). Distance traveled was recorded by the video tracking system for 10 min.

Immunohistochemistry

At the conclusion of testing, animals were deeply anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg) and transcardially perfused with 50 ml of heparinized saline followed by 400 ml of 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1 MPB (pH 7.4). After perfusion, brains were removed and postfixed in the same fixative. Following post-fixation, brains were cryoprotected in a gradient of sucrose solutions (containing 0.1% sodium azide) of 10% and 20% sucrose in 0.1 MPB for 1 h each and 30% sucrose for 48–72 h. Brains were immersed in O.C.T. Embedding Compound (Electron Microscopy Sciences, Hatfield, PA, USA) and frozen in dry ice. Coronal sections of the forebrain (35 μm) were cut using a Microm HM550 cryostat (Richard-Allan Scientific, Kalamazoo, MI, USA) in multiple sets and collected in 0.1 MPB. Every sixth section was processed for immunohistochemical visualization of DBH immuno-reactivity to verify the DSAP-induced lesion. Free-floating sections were treated with 1% sodium borohydride in 0.1 MPB for 30 min. They were then rinsed with 0.1 MPB and later washed in 0.1 M Tris saline buffer (TS, pH 7.6). The sections were blocked in 0.5% bovine serum albumin (BSA) in 0.1 M TS for 30 min and then washed for 5 min, twice. Sections were incubated overnight at room temperature with a mouse antibody for mouse monoclonal antibody recognizing DBH (1:1,000, Chemicon, Millipore) in 0.1% BSA/0.25% Triton-X 100 in 0.1 M TS. The sections were then washed in 0.1 M TS, three times for 10 min. Then, sections were incubated in a secondary biotin-conjugated donkey anti-mouse IgG (1:400, Jackson ImmunoResearch, West Grove, PA, USA) in 0.1% BSA/ 0.25% Triton-X 100 in 0.1 M TS for 30 min at room temperature. Then, sections were washed in 0.1 M TS, three times for 10 min. Sections were incubated in an avidin-biotin complex solution (1:200, VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) in 0.1 M TS for 30 min and then washed. Finally, a peroxidase reaction product was achieved by incubating sections in 22 mg of 3-3′ diaminobenzidine (Sigma-Aldrich) containing 0.05% hydrogen peroxide.

Data analysis

Quantification of noradrenergic fibers depletion

Noradrenergic fibers were identified using an antibody specific for DBH. Sections of SAP and DSAP animals were labeled for DBH as described above. Sections containing the Acb and BNST were visualized using a Leica DMRBE microscope (Wetzlar, Germany), and darkfield images were acquired (at ×10) using SPOT Advanced software

(Diagnostics Instruments, Inc., Sterling Heights, MI, USA). Light intensity was kept constant for all image acquisitions. To quantify the amount of fiber depletion, two methods were used. For sections containing the Acb, two to three sections per animal (comprising different levels of the Acb as exemplified in Fig. 1c) were used for analysis. Using Image-Pro Plus (Version 5.1, Media Cybernatics, Bethesda, MD, USA) the area of the Acb and the number of fibers per section and per side was quantified. Data was analyzed as the ratio of total number of fibers/total area analyzed and presented as percentage of control (SAP-injected animals). Since the BNST contains an extremely dense amount of noradrenergic fibers, it is not feasible to count individual DBH-immunoreactive fibers. Therefore, for sections containing the BNST, intensity of labeling was measured using Kodak Molecular Imaging Software (Version 4.5, Carestream Health Inc., Rochester, NY, USA). Two to three sections containing the anterior BNST (ranging from approximately 0.26 posterior to bregma to a few sections posterior to 0.40 mm, Fig. 1d) per animal was analyzed. Dorsal and ventral regions were analyzed separately. A region of interest (ROI) was set as a template and used to quantify all images so that the area analyzed remained constant. Thus, data is presented as percentage of control (SAP-injected animals) mean intensity. For every section analyzed, a background value was quantified in an area of the section lacking DBH-ir. The background value was subtracted to the intensity of the ROI.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 Graduate Student Version. Behavioral data were analyzed by a two-way ANOVA (toxin × drug). Repeated measures multi-variate analysis of variance (ANOVARM) with day or period of time as the within-subject factor was also used when appropriate. One-way ANOVA, t test and post hoc Bonferroni test were used to analyze differences between groups when appropriate. Significance was set at $p < 0.05$.

Results

Toxin depletion of noradrenergic fibers

Animals recovered rapidly from intracranial injections without evidence of illness or abnormal behavior. DSAP and SAP animals gained weight at the same rate (Fig. 1a and b).

Immunohistochemistry for DBH in the forebrain of DSAP and SAP-injected animals was performed to verify the localization and the extent of the lesion. Two animals,

Fig. 1 Effect of saporin conjugated with an antibody against DBH (DSAP) injection into the nucleus accumbens (Acb) and into the bed nucleus of stria terminalis (BNST). **a** and **b** Toxin and drug treatment had no significant effect on animals' weight throughout the experiment. **c** and **d** Schematics adapted from the rat brain atlas of Paxinos and Watson (1997) showing the approximated levels of the Acb (**c**) and BNST (**d**) used for NE depletion quantification (note: for the BNST, a more caudal section between −0.40 and −0.80 mm was analyzed). *Inset in c* represent the level of the photomicrographs in **e** and **g**. *Inset in d* represent the level of the photomicrographs in **f** and **h**. **e-h** Darkfield photomicrographs showing DBH immunoreactivity in the Acb (**e** and **g**) and in the BNST (**f** and **h**) after injection of saporin or DSAP. Injection of DSAP significantly reduced the amount of DBH immunoreactivity by about 75% in both the Acb (**i**) and BNST (**j**; ***$p < 0.0002$). *ac* anterior commissure, *LV* lateral ventricle, *L* lateral, *V* ventral. *Scale bar* 100 μm
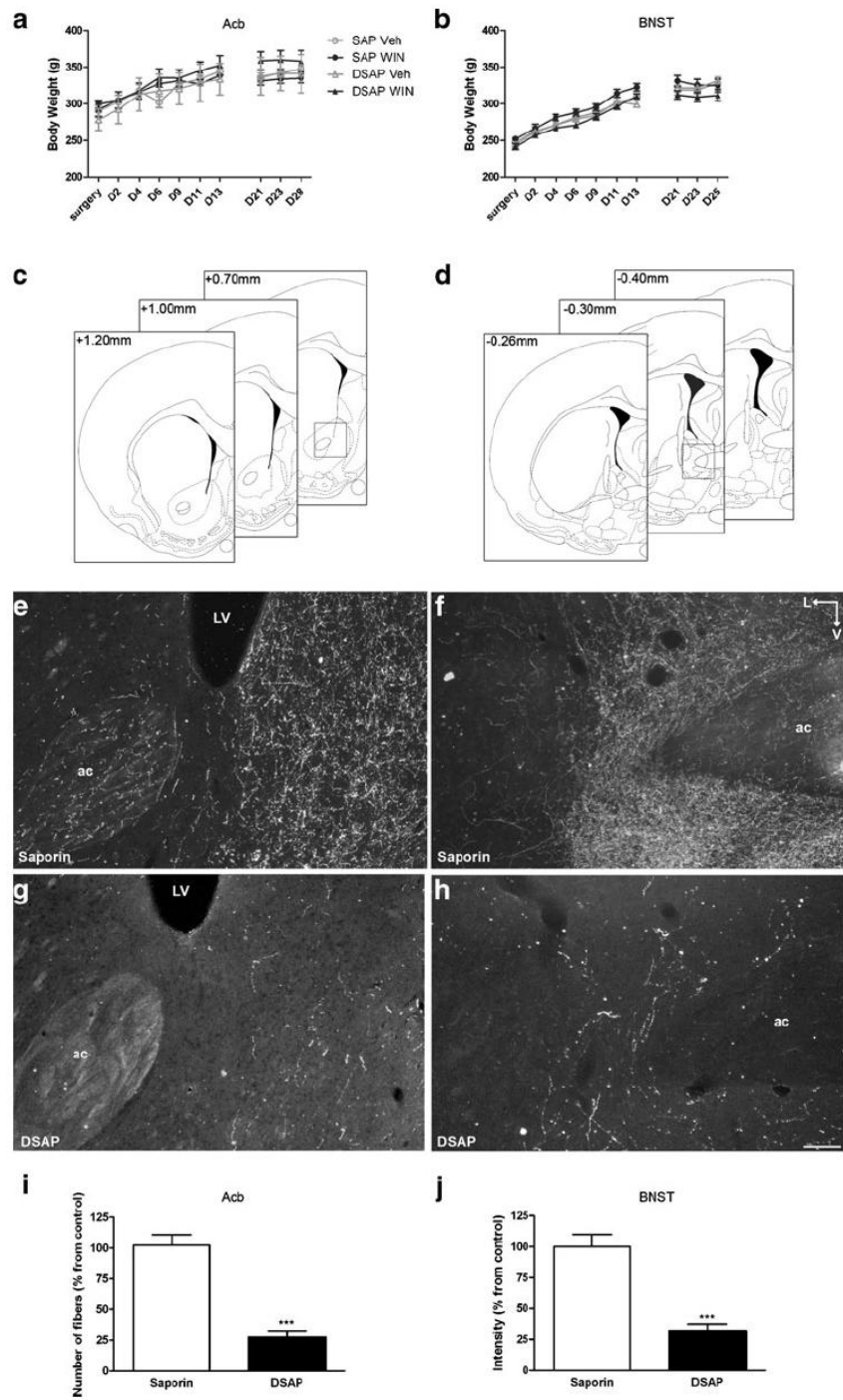
out of 56 without any baseline preference, were excluded from behavioral testing due to inaccurate placement of the toxin. Injection of SAP did not affect DBH immunoreactivity (ir) when compared to vehicle-injected animals, whereas DSAP-injected animals revealed a marked reduction of DBH-ir (Fig. 1e–h) in both the Acb and BNST. Surrounding areas, such as the septal nuclei for the Acb and the ventral pallidum and medial preoptic area for the BNST were intact. Depletion of DBH fibers was quantified as explained in the methods section and for both areas a significant depletion of about 75% of DBH-ir fibers was achieved using injection of DSAP when compared to SAP-injected animals (Fig. 1i and j).

Depletion of noradrenergic fibers in the Acb reverses the aversive effects of WIN 55,212-2

The place conditioning paradigm was used to assess the aversive effects of WIN 55,212-2. Animals were assigned to four groups: animals that received SAP injections and were injected with vehicle in both sessions (SAP/vehicle, seven animals); animals that received SAP injections and were conditioned with WIN 55,212-2 (SAP/WIN, six animals); animals injected with DSAP and received vehicle in both sessions (DSAP/veh, six animals) and animals that received DSAP injections and were conditioned with WIN 55,212-2 (DSAP/WIN, ten animals). Repeated measures analysis revealed that the there was an overall effect of time of testing ($F(1,25)=5.849$, $p=0.023$), meaning that the conditioning phase affected the performance of the animals on the test day. The analysis also showed an interaction between the treatments (toxin and drug) ($F(1,25)=4.350$, $p=0.047$). Further analysis showed that SAP-treated animals that received WIN 55,212-2 spent less time in the drug-paired chamber than the respective vehicle group ($t(11)=5.468$, $p<0.001$), indicating that WIN 55,212-2 induced aversive-like behaviors (Fig. 2a). On the contrary, animals depleted of NE in the Acb did not show aversion to WIN 55,212-2 when compared with DSAP/vehicle-treated animals ($t(14)=-0.471$, $p=0.645$) (Fig. 2a). This suggests that noradrenergic input to the Acb is important for the development of aversion to WIN 55,212-2.

Depletion of noradrenergic fibers in the BNST is not implicated in the aversive effects of WIN 55,212-2

Animals injected with DSAP or SAP in the BNST were assigned to four different groups as mentioned above for Acb injections and conditioned in the same manner (six to eight animals a group). Repeated measures analysis revealed an effect of time of testing ($F(1,21)=6.169$, $p=0.022$), meaning that the conditioning phase affected the
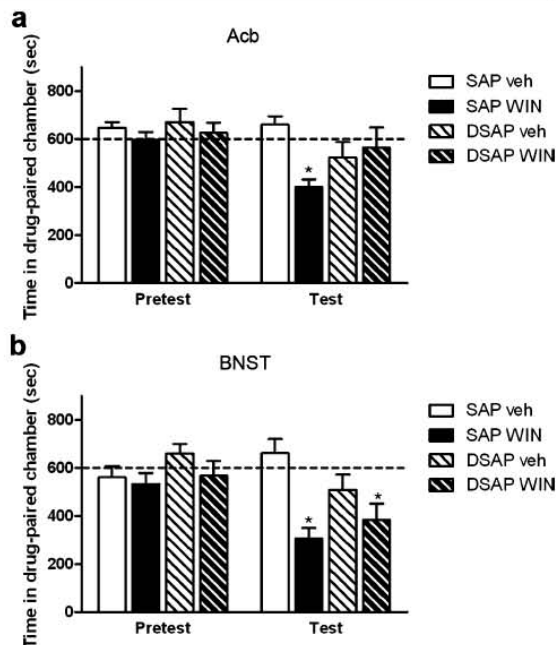

Fig. 2 Effect of DSAP on the development of WIN 55,212-2-induced place aversion. a Animals that received saporin injection in the Acb developed place aversion to WIN 55,212-2 (3.0 mg/kg, *$p<0.001$ compared to SAP/Veh). This effect was blocked by injection of DSAP into the Acb ($p>0.05$, compared to DSAP/Veh). b Animals that received toxin injection into the BNST developed place aversion to WIN 55,212-2 that was not blocked by DSAP injection (*$p=0.05$ compared to vehicle-treated animals)

performance of the animals on the test day. The analysis also revealed an interaction between time of testing and drug ($F(1,21)=4.324$, $p=0.050$; Fig. 2b) but not between time of testing and toxin ($F(1,21)=3.403$, $p=0.079$) suggesting that WIN 55,212-2 is aversive in both SAP and DSAP-treated animals and that depletion of NE in the BNST does not reverse the effects of WIN 55,212-2.

Spatial reference memory is intact

A spatial memory test was performed to ensure that depletion of NE from the target areas did not impair the animals' ability for recall that could impact findings from the place conditioning test. To evaluate acquisition in the water maze, a two-way ANOVA for repeated measures was performed to assess any overall effects of factors toxin and drug, or their interactions on latency to the platform, distance traveled, and swim velocity during task acquisition. The analysis revealed an overall effect of trial on latency to reach the platform ($F(3,36)=62.719$, $p<0.0001$) and distance traveled ($F(3,36)=55.930$, $p<0.0001$), indicating that all animals efficiently learned where the

platform was (Fig. 3a and b). No overall effect of trial on swim velocity was observed ($F(3,36)=1.571$, $p=0.213$; Fig. 3b), suggesting that speed was constant throughout the acquisition phase. Moreover, there were no statistically significant interactions between toxin and drug for the three parameters analyzed, indicating no difference between groups on memory acquisition, distance traveled, and swim velocity. Similar results were observed when the toxin was injected in the BNST (Fig. 3a–c, right column). There was an overall effect of trial on latency to reach the platform ($F(3,36)=55.930$, $p<0.0001$), distance traveled ($F(3,36)=13.348$, $p<0.0001$), and swim velocity ($F(3,36)=7.274$, $p<0.001$). Conversely, there were no significant interactions between toxin and drug for the three parameters analyzed showing that all groups had similar performances in the test.

To assess memory retention, a probe trial was performed after the last trial of the acquisition phase. Repeated measures with time spent in the target quadrant and the average time spent in the other three quadrants was performed with two independent factors: toxin and drug. The analysis showed an overall effect of time spent in the quadrants (Acb, $F(1,12)=410.008$, $p<0.0001$; BNST, $F(1,12)=53.960$, $p<0.0001$). There was no interaction between drug and toxin meaning that the two factors combined did not affect the animals' performance. T test analysis revealed that all groups spent significantly more time in the target quadrant comparing to the non-target quadrants (Acb, $p<0.001$; BNST, SAP/Veh and DSAP/WIN $p<0.001$ and SAP/WIN and DSAP/Veh $p<0.005$; Fig. 4a). Two-way ANOVA of distance traveled and swim velocity during the probe trial revealed no significant interaction between drug and toxin (Acb, distance traveled $F(1,12)=1.471$, $p=0.249$, swim velocity $F(1,12)=1.6$, $p=0.23$; BNST, distance traveled $F(1,12)=0.119$, $p=0.736$, swim velocity $F(1,12)=0.141$, $p=0.714$; Fig. 4b and c), suggesting no effect of drug or toxin on the animals' locomotor activity.

Fig. 3 Spatial memory acquisition is intact in animals injected with the toxin in the Acb (*left column*) and in the BNST (*right column*). Depletion of noradrenergic fibers in both the Acb and BNST did not impair memory acquisition. All groups of animals performed well in the acquisition phase of the Morris water maze test, showing low latency times to find the hidden platform by day 4 (a, ***$p<0.0001$, ANOVARM). All groups of animals showed similar locomotor activity, with no significant difference on distance (b) and speed of swim (c) ($p>0.05$, ANOVARM)
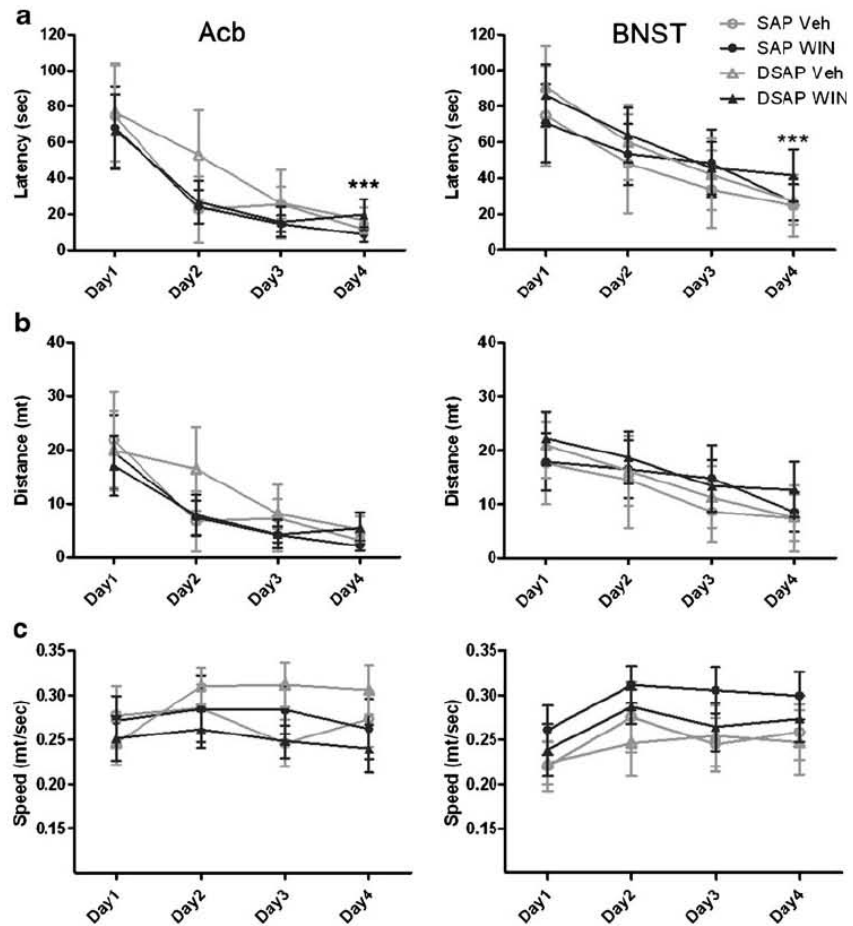
Fig. 4 Spatial memory retention is intact in animals injected the toxin in the Acb (*left column*) and in the BNST (*right column*). Depletion of noradrenergic fibers in both the Acb and BNST did not impair memory retention. During the probe trial (platform removed), all groups of animals spent significantly more time in the target quadrant (a) compared to the average time spent on the other three quadrants (*p<0.005, **p<0.001 compared to target quadrant). No significant effect was observed in the locomotor activity shown by no changes in the distance (b) and speed of swim (c) during the probe trial
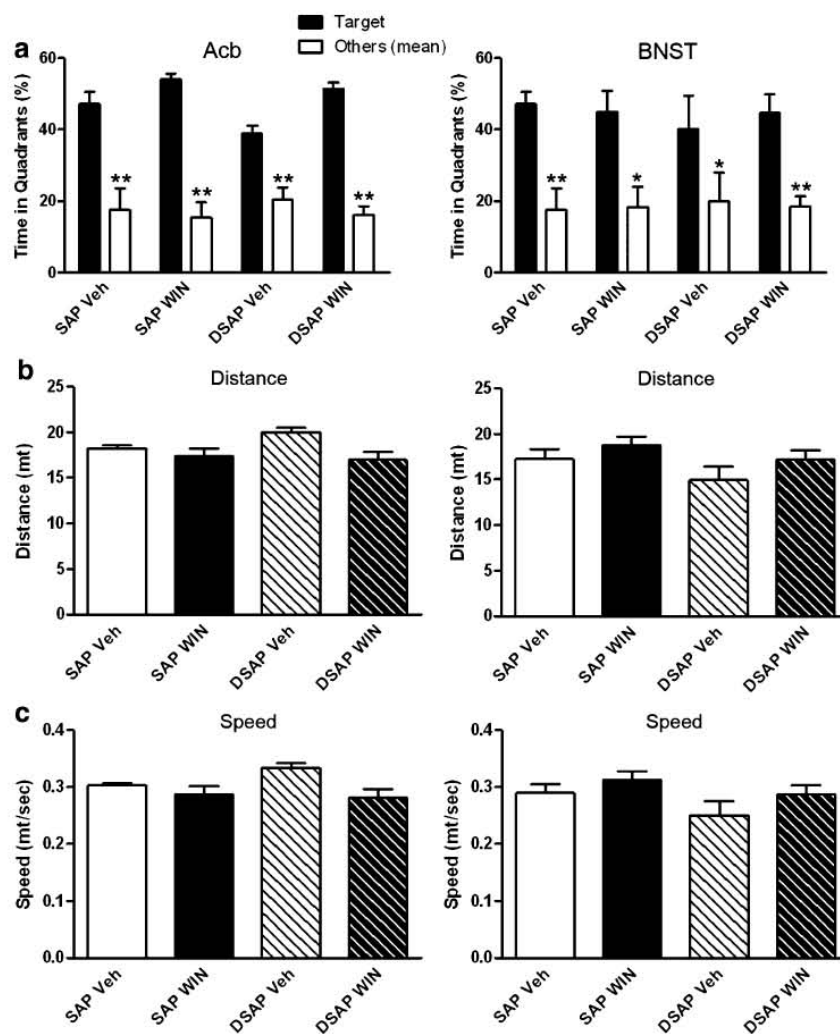

Depletion of noradrenergic fibers to the Acb or BNST has no effect on WIN 55,212-2-induced anxiety

Cannabinoid agonists are known to induce anxiety-like behaviors at high doses (Viveros et al. 2005; Rutkowska et al. 2006). To assess whether the reversal of aversive-like behaviors was due to changes in the level of anxiety, a group of animals was subjected to the EZM. A two-way ANOVA crossing toxin (SAP and DSAP) and drug (vehicle and WIN 55,212-2) treatment was performed to analyze changes in the percentage of time spent in the closed arms of the maze. This revealed an overall effect of drug in both the Acb (Fig. 5a) and BNST (Fig. 5b) experiments ($F(1,12)=36.686$, $p<0.0001$; $F(1,12)=34.372$, $p<0.0001$, respectively), indicating that WIN 55,212-2 was anxiogenic. There was no overall effect of toxin ($F(1,12)=$

3.047, $p=0.106$ (for the Acb); $F(1,12)=3.449$, $p=0.088$ (for the BNST)) in the time spent in the closed arm. The analysis revealed no interaction between drug and toxin treatment (Acb, $F(1,12)=0.22$, $p=0.647$; BNST, $F(1,12)=0.199$, $p=0.663$), revealing that depletion of NE from the Acb and BNST did not affect the anxiety-like behavior induced by WIN 55,212-1.

In addition, similar results were observed when analyzing the total number of entries into the arms of the maze (Table 1). There was an overall effect of drug in both the Acb and BNST experiments ($F(1,12)=72.104$, $p<0.001$), ($F(1,12)=11.108$, $p<0.01$, respectively). There was no effect of toxin (Acb, $F(1,12)=1.182$, $p=0.298$; BNST, $F(1,12)=0.184$, $p=0.184$) and no interaction between drug and toxin (Acb, $F(1,12)=1.748$, $p=0.211$; BNST, $F(1,12)=0.094$, $p=0.765$). These results indicate an anxiogenic

Fig. 5 Anxiety-like behavior (**a** and **b**) and locomotor activity (**c** and **d**) of animals injected with the toxin in the Acb (**a** and **c**) and in the BNST (**b** and **d**). Animals injected WIN 55,212-2 (3.0 mg/kg) showed higher anxiety levels as measured by more time spent in the close arms of the elevated zero maze (**a** and **b**; *$p<0.0001$). Injection of DSAP had no effect of the anxiety levels. Toxin and drug administration in the Acb group had no effect on locomotor activity (**c**). DSAP injection into the BNST increased locomotor activity independently of drug administration (**d**, *$p<0.005$)
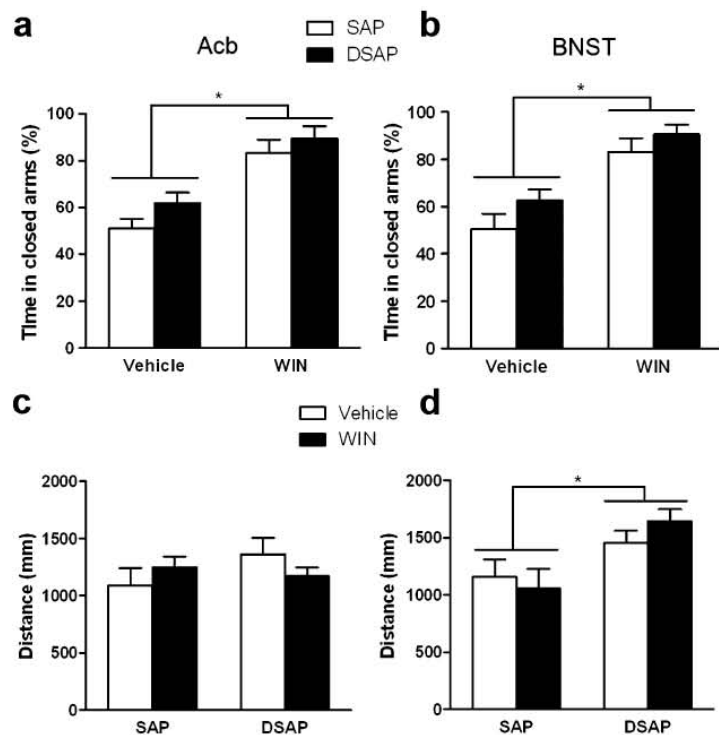
effect of WIN 55,212-2 that was not affected by depletion of NE.

Locomotor activity

After completion of the EZM, the animal's locomotor activity was assessed. A two-way ANOVA crossing toxin (SAP and DSAP) and drug (vehicle and WIN 55,212-2) treatment revealed no effect of toxin ($F(1,12)=0.539$, $p=0.477$) or drug ($F(1,12)=0.727$, $p=0.411$) treatment in the animals injected in the Acb (Fig. 5c). However, in the animals injected in the BNST it was observed an overall effect of toxin ($F(1,12)=12.387$, $p=0.004$) but not drug ($F(1,12)=0.219$, $p=0.648$) in the distance traveled, suggesting that depletion of NE from the BNST increases locomotor activity (Fig. 5d).

Table 1 Total number of entries in the elevated zero maze

	Vehicle	WIN 55,212-2	Total
Acb			
SAP	38 (±4.183)	7.5 (±2.63)	45.5
DSAP	48.25 (±5.921)	6.5 (±3.594)	46.75
Total	78.25**	14	
BNST			
SAP	49 (±8.399)	25 (±11.195)	74
DSAP	40.25 (±6.575)	11.4 (±4.874)	51.64
Total	89.25*	36.4	

Data represent mean of total number of entries (into opened and closed arms), ± SEM

*$p<0.01$, vehicle-treated animals compared to WIN 55,212-2-treated animals

**$p<0.001$, vehicle-treated animals compared to WIN 55,212-2-treated animals

Discussion

This study examined the neurochemical and regional substrates involved in cannabinoid-induced aversion and anxiety. The results indicate that administration of a CB1r/CB2r agonist induces conditioned place aversion and anxiety. It is reported that noradrenergic transmission within the Acb is a critical determinant for the expression of aversion-like behavior (as measured by the place conditioning paradigm) following exposure to a cannabinoid agonist. Moreover, norepinephrine depletion from the Acb and BNST did not affect anxiety-like behaviors, underscoring the involvement of differential circuitry in the expression of aversion and anxiety to a cannabinoid receptor agonist.

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WIN 55,212-2-induced aversion: role of limbic circuitry

The present results are in agreement with previous findings that report cannabinoid receptor agonists to be aversive to rats, as shown by the induction of conditioned place aversion (McGregor et al. 1996; Sanudo-Pena et al. 1997; Mallet and Beninger 1998; Pandolfo et al. 2009). Aversive behaviors require emotional learning and association of emotions with a context, therefore limbic areas such as the PFC, BNST, and Acb have been involved in eliciting these behaviors (Gracy et al. 2001; Levita et al. 2002; Delgado et al. 2008). Gracy and colleagues (2001) have shown that place aversion to naltrexone-induced opiate withdrawal is related to neuronal activation of the shell subregion of the Acb and the central nucleus of the amygdala (CeA). Moreover, monoaminergic transmission in areas such as the amygdala, PFC, BNST, and Acb has been implicated in the development of aversive behaviors (Aston-Jones et al. 1999; Delfs et al. 2000; Ventura et al. 2007; Kerfoot et al. 2008). For instance, Aston-Jones and colleagues (1999) have shown that blockade of beta adrenergic receptors in the CeA attenuates the morphine withdrawal-induced place aversion. Herein, we explored the hypothesis that NE in the Acb and BNST is a critical determinant for the establishment of cannabinoid-induced aversion. Using an immuno-toxin lesion approach of two limbic areas (Acb and BNST), we were able to establish the role of selected circuits involved in the expression of aversion to cannabinoids. Both areas are important nuclei of the limbic system, integrating information arising from the amygdala (concerning affective components of the behavior), from the hippocampus and PFC (conveying contextual features from the environment), and from the ventral tegmental area (regarding reward related components of learning experiences; Forray and Gysling 2004; Kerfoot et al. 2008). Moreover, both the Acb and BNST receive direct input from the NTS (Delfs et al. 1998; Forray et al. 2000; Forray and Gysling 2004) that conveys information regarding peripheral signals (e.g., arousal) with limbic structures. We show that this noradrenergic input from the NTS to the Acb is critical for the expression of place aversion to WIN 55,212-2. To our knowledge, it is not known whether the noradrenergic neurons projecting to the Acb have collateral projections to other areas. This is a potential caveat as, when lesioning NE neurons projecting to the Acb, collateral projections to other areas could be affected. However, we consider this a remote possibility because depletion of NE from the BNST, which receives much more NE than the Acb and does have collaterals to the CeA (Roder and Ciriello 1994) and paraventricular nucleus of the hypothalamus (Terenzi and Ingram 1995), did not affect any of the behaviors analyzed. A more important consideration regarding interpretation of findings from this study relates to the fact that these animals lack NE

throughout the conditioning phase and, therefore, it is not possible to discern whether NE is critical for the establishment and/or, on the other hand, for the recalling of the motivational association. However, previous studies show that impairment of NE transmission after the learning phase does not impact the expression of the behavior (Miranda et al. 2007; Kerfoot et al. 2008) suggesting that NE is not required for recalling learned associations. Nevertheless, pharmacological approaches would be needed to better clarify the time point in which NE is important for cannabinoid-induced aversion.

The ability of WIN 55,212-2 to induce aversion is most likely mediated by activation of CB1r as it has been shown that prior administration of the CB1r antagonist AM 251 prevents WIN 55,212-2-induced aversion (Pandolfo et al. 2009). CB1r has been localized to GABAergic neurons (Matyas et al. 2006) in the Acb but seldom on noradrenergic neurons (Carvalho et al. 2010). Moreover, cannabinoids have been shown to affect both glutamate and GABA transmission in the Acb (Manzoni and Bockaert 2001; Robbe et al. 2001). Interestingly, CB1r is found in noradrenergic neurons of the NTS (Carvalho et al. 2010) and WIN 55,212-2 has been shown to activate NTS neurons (Himmi et al. 1998; Jelsing et al. 2009).

We interpret the results of the present study in the following way: WIN 55,212-2 may act on CB1 receptors that are localized to noradrenergic neurons of the NTS, increasing their firing rate and subsequently increasing release of NE in the Acb. Consistent with this interpretation, WIN 55,212-2 has been shown to lead to changes in adrenergic receptor expression in the Acb (Carvalho et al. 2010). Moreover, one could speculate that activation of CB1r in glutamatergic and GABAergic terminals in the Acb may decrease the release of these amino acids, making Acb medium spiny neurons more sensitive to NE. In addition, other systems may be involved. For example, kappa opioid receptors (KOR) have been shown to be critical for THC-induced aversion (Zimmer et al. 2001; Ghozland et al. 2002). Mice lacking KOR do not show aversion to THC in the place conditioning paradigm. Dynorphin, the endogenous KOR agonist, is distributed throughout the Acb, in axon terminals that form mostly symmetric synapses (Khachaturian et al. 1982; Van Bockstaele et al. 1994). Interestingly, dynorphin is also found within NTS neurons and fibers (Fodor et al. 1994) and acute administration of the KOR synthetic agonist U-50,488H has been shown to increase c-fos activation of catecholaminergic NTS neurons (Laorden et al. 2003). This can be a potential mechanism by which dynorphin and KOR facilitate aversion to cannabinoids. Taken together, there are a number of potential interpretations and future studies are required to carefully parse out the nature of cannabinoid actions on the NTS/Acb circuit.

Others have shown that blockade of NE transmission within the BNST impairs place aversion to opiate withdrawal (Aston-Jones et al. 1999; Delfs et al. 2000). Although, in the

present study, depletion of NE in the BNST did not affect WIN 55,212-2-induced aversion, the possibility exists that upon withdrawal from cannabinoid exposure, NE transmission in the BNST becomes engaged in a fashion similar to opiate withdrawal. Future studies are required to test this possibility.

Anxiogenic effects of WIN 55,212-2

Cannabinoid agonists have been shown to exert anxiogenic effects in both animals and humans (Onaivi et al. 1990; Childers and Breivogel 1998; Arevalo et al. 2001; Marco et al. 2004; Witkin et al. 2005). Taking this into consideration, we hypothesized, along with others (McGregor et al. 1996), that reduction of the aversive effects of WIN 55,212-2 observed in the present study could be due to a reduction in anxiety levels. In order to examine this, animals were tested in the EZM. Our results are in agreement with others that showed that WIN 55,212-2 administration induces anxiety-like behaviors as seen by an increased time spent in the closed arms of the maze and decreased exploration measured by a reduction in the total number of entries. None of these EZM outputs was affected by NE depletion in both the Acb and BNST. These results dissociate anxiety-like behaviors from aversive behaviors. The results show that the same lesion that reverses the aversive behavior (depletion of NE in the Acb) had no effect on anxiety-like behavior. Though this fact cannot rule out an association between anxiety and aversion to WIN 55,212-2, it clarifies the nuclei involved in these two behaviors.

Nevertheless, it is surprising that disrupting noradrenergic transmission in the Acb, but especially in the BNST, does not affect anxiety-like behavior. The BNST is known to be a key nucleus in the expression of anxiety (Davis 1998; Davis 2006) and it is a "hot spot" of noradrenergic innervation (Forray and Gysling 2004). Hence, it is surprising that depletion of NE did not affect the expression of anxiety. However, little is known about the circuitry involved in cannabinoid-induced anxiety. The fact that other stimuli (stress, drug withdrawal) increases NE release in the BNST and this may trigger anxiety may not hold true for cannabinoid based agents. Moreover, the possibility exists that a 75% reduction of noradrenergic fibers was not sufficient to remove the noradrenergic basal tone in the BNST. Although further studies are required, the present results seem to suggest that CB1R-induced anxiety is not dependent on noradrenergic transmission.

Concluding remarks

The endocannabinoid system is widely expressed in the central and peripheral nervous system as well as immune system. Thus, it is involved in numerous physiological processes. Understanding how cannabinoids impact multiple systems will help us to better manipulate the endocannabinoid system without engaging unwanted side effects. The present study provides new information about the neural circuits involved in cannabinoid-induced behaviors that may lead to the development of potential new pharmacotherapies for the treatment of psychiatric disorders.

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Chapter 2.3

Carvalho AF and Van Bockstaele EJ

Direct intra-accumbal infusions of betaxolol abolish WIN 55,212-2-induced aversion

Direct intra-accumbal infusions of betaxolol abolish WIN 55,212-2-induced aversion.

Ana F. Carvalho[1,2] and Elisabeth J. Van Bockstaele, Ph.D.[1]

1 Department of Neurosciences, Farber Institute for Neurosciences
Thomas Jefferson University
Philadelphia, PA, USA

2 Life and Health Sciences Research Institute (ICVS)
School of Health Sciences, University of Minho
Braga, Portugal

Corresponding author: Ana Franky Carvalho
                             Department of Neurosciences,
                             Farber Institute for Neurosciences
                             Thomas Jefferson University
                             900 Walnut Street, Suite 417
                             Philadelphia, PA 19107
                             Phone: (215) 503 9147
                             Fax: (215) 503 9238
                             e-mail: arfranky@gmail.com

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Abstract

The cannabinoid system is known to interact with a variety of neuromodulators in the central nervous system and impacts diverse behaviors. Previous studies have demonstrated that limbic norepinephrine is a critical determinant in the behavioral expression of cannabinoid-induced aversion. The present study was carried out to define the adrenergic receptor subtype involved in mediating cannabinoid-induced behavioral responses. An acute microinjection of the beta-adrenergic blocker, betaxolol, directly into the nucleus accumbens was able to prevent WIN 55,212-2-induced aversion as measured in a place conditioned paradigm. These results suggest that noradrenergic transmission in the nucleus accumbens is important for cannabinoid-induced aversion and that beta-adrenergic antagonists may be effective in counteracting unwanted side effects of cannabinoid-based agents.

Keywords: Cannabinoids, adrenergic receptors, place conditioning

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Introduction

Previous studies have shown an anatomical and functional interaction between the cannabinoid and noradrenergic systems in the brain. The cannabinoid receptor type 1 (CB1r) has been found in noradrenergic neurons and terminals in brain regions such as the prefrontal cortex (PFC) (Oropeza *et al.*, 2007), nucleus accumbens (Acb) (Carvalho *et al.*, 2010a), locus coeruleus (LC) (Scavone *et al.*, 2010) and the nucleus of the solitary tract (NTS) (Carvalho *et al.*, 2010a). Moreover, administration CB1r agonist WIN 55,212-2 (3.0mg/kg) has been shown to increase norepinephrine (NE) release in the PFC as well as to increase c-fos expression in the LC (Oropeza *et al.*, 2005; Page *et al.*, 2007). Cannabinoids are known to dose-dependently affect several behaviors. While low doses usually induced reward and have anxiolytic effects, high doses (namely WIN 55,212-2 at the dose of 3.0mg/kg) usually induce aversive and anxiety-like behaviors (Degroot, 2008; Murray & Bevins, 2010). In line with this, in a previous study, we have investigated the contribution of NE to cannabinoid-induced aversion and anxiety (Carvalho *et al.*, 2010b). It was shown that NE in the Acb is critical for cannabinoid-induced aversion but not anxiety. Although the study showed an important role for NE in the aversion induced by a cannabinoid agent, it did not provide the adrenergic receptor (AR) subtype involved. The present study was designed to investigate the role of the β1-AR in cannabinoid-induced aversion and whether blockade of β1-AR after conditioning and prior to testing is sufficient to abolish this WIN 55,212-2-induced behavior. Animals were conditioned to the CB1r agonist, WIN 55,212-2, using a place conditioning paradigm and an intra-cerebral microinjection of a β1-AR blocker, betaxolol, was given prior to testing the animals.

Methods

Subjects

Twelve male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 220-250g were housed separately in a controlled environment (12-hour light schedule, temperature at 20°C). Food and water were provided *ad libitum*. The care and use of animals were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and were conducted in accordance with the NIH *Guide for the care and use of laboratory animals.* All efforts were made to minimize animal suffering and reduce the number of animals used.

Cannulae Implantation and Intracerebral Microinjections

Rats were anesthetized with an intraperitoneal (i.p.) injection of a saline solution containing a cocktail of Ketamine HCl (100mg/kg; Phoenix Pharmaceutical, Inc. St. Joseph, MO) and Xyla-Ject (2mg/kg; Phoenix Pharmaceutical, Inc.) and subsequently placed in a stereotaxic surgical frame (Stoelting Corp., Wood Dale, IL). The anesthesia was maintained by administration of isoflurane (Webster Veterinary Supply, Inc., Sterling, MA) through a nose cone. Bilateral cannulae (22 gauge, 8 mm long, from PlasticOne) were implanted into the Acb (AP: 1.5mm rostral to bregma, ML: +/- 0.9mm , DV: -6.4mm), according to Rat Brain Atlas of Paxinos and Watson (Paxinos, G. and Watson, C., 1997) coordinates. Cannulae were affixed to the skull using acrylic cement and double stylets were placed in the cannulae to prevent blockage. Animals were given a week to recover from surgery before behavioral testing. For intracerebral microinjections, the obturators were removed and 28 gauge injector cannulae were lowered to the final site (1 mm past the guide). Infusions of 0.5 μL per side were made using a Hamilton syringe.

Drug preparation and administration

WIN 55,212-2 (Sigma-Aldrich, St. Louis, MO) was dissolved in 5% dimethyl sulfoxide (DMSO)(Fisher Scientific, Fair Lawn, NJ) in saline and injected i.p. (3.0mg/kg) in a volume of 1ml/kg body weight. Vehicle injections consisted of 5% DMSO in saline. Betaxolol (Sigma-Aldrich) was dissolved in saline (1nmol/0.5µl); betaxolol or saline were microinjected in a volume of 0.5 µl per side (as previously described (Aston-Jones *et al.*, 1999)).

Place conditioning

An unbiased place conditioning procedure was used, so that the side of the apparatus used to conditioned animals was counterbalanced in all the groups. The paradigm consisted of three phases: pre-test, conditioning and test. On pre-test day (day 1), animals were placed in the apparatus and allowed to freely explore both sides of the apparatus for 20 min. The time spent in each side was recorded by an investigator. During the conditioning phase (days 2–6), the rats were injected twice daily. In the morning, animals were injected with vehicle and confined to one side of the apparatus for 45 min. In the afternoon, animals were injected with WIN 55,212-2 (3.0mg/kg) and confined to the opposite side for 45 min. On the test day (day 7), animals received a microinjection of betaxolol in the Acb five minutes before being place in the apparatus and allowed to explored both sides for 20 min. Control animals received a microinjection of saline in the Acb. The time spent in each side was measured by an investigator. No WIN 55,212-2 or vehicle injection was given to the animals on the test day.

Verification of cannula placement

At the conclusion of testing, animals were anesthetized with isoflurane (Isoflurane, USP, Webster Veterinary, Sterling, MA) and decapitated. Brains were removed and placed in 10% buffered

formalin (Fisher Scientific) for about two hours and then immersed in O.C.T. Embedding Compound (Electron Microscopy Sciences, Hatfield, PA) and frozen in dry ice. Coronal sections of the forebrain (35um) were cut using a Microm HM550 cryostat (Richard-Allan Scientific, Kalamazoo, MI) and every other section was collected on slide. Slides were allowed to dry and then stained with neutral red. Slides were visualizes using a Leica DMRBE microscope (Wetzlar, Germany), and images were acquired using SPOT Advanced software (Diagnostics Instruments, Inc., Sterling Heights, MI). Figures were then assembled and adjusted for brightness and contrast in Adobe Photoshop CS2.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 Graduate Student Version. Behavioral data were analyzed using a repeated measures multivariate analysis of variance with "time of testing" as the within-subject factor and "treatment" as the between-subject factor. Post-hoc analyses included paired and independent t-tests. Significance was set at $p < 0.05$.

Results

Verification of cannula placement

Coronal sections from the forebrain (ranging from plates 20-22 of the rat brain atlas of Paxinos and Watson (Paxinos, G. and Watson, C., 1997)) were visualized using light microscopy for accuracy of cannulae placement. Of the twelve subjects, eleven exhibited cannulae placements that were restricted to the Acb. Specifically, these did not significantly encroach on surrounding areas (e.g. PFC, BNST, lateral septum, dorsal striatum, ventral pallidum). Figure 1*a* shows a photomicrograph of a representative cannula placement. For simplicity, Fig. 1*b* shows a schematic representation of all cannulae placements for the eleven animals included in the behavioral analysis (plate 13 of the brain atlas (Paxinos, G. and Watson, C., 1997)). All placements are within the medial Acb, most of which are located in the shell subregion, others are located in the core subregion or border region.

Intra-accumbal injection of betaxolol prevents WIN 55,212-2-induced aversion

The place conditioning paradigm was used to assess the aversive effects of WIN 55,212-2 at the dose of 3.0mg/kg (Carvalho *et al.*, 2010b). All animals were conditioned to WIN 55,212-2 during the conditioning phase. Animals were assigned to two groups: animals that received betaxolol (n=6) or saline (n=5) prior to the test. Repeated measures analysis revealed that the there was an overall effect of time of testing ($F_{(1,9)}=10.79$, $p=0.009$), suggesting that the conditioning phase affected the performance of the animals on the test day (Figure 2). The analysis also showed an interaction between the treatment and time ($F_{(1,9)}=6.043$, $p=0.036$). Further analysis showed that animals that were given saline prior to the test spent significantly less time in the side paired with WIN 55,212-2 in the test day when compared to the pre-test (paired t-test,

t(4)=4.635, p=0.01), showing that WIN 55,212-2 induced aversion. On the contrary, the time spent in the side paired with WIN 55,212-2 in the test day did not differ from the pre-test in the animals that were given betaxolol (paired t-test, t(5)=0.551, p>0.05), suggesting that betaxolol injection prevents WIN 55,212-2-induced aversion. Moreover, the animals given saline spent less time spent in the side paired with WIN 55,212-2 in the test day than the animals that were given betaxolol (independent t-test, t(9)=-2.671, p=0.026). This suggests that β1-ARs in the Acb are important for the development of aversion to WIN 55,212-2.

Discussion

In this study, blockade of β1-ARs in the Acb prior to testing abolished aversion to systemic WIN 55,212-2 administration, using a place conditioning paradigm. We have previously shown that WIN 55,2121-2-induced aversion was abrogated by depletion of accumbal NE (Carvalho *et al.*, 2010b). In this previous study, depletion of accumbal NE was achieved using an immunotoxin approach, allowing us to deplete NE specifically in the Acb. Thus, animals lacked accumbal NE during the entire conditioning protocol. The present study adds to these previous results by identifying the β1-AR as a target involved in NE signaling. Moreover, we have shown that an acute injection of betoxolol in the Acb prior to testing was sufficient to inhibit the expression of aversion. However, this study has not explored whether the effect of betaxolol is long-lasting.

The β1-AR is a G-protein coupled receptor that stimulates Gs, and whose activation can increase glutamate-mediated excitation of medium spiny neurons (MSN) in the Acb (Kombian *et al.*, 2006). It is hypothesized that activation of MSN can trigger the development of aversive responses while inactivation of MSN can trigger reward responses (Carlezon & Thomas, 2009). Accordingly, inactivation of β1-AR by betaxolol may inhibit Acb activation by WIN 55,212-2, preventing the expression of aversion.

In recent years, cannabinoid based agents have been explored as potential new therapeutics for several disorders, from pain to neurodegenerative diseases and psychiatric disorders (Kano *et al.*, 2009; Crippa *et al.*, 2010). However, due to the wide distribution of the endocannabinoid system (Piomelli, 2003), unwanted effects may occur after manipulation of this system. For this reason, it is important to understand targets of the cannabinoid system and their functional consequences. Our previous and present studies identify the noradrenergic system, specifically limbic NE, as a critical player in the expression of cannabinoid-induced aversion. The ability to

block the expression of aversion with an acute microinjection of betaxolol after conditioning can be seen as a potential tool to reduce unwanted effects following administration of systemic cannabinoid agents.

Acknowledgments

Interest Statement

None.

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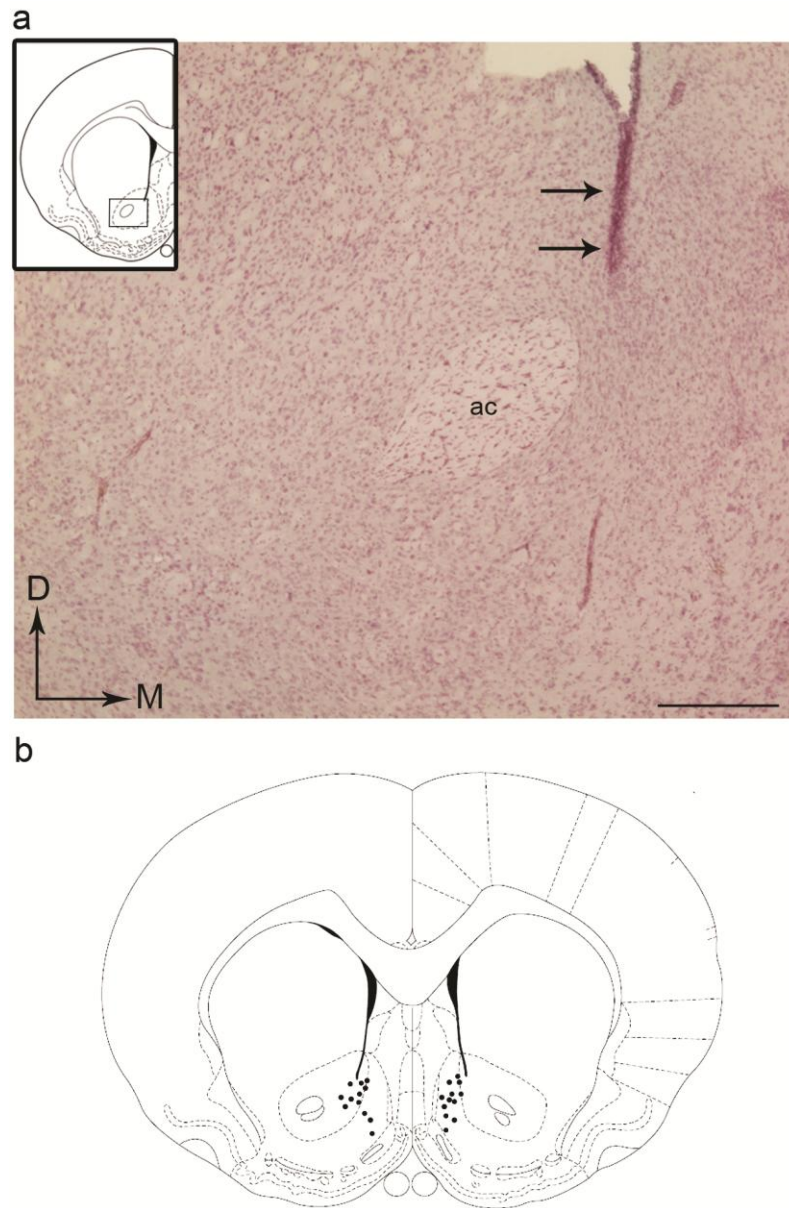
Figure 1. *a)* Photomicrograph of the Acb showing the placement of the cannula (arrows). *b)* Schematic diagram of a coronal section through the rostral forebrain adapted from the rat brain atlas (Paxinos, G. and Watson, C., 1997) showing sites of bilateral cannulae placements into the Acb. Dots represent the tip of the cannulae. Scale bar, 25 µm.
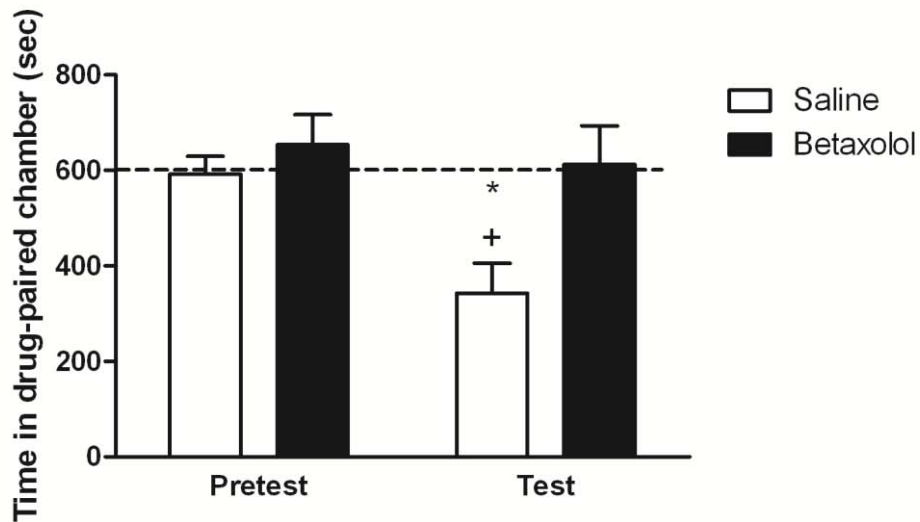
Figure 2. Effect of betaxolol on the development of WIN 55,212-2-induced place aversion. Animals that received a vehicle injection in the Acb prior to testing developed place aversion to WIN 55,212-2 (* $p<0.01$ compared to saline in pre-test day), as seen by decreased time spent in the drug-paired chamber. Conversely, microinjection of betaxolol prevented the development of aversion (+ $p<0.05$ compared to saline in test day).

Discussion

The present work aimed to clarify a potential interaction between the endocannabinoid and noradrenergic system in the brain and its implications in behavior. First, an anatomical characterization of the CB1r in the Acb and NTS was performed. Subsequently, it was shown that the systemic CB1r agonist, WIN 55,212-2, affected the expression of adrenergic receptors in the Acb. Finally, the importance of limbic NE to cannabinoid-induced aversion and anxiety was assessed.

3.1 Anatomical characterization of the endocannabinoid system

The endocannabinoid system can be targeted through its receptors (CB1r and CB2r), its endogenous ligands (anandamide, 2-AG) and its anabolic and catabolic enzymes (DAGL, FAAH, MAGL, among others). In addition, there is evidence for the existence of an endocannabinoid membrane transporter (EMT) (Piomelli, 2003; De Petrocellis *et al.*, 2004) but, since it has not been cloned yet, its precise localization cannot be determined. Since endocannabinoids are derived from cell membrane-derived lipids precursors, it is possible that any cell type has the ability to produce endocannabinoids. Thus, it is important to identify which cells have the enzymatic machinery to produce endocannabinoids. However, it must be taken into account that the synthetic pathways of endocannabinoids are complex and not yet fully understood which makes of the interpretation of any anatomical data a difficult task. Nevertheless, some studies have looked at the localization of three enzymes, NAPE-PLD, DAGL and PLCβ1. PLCβ1 mRNA has been detected by *in situ* hybridization in the whole rat brain, with higher expression in the olfactory bulb, cortex, caudate-putamen, piriform cortex, lateral septum and hippocampal formation (Watanabe *et al.*, 1998). Two isoforms of DAGL have been described (α and β); both are expressed in neurons with an interesting localization to axonal profiles during development and to somato-dendritic profiles in adults (Bisogno *et al.*, 2003). Higher expression of DAGL mRNA was reported in the olfactory bulb, cortex, caudate-putamen, thalamus and Purkinje cells of the cerebellum (Yoshida *et al.*, 2006; Uchigashima *et al.*, 2007). NAPE-PLD was quantified by western blot and RT-PCR in several brain regions; these studies identified the presence of NAPE-

PLD in higher amounts in the olfactory bulb, brainstem, cerebellum, frontal cortex, basal ganglia, hippocampus, hypothalamus, occipital cortex and thalamus (Morishita *et al.*, 2005).

In contrast, the catabolic pathway is simpler and better conclusions can be withdrawn from localization studies. FAAH is known to be the main enzyme involved in degradation of anandamide, while MAGL seems to be the main enzyme in the degradation pathway of 2-AG (Dinh *et al.*, 2002b; Piomelli, 2003; Dinh *et al.*, 2004). Interestingly, FAAH seems to be more abundant post-synaptically while MGL is found mainly pre-synaptically (Gulyas *et al.*, 2004). This way, FAAH is often found juxtaposed to CB1r-containing terminals whereas MAGL may co-exist in CB1r-containing terminals. The regional distribution of these two enzymes has been described with FAAH mRNA being more abundant in the cortex, hippocampus and cerebellum (Thomas *et al.*, 1997). MAGL mRNA expression has been reported to be high in the cortex, hippocampus, cerebellum and thalamus and moderate in the Acb, islands of Calleja and pontine nuclei (Dinh *et al.*, 2002a). From these localization studies, two findings are worth noting. First, FAAH and MAGL show a very striking subcellular distribution, with FAAH being more abundant and almost exclusively found in somato-dendritic profiles while MAGL is found at axon terminals, thus suggesting that the two endocannabinoids, anandamide and 2-AG, may have different physiological roles. Second, the fact that the distribution of these enzymes is not always consistent with the distribution of CB1r (see below) suggests that endocannabinoids produced by neighbor cells can diffuse to receptors on other cells. Future studies providing better characterization of the neurochemical properties of the expressing neurons together with the clarification of whether there are different isoforms of these enzymes that could help predict their localization will be very insightful to the function of the endocannabinoid system.

Measurement of endocannabinoid tissue content has also been used to map areas rich in endocannabinoids. However, contradicting and inconsistent results have been reported using both purification from tissue and consequent quantification by liquid or gas chromatography coupled with mass spectrometry and in vivo microdialysis. This is probably due to the limitations and caveats of each technique but also due to the fact that endocannabinoids are thought to be produced "on demand" and low levels of endocannabinoids are expected at basal levels. The process of extraction and purification of endocannabinoids from tissue is delicate and is one cause for variability in endocannabinoid quantification between studies (Buczynski & Parsons, 2010). In addition, increases in endocannabinoid content post-mortem have been reported in rat, mouse, sheep, cow and pig brain tissue (Schmid *et al.*, 1995; Kempe *et al.*, 1996), indicating

that time between sacrifice and sample processment is a critical determinant of endocannabinoid content. It is not known whether euthanasia itself alter brain endocannabinoid content. The use of *in vivo* microdialysis circumvents the effects of post-mortem time on the levels of endocannabinoids and the need to extract and purify endocannabinoids from tissue. However, the aqueous environment within the dialysis probe minimizes diffusion of lipophilic endocannabinoids, resulting in very low collection efficiencies and subsequent low endocannabinoids concentrations. These low concentrations of endocannabinoids may not reflect the real physiological importance one would attribute to such low levels (Buczynski & Parsons, 2010). Moreover, these quantification techniques do not allow for the identification of the producing and target cells.

To date, localization of CB1r has been the main tool to map the endocannabinoid system in the CNS. Localization of CB1r using antibodies, radioactive ligands or oligonucleotide probes has provided great insights into the functions of the system. It is worth noting that receptor density may not correlate with receptor activity as measured by cannabinoid-stimulated [^{35}S]GTPγS binding (Breivogel *et al.*, 1997; Childers & Breivogel, 1998). This suggests that the amount of cannabinoid activity in a brain region cannot be predicted solely based on relative receptor density. The first studies focusing on the distribution of CB1r used an autoradiographic approach with the radiolabeled potent agonist CP 55,940 (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991). High levels of binding were detected in the globus pallidus, substantia nigra, caudate-putamen, olfactory bulb, cerebellum and hippocampus. Moderate levels were found in the cortex, Acb, caudal NTS and LC (Herkenham *et al.*, 1991). The use of antibodies allowed the identification of the subcellular localization of CB1r, which was found mainly, but not exclusively, in axon terminals (Freund *et al.*, 2003). The identification of regions with high levels of CB1r has also helped to understand the role of the endocannabinoid system. For instance, localization of CB1r in the hippocampus and cortex underscores the effects of cannabinoids in memory and cognition. Similarly, the presence of CB1r in the basal ganglia reflects the effects of cannabinoids on movement. However, to fully understand how cannabinoids affect behavior it is relevant to identify the neurochemical properties of the target cells. For example, many studies have localized CB1r to glutamatergic and GABAergic neurons where CB1r activation was shown to induce DSE and DSI (Hajos *et al.*, 2000; Manzoni & Bockaert, 2001; Robbe *et al.*, 2001; Piomelli, 2003).

3.1.1 Anatomical localization of CB1r with respect to noradrenergic system in the limbic circuitry

With respect to the noradrenergic system, binding radioautographic studies have shown the existence of moderate density of CB1r protein and mRNA in the LC and NTS (Herkenham *et al.*, 1991; Mailleux & Vanderhaeghen, 1992; Matsuda *et al.*, 1993; Derbenev *et al.*, 2004; Jelsing *et al.*, 2008). Some studies have shown, by dual immunohistochemistry with DBH or tyrosine hydroxylase (TH), that some of the CB1r-positive neurons in the LC (Scavone *et al.*, 2006; Scavone *et al.*, 2010) and NTS (Chapter 2.1) are noradrenergic. Interestingly, the PFC and the Acb, two brain regions involved in some of the symptoms of psychiatric disorders which receive noradrenergic afferents from the LC and NTS respectively, show a very different pattern of CB1r distribution with respect to noradrenergic terminals. In the PFC, CB1r can be found in noradrenergic terminals (approximately 30% of CB1r-positive fibers were noradrenergic) (Oropeza *et al.*, 2007) while in the Acb the percentage of co-localization of CB1r and DBH is very low (Chapter 2.1). This may reflect different modulation of NE by endocannabinoids in these two regions. In line with this, the impact of systemic WIN 55,212-2 administration in the adrenergic receptors expression in the PFC and Acb is different (Chapter 2.1, see discussion below).

Interestingly, CB1r shows an interesting topography distribution in the Acb. The heterogeneous distribution of CB1r throughout the Acb may reflect different abilities of the cannabinoid system to modulate behavior in the Acb. It is proposed that the Acb subregions (shell and core) can be further subdivided with respect to function (Zahm, 1999). For instance, anatomical and behavioral studies support a rostro-caudal gradient for appetitive versus aversive behaviors (Reynolds & Berridge, 2001; Reynolds & Berridge, 2002; Reynolds & Berridge, 2003). In line with this, the possibility exists that cannabinoids, due to the heterogeneous distribution of CB1r, can have a bigger impact on certain behaviors over others.

3.2 Effects of cannabinoids on noradrenergic transmission

3.2.1 The effects on LC activity

Several studies have reported an effect of cannabinoids on LC activity. Namely, cannabinoids have been shown to increase LC spontaneous firing (Mendiguren & Pineda, 2004; Mendiguren & Pineda, 2006; Muntoni *et al.*, 2006). Patel and Hillard show increased Fos labeling in noradrenergic neurons in the LC following systemic injection of CP55940 and WIN 55,212-2 (Patel & Hillard, 2003). In this study, it is also shown that both CB1r agonists increase Fos expression in dopaminergic neurons. However, this activation of dopaminergic neurons by cannabinoid agonists is blocked by an α1-AR antagonist and by an α2-AR agonist, suggesting that CP55940 and WIN 55,212-2 may be activating dopaminergic neurons by acting on LC-NE neurons. On another study, Oropeza and colleagues (2005) have shown that systemic WIN 55,212-2 (at 15 and 3mg/kg) induces Fos expression in noradrenergic neurons of the LC. This effect was blocked in the presence of the CB1r antagonist SR 141716A, suggesting an effect mediated by CB1r.

Recordings from LC-NE neurons in anaesthetized rats have shown that systemic and central administration of cannabinoids, dose-dependently, increases the firing rate of the LC (Mendiguren & Pineda, 2006; Muntoni *et al.*, 2006). This effect was blocked by administration of the CB1r antagonist SR141716A. Interestingly, administration of SR141716A alone caused a significant reduction of LC spontaneous firing, suggesting that LC is under the control of an endogenous cannabinoid tone. This hypothesis is further supported by evidence showing that URB597, a selective inhibitor of FAAH (the enzyme responsible for degradation of anandamide) is able to enhance the spontaneous firing rate of LC-NE neurons (Gobbi *et al.*, 2005).

Cannabinoids have also been shown to inhibit KCL-evoked excitation of the LC (Mendiguren & Pineda, 2007), indicating that cannabinoids may have a protective role in the LC by preventing overactivation of this nucleus. Overactivation of the LC has been proposed to alter behavioral flexibility and disable focused or selective attention (Aston-Jones *et al.*, 1999b; Usher *et al.*, 1999; Aston-Jones, 2002). On the other hand, the phasic firing of the LC is important for a good performance on tasks that require focused attention. Thus, an excess in inhibition by cannabinoids may lead to a decrease of the phasic activation of the LC which could result in an overall disruption of attention in both animals and humans (Jentsch *et al.*, 1997; Solowij *et al.*, 2002; Arguello & Jentsch, 2004).

3.2.2 The effects on NTS activity

There is also compelling evidence for the action of cannabinoids in the NTS. However, the cannabinoid effects on NTS activity appear to be more complex than in the LC. In the NTS not all neurons are sensitive to Δ9-THC or other cannabinoids analogs (Himmi *et al.*, 1996; Himmi *et al.*, 1998). About 50% of the neurons of the NTS are responsive to cannabinoids analogs, a response apparently mediated by CB1r. Interestingly, some NTS neurons have their activity increased after cannabinoid treatment, while others display decreased neuronal activity. Moreover, both WIN 55,212-2 and the antagonist rimonabant were able to increase Fos expression in the NTS, albeit apparently in different set of neurons (Jelsing *et al.*, 2009). In a different study, analyzing the cardiovascular regulation by the NTS, a subset of NTS neurons with baroreceptive properties was found to increase discharge after application of anandamide and the endocannabinoid uptake inhibitor AM404 (Seagard *et al.*, 2005), similarly to conditions in which there is increase in blood pressure. The different responses to cannabinoid analogs observed in the NTS may be due to the fact that the NTS is a very heterogenous nucleus containing a large variety of neurotransmitters and neuropeptides. Catecholaminergic, serotoninergic, dopaminergic, GABAergic and cholinergic neurons can be found within the same subregions of the NTS (Barraco *et al.*, 1992). Since most studies fail to identify the neurochemical properties of the neuronal population analyzed it is hard to speculate about the meaning of these findings. In any case, the different studies reveal that cannabinoids can strongly influence activity of NTS neurons. With respect to NTS-NE neurons, this thesis shows that noradrenergic neurons in the NTS are positive for CB1r (Chapter 2.1), providing anatomical evidence for a potential action of cannabinoids in noradrenergic neurons. In addition, some Δ9-THC-sensitive neurons were depressed when clonidine, a α2-AR agonist, was co-administered, suggesting that these neurons are likely noradrenergic (Himmi *et al.*, 1996).

3.2.3 The effects of cannabinoids on NE release in target regions

Several studies have reported that systemic and local administration of cannabinoid analogs alters the release of NE in specific areas of the brain. Systemic administration of WIN 55,212-2 or Δ9-THC has been shown to increase the release of NE in the PFC and in the Acb (Jentsch *et*

al., 1997; Oropeza *et al.*, 2005; Page *et al.*, 2007). Jentsch and colleagues showed an increase in NE turnover in the PFC and Acb of rats after systemic injection of Δ9-THC. They also show that Δ9-THC also increased dopamine turnover but only in the PFC; no effects were observed on serotonin turnover. Oropeza and colleagues (Oropeza *et al.*, 2005) report an increase of NE release in the PFC with concomitant Fos activation in noradrenergic neurons of the LC; importantly, these effects were blocked by the CB1r antagonist SR 141716A. In another study, repeated administration of WIN 55,212-2 increased the release of NE in PFC with increased TH expression in the LC (Page *et al.*, 2007). Consistent with this, an increased activity rate of TH in rats given Δ9-THC and WIN 55,212-2 has been reported, resulting in increased levels of NE in the LC, hippocampus, cortex, hypothalamus and cerebellum (Moranta *et al.*, 2004). In addition, decreased synthesis of serotonin and dopamine were observed upon Δ9-THC and WIN 55,212-2 administration. In line with the NE increased release in the PFC and in the Acb, another study has reported alterations in the expression of ARs, as well as in the NE transporter (NET) (Reyes *et al.*, 2009). Reyes and colleagues have shown that acute administration of WIN 55,212-2 decreases NET expression in the PFC, which in addition to LC activation (Oropeza *et al.*, 2005) and increased TH activity in the LC (Moranta *et al.*, 2004; Page *et al.*, 2007) may account for the increased release of NE. Furthermore, repeated systemic administration of WIN 55,212-2 was shown to decrease the levels of β1-AR in the PFC.

On the contrary, abstinence from WIN 55,212-2 induced an upregulation of β1-AR which can be seen as a rebound effect probably due to returning of NE to basal levels after abstinence. No changes were observed in α2A-AR levels. In the Acb, we have shown that β1-AR expression was decreased with acute or repeated administration of WIN 55,212-2 (Chapter 2.1). Additionally, α2A-AR was decreased but only after repeated administration; this effect persisted with abstinence from WIN 55,212-2 (Chapter 2.1). The lower levels of β1-AR may represent an adapting mechanism to the rise in extracellular NE in the Acb after WIN 55,212-2 treatment. The decreased in α2A-AR expression only after repeated exposure to WIN 55,212-2 may reflect a secondary mechanism to increase NE release. Activation of α2A-AR is known to decrease cAMP production in the axon terminal, decreasing the release of vesicular NE (Wozniak *et al.*, 2000).

Interestingly, some reports have also shown that the CB1r antagonist, SR141716A is capable of increasing NE release in the PFC (Tzavara *et al.*, 2003) and in the hypothalamus (Tzavara *et al.*, 2001), and the administration of SR141716A is accompanied with antidepressant effects in the forced swim test. However, in another study, SR141716A alone did not trigger an effect in the

levels of NE compared to vehicle treated animals; however, in this study, it was observed that SR141716A blocked the effects of WIN 55,212-2-induced NE release (Oropeza *et al.*, 2005). These contradictory effects can be explained in part by the different doses used in these studies. In the latter, SR141716A was used at 0.2mg/kg while in the former study the doses applied ranged from 1mg/kg to 10mg/kg. The findings from CB1r antagonism can also reflect the existence of a basal tone of endocannabinoids in these regions.

In line with the known effects of cannabinoids on NE transmission, this thesis investigated the importance of NE to cannabinoid-induced behavior (Chapters 2.2 and 2.3).

3.3   Contribution of norepinephrine to cannabinoid-induced behaviors

3.3.1   Limbic norepinephrine and behavior

Many studies have revealed an important role for NE in mental function and dysfunction. While, for many decades, the LC-NE system was seen as the main NE source of the CNS and was implicated in attention, memory and behavior, increased interest in the NTS is now evident. Several studies have reported the existence of direct ascending projections from the NTS to areas such as the bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (Ricardo & Koh, 1978; Reyes & Van Bockstaele, 2006) or Acb (Delfs *et al.*, 1998). In fact, NTS ascending projections have been shown to impact behavior (Aston-Jones *et al.*, 1999a; Delfs *et al.*, 2000). Pharmacological studies have provided great input about the functional implications of NE. In fact, blockade of β-ARs is known to impair memory, decrease anxiety and increase depressive symptoms (Gottschalk *et al.*, 1974; Sternberg *et al.*, 1986; Patten, 1990). These effects of NE modulation can be understood as region specific or, due to the highly intricate neurocircuitries of the limbic system, it is possible that NE has a more general, regulatory function. Region specific studies have shown that NE by acting in regions like the hippocampus, PFC, amygdala or BNST is important for memory, aversion and anxiety (Delfs *et al.*, 2000; Aston-Jones, 2002; Tully & Bolshakov, 2010).

3.3.2 Place conditioning and aversion

Cannabinoid agents have been shown to produce both preference and aversion in the place conditioning paradigm. Many variables can account for such different behavior. This issue was recently revised by Murray and Bevins (Murray & Bevins, 2010). The most consistent factor to affect test outcome seems to be the dose of the cannabinoid agent used. Low doses have tendency to induce preference while high doses have tendency to induce aversion. In addition, the number of pairings and duration of sessions are also important variables that can influence test outcome. In the studies of the present thesis, it is shown that WIN 55,212-2 induces conditioned place aversion. Place conditioning is a classical conditioning paradigm in which animals learn to associate the effects of a drug (or other discrete treatment) with particular environmental (contextual) cues. Place conditioning can identify both conditioned place preference (CPP) and conditioned place aversion (CPA), and thus it can be used to study both rewarding and aversive drug effects (Bardo & Bevins, 2000; Carlezon, 2003). Conditioning involves an animal receiving repeated access to the drug in one context and receiving the control drug in another context. The outcome of the place conditioning is based in the assumption that animals have a tendency to approach and remain in contact with environments in which they have experienced rewarding drug effects, and they have a tendency to avoid environments in which they have experienced aversive drug effects. This requires that the animals are able to distinguish between the two environments; the power of the place conditioning assay is maximized when the animals do not have an *a priori* preference for either environment. *A priori* preferences can often be detected by a pretest, in which animals have access to the entire place conditioning apparatus. An apparatus is considered "unbiased" when there is no evident initial preference for one of the environments.

Place conditioning is useful in probing neural circuits involved in reward and aversion. For example, microinjection of amphetamine into the Acb produces CPP, whereas microinjection of amphetamine into the area postrema produces a conditioned taste aversion (CTA) (Carr & White, 1983; Carr & White, 1986). Other studies have shown that microinjection of μ opioids into the VTA produces CPP, whereas microinjection of k opioids into the VTA, Acb, medial PFC or lateral hypothalamus produces CPA (Shippenberg & Elmer, 1998). Hence, place conditioning studies allow parsing out the neural circuits involved in drug reward and aversion and perceiving that drugs can induce reward and aversion depending on the region and receptor subtypes being

activated. Accordingly, in the studies provided herein (Chapters 2.2 and 2.3) we were able to partially dissect the neural circuitry involved in cannabinoid-induced aversion. Monoaminergic transmission in several limbic structures (e.g. amygdala, PFC, BNST and Acb) has been reported to be important for the expression of aversive behaviors (Aston-Jones *et al.*, 1999a; Delfs *et al.*, 2000; Ventura *et al.*, 2007; Kerfoot *et al.*, 2008). In Chapter 2.2, the hypothesis that NE in the Acb and BNST is critical for WIN 55,212-2 aversion was investigated. Both the Acb and BNST receive direct noradrenergic projections from the NTS (Delfs *et al.*, 1998; Forray *et al.*, 2000; Forray & Gysling, 2004). Activation of the NTS has been shown to occur when CTA acquisition and expression occur (Sakai & Yamamoto, 1997; Swank, 2000). Although these studies showed no neurochemical characterization of the activated neurons, since the highest neuronal activation was seen in the caudal and intermediate NTS, the possibility exists that some of the activated neurons are noradrenergic. The localization of CB1r to noradrenergic neurons in the NTS (Chapter 2.1) and the ability of WIN 55,212-2 to induced NTS activation (Jelsing *et al.*, 2009) underlie the hypothesis that WIN 55,212-2 induces aversion by increasing NE release in target regions. Our results show that NE in the Acb is critical for WIN 55,212-2-induced aversion, as decreasing NE signaling in the Acb, either by immunotoxin depletion of noradrenergic fibers (Chapter 2.2) and by blockade of β1-ARs (Chapter 2.3), impaired its expression. In addition, it is known that blockade of β1-AR reduces the excitability of accumbal neurons which may signal aversion (Kombian *et al.*, 2006; Carlezon & Thomas, 2009).

Noradrenergic transmission in the BNST has been implicated in the signaling of negative affective effects (aversion) of opiate withdrawal (Delfs *et al.*, 2000; Cecchi *et al.*, 2007) and visceral pain (Deyama *et al.*, 2009; Minami, 2009). However, our results seem to suggest that NE in the BNST is not critical for WIN 55,212-2-induced aversion (Chapter 2.2). While technical limitations should be taken into consideration, as the noradrenergic depletion achieved may have not been sufficient to remove all norepinephrine basal tone, the possibility that NE in BNST is not require for the expression of WIN 55,212-2 aversion is also plausible. Indeed, NE in the BNST may only be necessary for the expression of cannabinoid withdrawal-induced negative effects, as it happens in the case of opiate withdrawal (Delfs *et al.*, 2000; Cecchi *et al.*, 2007).

3.3.3 Elevated zero maze and anxiety

In the studies provide herein, the effects of WIN 55,212-2 on anxiety levels were measured in the elevated zero maze (EZM). The EZM is a modification of the well established elevated plus maze (EPM). EZM´s design comprises an elevated annular platform with two opposite enclosed quadrants and two open, removing any ambiguity in interpretation of time spent on the central square of the traditional design and allowing uninterrupted exploration. The EZM is a reliable and sensitive model of anxiety-like behavior in rodents (Shepherd *et al.*, 1994). The test is based on the natural conflict of rodents to explore a novel environment and their innate aversion to open, elevated and brightly lit spaces. As a consequence of the aversive properties of the open arms, subjects spend a greater amount of time on the closed arms and the proportion of total exploration in the open arms provides a measure of anxiety, such that increases in percent time spent on the open arms is considered to be indicative of anxiolytic drug action ((Handley & Mithani, 1984; Pellow & File, 1986). Conversely, decreases in percent time spent on open arms reflect an anxiogenic effect of the drug.

Cannabinoids have been shown to trigger anxiolytic and anxiogenic effects. The contradictory results of cannabinoid agents may be due to some of the following variables: prior drug use, dose used, basal anxiety levels and regional endocannabinoid basal tone (Degroot, 2008). Generally, the anxiogenic properties of cannabinoid agents occur more frequently in drug-naïve subjects and in novel/stressful environments (Haller *et al.*, 2004; Viveros *et al.*, 2005; Degroot, 2008). This suggests that basal endocannabinoid tone is important to the response to exogenous cannabinoids. In fact, anxiety provoking stimuli have been shown to increase endocannabinoid levels in the brain (Marsicano *et al.*, 2002). In this scenario, endocannabinoids are thought to work as a coping mechanism, important to decrease anxiety levels. While in physiological situations this increase in endocannabinoids may be restrict to specific brain regions, such as the amygdala (Marsicano *et al.*, 2002), in cases where exogenous cannabinoids are administered, the different pattern of cannabinoid receptor activation may exert an anxiogenic effect. In the present work, it is shown that WIN 55,212-2 induces an anxiety-like behavior as measured in the EZM. It is also shown that decreasing NE signaling in the Acb and BNST did not affect the anxiogenic effect of WIN 55,212-2 (Chapter 2.2 and Chapter 2.3). In fact, decreased NE tone in the Acb was able to reverse WIN 55,212-2-induced aversion, but it was not sufficient to block WIN 55,212-2-induced anxiety. These results suggest that WIN 55,212-2-induced anxiety is not

mediated by NE input to the Acb. These findings are not surprising as the Acb has not been implicated in the development of anxiety-like behaviors. On the other hand, the results obtained from NE depletion from the BNST are quite fascinating. The BNST is seen as an important nucleus for the expression of anxiety (Davis, 1998; Walker *et al.*, 2003; Davis, 2006) and is one of the richest areas in NE in the CNS (Forray & Gysling, 2004). Although NE in the BNST has been shown to mediate anxiety to certain stressors, it does not mediate anxiety in response to all types of stressors (Cecchi *et al.*, 2002). This way, it has been proposed that NE effects on anxiety are stimuli-specific. NE is also known to be important to drug withdrawal-induced anxiety (Smith & Aston-Jones, 2008). However, in our studies WIN 55,212-2 withdrawal was not induced and that could explain why NE depletion did not affect WIN 55,212-2-induced anxiety. Moreover, other neurotransmitters have also been implicated in signaling anxiety in the BNTS, such as CRF (Smith & Aston-Jones, 2008). In line with this, there is the possibility that WIN 55,212-2-induced anxiety is mediated by other neurotransmitters. Taken all together, the results suggest that WIN 55,212-2-induced anxiety is independent of noradrenergic transmission.

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Chapter 4

Conclusions

In conclusion, the studies provided in the present thesis show an interaction between the endocannabinoid and noradrenergic systems. This interaction is functional and has behavioral implications. Using anatomical, biochemical and behavioral tests it was shown that endocannabinoids can modulate noradrenergic transmission and that noradrenergic transmission is important for cannabinoid-induced aversion.

Briefly, the results in the present thesis show that:

1. CB1r are present in the Acb and in the NTS. Localization of CB1r to noradrenergic profiles in the Acb is sparse but abundant in the NTS.

2. The synthetic cannabinoid WIN 55,212-2 is able to alter adrenergic receptors expression in the Acb.

3. WIN 55,212-2 induces aversion, and this behavioral effect is dependent on noradrenergic transmission in the Acb.

4. Noradrenergic transmission is not critical for WIN 55,212-2-induced anxiety.

Future perspectives

The present work has shown that the endocannabinoid and noradrenergic systems interact. The interactions were explored mainly in the limbic system. Ultimately, the present work adds to the literature and opens new possibilities in the understanding of cannabinoid-induced central effects and new ways to modulate the noradrenergic system. However, several questions arise from the present work and should be addressed.

Future studies should examine:

1. The functional implications of the topographic distribution of CB1r and NE in the Acb. The Acb is known to mediate both aversion and reward. It would be interesting to analyze whether norepinephrine is also critical for cannabinoid-induced reward.

2. The mechanism by which cannabinoids activate noradrenergic neurons in the NTS. The existence of CB1r in noradrenergic neurons suggests that this activation can be by direct activation of these receptors. In addition, CB1r was also localized to non-noradrenergic neurons which could also modulate the activation of noradrenergic neurons. Moreover, the possibility exists that afferents of the NTS are under control of cannabinoids.

3. The ability of exogenous cannabinoid to modulate noradrenergic transmission in pathologic situations. WIN 55,212-2 administration induced changes in the expression of adrenergic receptors. It would be of great interest to examine whether these effects are present in a situation of disease.