ABSTRACT

Badr Mostafa Sadek Ibrahim**. MECHANISMS FOR CENTRAL CANNABINOID RECEPTOR 1 EVOKED SYMPATHOEXCITATION/PRESSOR RESPONSE IN CONSCIOUS RATS** (Under the direction of Dr. Abdel A. Abdel-Rahman) Department of Pharmacology and Toxicology, November, 2010

The main goal of the current study was to elucidate the molecular mechanisms implicated in central cannabinoid receptor subtype 1 (CB_1R)-evoked sympathoexcitation/pressor response. In pursuit of this goal, the candidate first characterized the centrally elicited hemodynamic effects of CB_1R activation (WIN55,212-2) in a conscious rat model. The results showed that the pressor response elicited by central CB_1R stimulation was associated with enhanced neuronal activity of presympathetic neurons in the rostral ventrolateral medulla (RVLM). The findings of multiple *in vivo* and *in vitro* studies have suggested a functional crosstalk between central CB_1R and orexins. Therefore, the candidate hypothesized that orexin-A/orexin receptor 1 (OX_1R) signaling in the RVLM is essential for the central CB_1R-mediated pressor response. In support of this hypothesis, central CB_1R activation elevated orexin-A levels in the RVLM and inhibition of orexin-A/OX_1R signaling abrogated the CB_1R-evoked pressor response. Colocalization studies have unraveled close proximity of orexin-A/OX_1R to CB_1R immuno-reactive neurons and punctate-like structures in the RVLM, which support the pharmacological findings. Furthermore, the present study delineated a novel role for PI3K/Akt/ERK1/2 signaling in RVLM and nucleus tractus solitarii (NTS) in the sympathoexcitation elicited by central CB_1R activation in conscious rats. The latter findings inferred a causal role for a down-regulated PI3K/Akt signaling in the RVLM and NTS in the central CB_1R-evoked pressor response. This conclusion is supported by the exacerbation of the WIN55,212-2-evoked pressor response following PI3K/Akt inhibition (wortmannin). By contrast, ERK1/2 phosphorylation was enhanced in the same neuronal pools

and the pharmacological and molecular studies suggest that this effect, which is mediated, at least partly, via the reduction in PI3K/Akt signaling, contributes to the central CB_1R-evoked pressor response. Finally, the findings demonstrated that direct CB_1R activation in the RVLM enhanced the activity of two distinct neuronal pools (catecholaminergic and nitroxidergic), which may be linked to the modulation of brainstem GABAergic neurotransmission ($GABA_AR$) and subsequently to the central CB_1R-evoked sympathoexcitatory and pressor response. The findings yield new insight into a functional crosstalk between CB_1R and OX_1R signaling in the RVLM, a neuronal pool that is heavily implicated in blood pressure control and in hypertension.

MECHANISMS FOR CENTRAL CANNABINOID RECEPTOR 1 EVOKED

SYMPATHOEXCITATION/PRESSOR RESPONSE IN CONSCIOUS RATS

A Dissertation

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by

Badr Mostafa Sadek Ibrahim

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This work is dedicated to my loving parents, my first teachers and best parents anyone could ask for! Your inspiration, knowledge, wisdom, and support were the keys to reach (our) dreams.

And

To my wife, Hoda and my sons: Mohammed and Omar, the highlights of my day, the love of my life. I could not have endured or completed graduate school without your unwavering support.

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LIST OF SYMBOLS OR ABBREVIATIONS

ABC	avidin biotin complex
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
ANOVA	analysis of variance
AP	arterial blood pressure
BP	blood pressure
CB$_1$R	Cannabinoid receptor 1
CB$_2$R	Cannabinoid receptor 2
CNS	central nervous system
Δ^9-THC	Δ^9- tetra-hydrocannabinol
DMSO	dimethylsulfoxide
FITC	fluorescein isothiocyanate
GPCR	G-protein coupled receptor
HR	heart rate
I.C.	intracisternal
I.C.V.	intracerebroventricular
I.V.	intravenous
MAP	mean arterial pressure

nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NTS	Nucleus Tractus Solitarii
OX$_1$R	Orexin receptor 1
OX$_2$R	Orexin receptor 2
PD98059	2´-amino-3´-methoxyflavone
pERK1/2	phosphorylated extracellular signal-regulated kinase
PI3K	Phosphotidylinositol 3-kinase
RVLM	rostral ventrolateral medulla
SB-408124	[N-(6,8-Difluoro-2-methyl-4-quinolinyl)-N'-[4-(dimethylamino) phenyl] urea
SD	Sprague Dawley rats
SHR	spontaneously hypertensive rat
TH	Tyrosine Hydroxylase
WIN55,212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo [1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate salt
WKY	Wistar Kyoto rats
Wortmannin	(1S,6br,9aS,11R,11bR) 11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(metho xymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5,- h]-2-h]-2-benzopyran-3,6,9-trione.

CHAPTER ONE-GENERAL INTRODUCTION

1. 1. Cannabinoid CB$_1$ Receptor

Cannabinoid receptors are the site of action of a heterogeneous group of compounds broadly termed cannabinoids. These compounds include those isolated from the plant *Cannabis sativa* (marijuana)-derived Δ^9-tetra-hydrocannabinol (Δ^9-THC), the naturally occurring prototype compound, as well as other synthetic compounds (discussed below). *Cannabis* has long been used for recreational and medical purposes since at least 2000 B.C. It is now known that cannabinoids exert their actions via two subtypes of G protein-coupled receptors: CB$_1$ and CB$_2$. Our study focuses on the CB$_1$R, which is found primarily in the CNS, including the cardiovascular regulatory nuclei in the brainstem, unlike CB$_2$R, which is present mainly peripherally.

1. 1. 1. Topography of CB$_1$ receptor

The CB$_1$ receptor, a 473-amino-acid protein, was first cloned from a rat cerebral cortex cDNA library (Matsuda, Lolait et al. 1990) and a human brainstem library (Gerard, Mollereau et al. 1991), which maintains the essential topographical features for a G-protein coupled receptor (GPCR) as shown in Fig. 1. 1, published by Howlett A.C. (Howlett 1998). This two-dimensional model of CB$_1$R includes; (i) seven hydrophobic transmembrane domain regions that extend through the plasma membrane; (ii) three extracellular loops (EL1/EL2/EL3); (iii) three intracellular loops (IL1/IL2/IL3); (iv) an extracellular N-terminal (NT); (v) and an intracellular C-terminal (CT).

Fig. 1. 1. Topography of cannabinoid receptor 1. [Taken from Howlett, A.C. 1998: with permission].

Extracellular

Intracellular

N-ter.

C-ter.

3
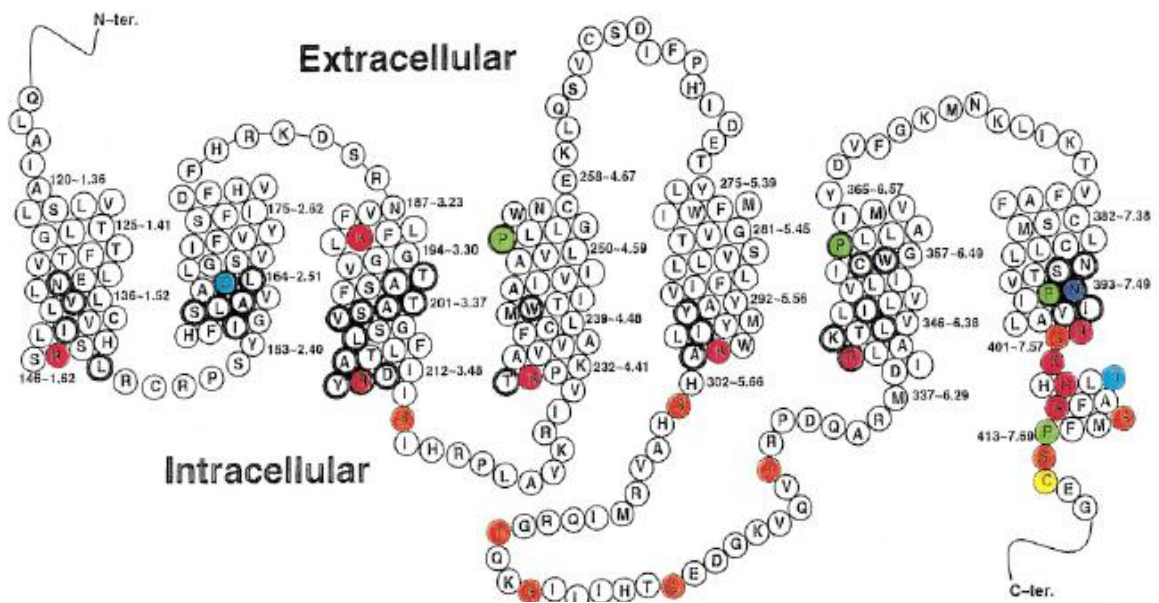
1. 1. 2. Signal transduction following CB$_1$R activation

Activation of CB$_1$R results in activation or inhibition of several downstream signaling molecules, which are site-specific e.g. inhibition of adenylyl cyclase activity, stimulation of inwardly rectifying potassium channels, inhibition of N- and P/Q-type voltage-dependent calcium channels (Twitchell, Brown et al. 1997; Mu, Zhuang et al. 1999), and activation of mitogen-activated protein (MAP) kinase pathway, Fig. 1. 2.

1. 1. 2. 1. Inhibition of adenylyl cyclase

Multiple lines of evidence suggest that cannabinoids act on CB$_1$R to inhibit adenylyl cyclase resulting in the reduction of cAMP production (Howlett, Qualy et al. 1986; Bidaut-Russell, Devane et al. 1990; Childers, Fleming et al. 1992). Further, it has been shown that the selective CB$_1$R antagonists, SR141716A and LY320135 can antagonize the inhibition of cAMP production induced by cannabinoids (Felder, Joyce et al. 1998).The involvement of an inhibitory G-protein (G$\alpha_{i/o}$) through which the CB$_1$R is negatively coupled to adenylyl cyclase has been demonstrated through cannabinoid-induced inhibition of adenylyl cyclase. This cyclase inhibition was blocked by G$\alpha_{i/o}$-selective pertussis toxin in mammalian brain and in cultured neuronal cells (Howlett, Qualy et al. 1986; Bidaut-Russell, Devane et al. 1990; Childers, Fleming et al. 1992).

1. 1. 2. 2. Modulation of ERK1/2 and PI3K/Akt signaling

Cannabinoids are highly potent activators of extracellular-signal regulated kinase1/2 (ERK1/2), which was evident in stably transfected Chinese hamster ovary cells expressing human CB$_1$R. This effect was (i) inhibited by SR141716A; (ii) sensitive to pertussis toxin; (iii)

and independent of the cannabinoid-induced inhibition of cAMP production (Bouaboula, Poinot-Chazel et al. 1995). Importantly, in U373 MG human astrocytoma cells (Galve-Roperh, Rueda et al. 2002) and hippocampal slices (Derkinderen, Valjent et al. 2003) CB_1R activation rapidly led to ERK1/2 phosphorylation via the phosphotidylinositol 3-kinase (PI3K) pathway in the U373 MG human astrocytoma cells but not in the hippocampus. Notably, studies on the neuroprotective and/or anti-oncogenic effects of cannabinoids via PI3K/Akt signaling pathway have yielded controversial results. First, intraperitoneal injection of Δ^9-THC activated PI3K/Akt pathway in mouse hippocampus, striatum, and cerebellum via a mechanism that was ERK1/2-independent (Ozaita, Puighermanal et al. 2007). Second, THC-mediated anti-cancer effect in human prostate cells involved PI3K/AKT and ERK1/2 signaling pathway activation (Sanchez, Ruiz-Llorente et al. 2003). On the other hand, it was demonstrated in multiple cancer cell lines that CB_1R activation down regulates both PI3K/Akt and ERK1/2 signaling pathway (Ellert-Miklaszewska, Kaminska et al. 2005; Greenhough, Patsos et al. 2007). Whether PI3K/Akt and or ERK1/2 signaling pathways are involved in central CB_1R-elicited sympathoexcitation has yet to be elucidated.

1. 1. 2. 3. Other CB_1R signaling

Many of CB_1R-mediated physiological functions are G protein $G\alpha_{i/o}$ mediated (Howlett, Qualy et al. 1986; Howlett and Mukhopadhyay 2000; Piomelli 2003). However, the diverse, sometimes opposing, CB_1R-evoked physiological functions that are not completely attributable to simply lowering intracellular cAMP levels, have led to investigations of the role of other non-$G\alpha_{i/o}$ signaling mechanisms (Lauckner, Hille et al. 2005).

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Fig. 1. 2. Main signaling transduction pathways of CB$_1$ receptor activation. Modified with permission from www.CNSforum.com
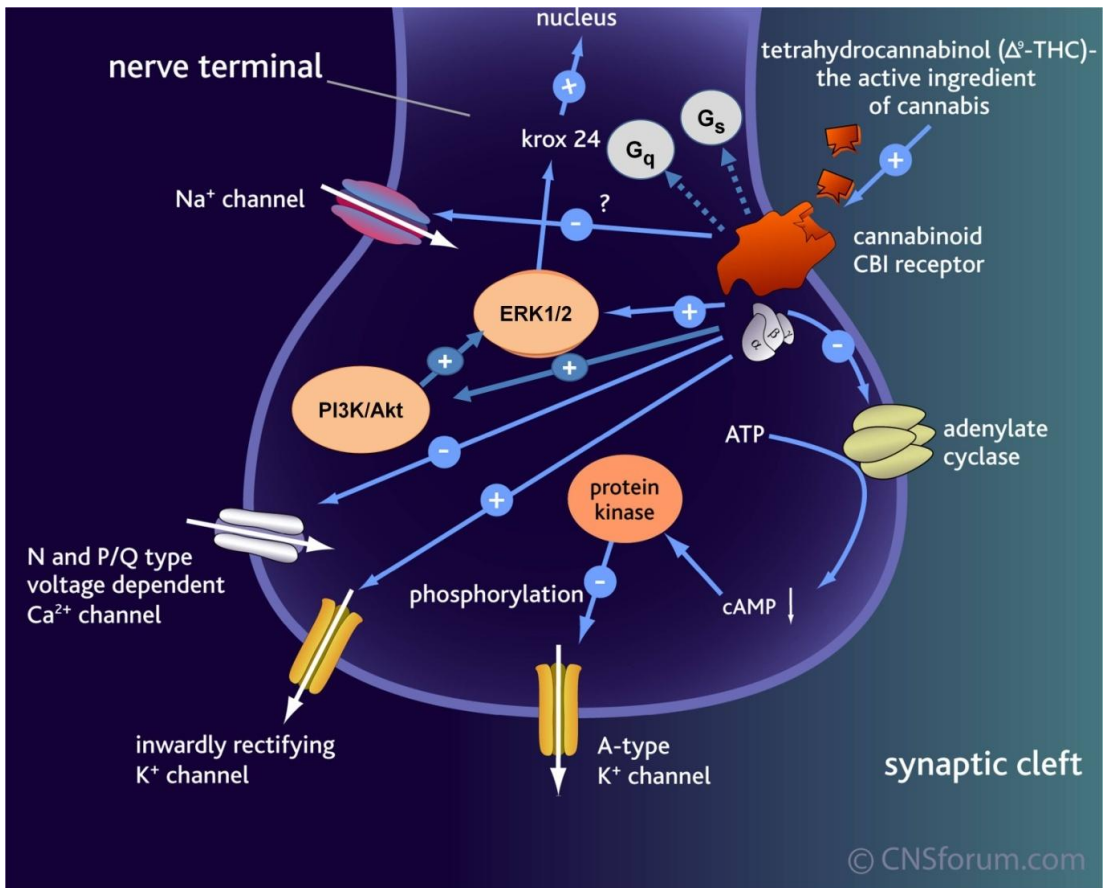
For instance, central CB_1R activation evokes sympathoexcitation/pressor responses while activation of $G\alpha_{i/o}$ coupled receptors in the brainstem α_{2A} adrenergic receptors (El-Mas and Abdel-Rahman 2004; Li, Wang et al. 2005) evokes depressor responses. In this line, recent studies have linked CB_1R coupling to activation of $G\alpha_{q/11}$ or $G\alpha_s$. It is possible that heterodimerization between CB_1R and other receptor(s) contribute, at least partly, to this divergent signal transduction. This notion is supported by the reported interaction between CB_1R and other co-localized receptors. First, evidence of CB_1R coupling with $G\alpha_s$ was demonstrated by simultaneous activation of dopamine D_2R, which resulted in accumulation of cAMP (Glass and Felder 1997; Kearn, Blake-Palmer et al. 2005). Second, CB_1R behaves as a $G\alpha_{q/11}$- G protein-coupled receptor in cultured hippocampal neurons and trabecular meshwork cells (Lauckner, Hille et al. 2005; McIntosh, Hudson et al. 2007). Most pertinent to the proposed studies are the findings that heterodimerization between CB_1R and OX_1R resulted in enhanced $G\alpha_{q/11}$-dependent OX_1R signaling in presence of CB_1R (Ellis, Pediani et al. 2006) (discussed below)

1.1. 2. 4. Retrograde CB₁R-mediated signaling

An accumulating body of evidence suggests that CB_1 receptors are located mostly presynaptically and play crucial roles in controlling the release of neurotransmitters at both excitatory and inhibitory synapses. As illustrated by Scotter et al. (Scotter, Graham et al. 2009) this mechanism is defined as retrograde signaling (Fig. 1.3); in this regards, endocannabinoids, which are postsynaptically released upon depolarization, activate presynaptic CB_1R which in turn inhibits Ca^{2+}-dependent exocytosis of various neurotransmitters (Freund, Katona et al. 2003; Piomelli 2003). For example, WIN55,212-2 inhibited GABA release from presynaptic terminals

in cultured hippocampal or RVLM neurons following postsynaptic depolarization, a phenomenon termed depolarization induced suppression of inhibition or (DSI); this effect was completely abolished in presence of selective CB_1 receptor antagonists (Vaughan, McGregor et al. 1999; Ohno-Shosaku, Maejima et al. 2001).

It seems that postsynaptically-released endocannabinoids act as retrograde secondary messengers at both inhibitory, as well as, excitatory synapses. In support of this notion, findings from cerebellar Purkinje cells, which upon elevation of calcium levels following depolarization, release endocannabinoids, which stimulate presynaptic CB_1R and ultimately suppress presynaptic calcium-induced glutamate release (Kreitzer and Regehr 2001). The latter phenomenon is termed depolarization-induced suppression of excitation or (DSE). Both CB_1R mediated DSE and DSI are considered key mechanisms for many of the central effects of endogenous and exogenous cannabinoids. For this reason, it was proposed that central CB_1R-elicited sympathoexcitation is mediated via indirect stimulation of the tonically active presympathetic neurons in the RVLM regulated by an array of tonic excitatory and inhibitory inputs (Padley, Li et al. 2003). Although, this hypothesis has not been investigated *in vivo*, the findings that CB_1R activation inhibited GABAergic transmission in cultured RVLM neurons (Vaughan, McGregor et al. 1999) further support this hypothesis.

Fig. 1. 3. CB₁R-mediated retrograde signaling at both excitatory and inhibitory synapses through postsynaptically released endocannabinoids. 2-AG: 2-Arachidonoylglycerol; DSI: depolarization induced suppression of inhibition; DSE: depolarization induced suppression of excitation. Taken from (Scotter, Graham et al. 2009) with permission.
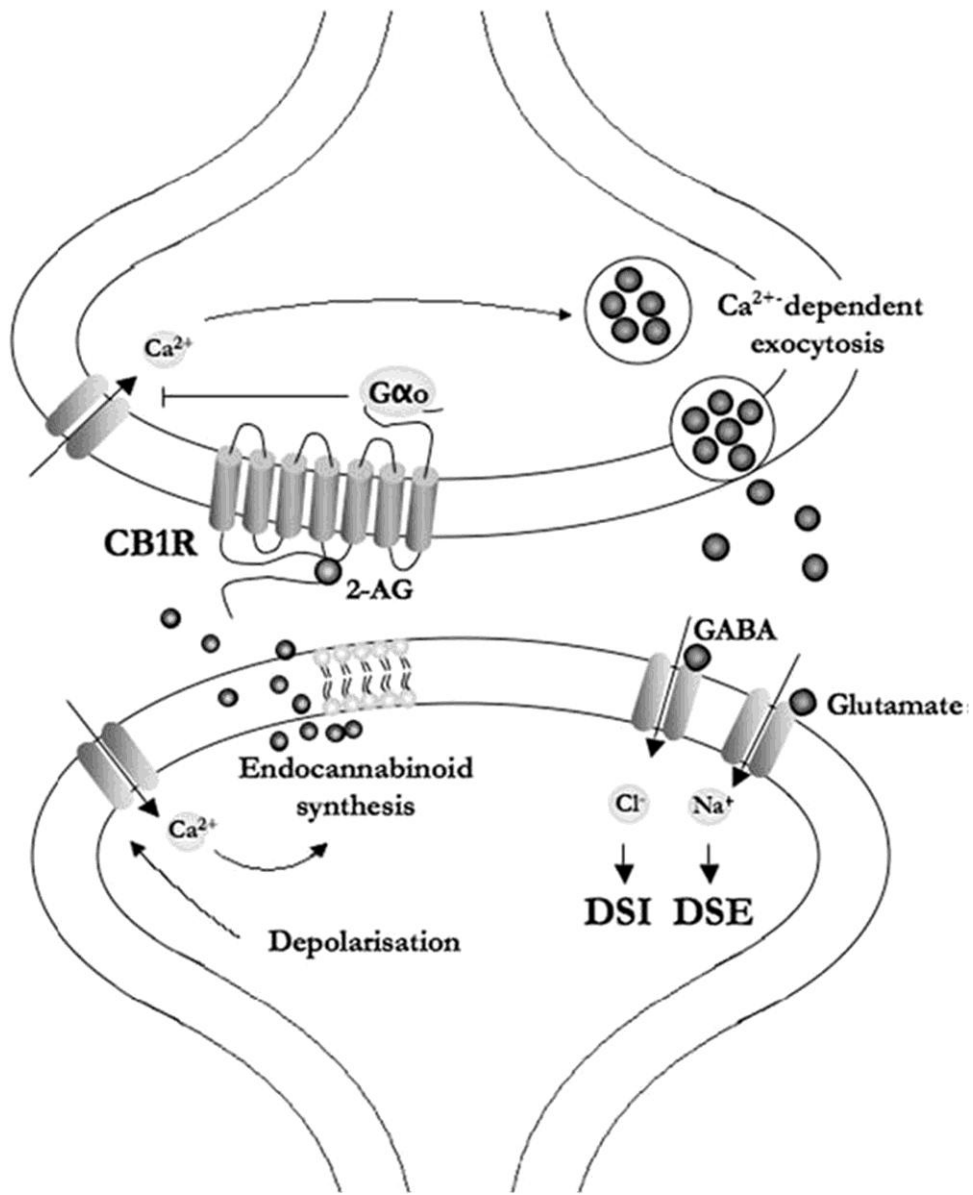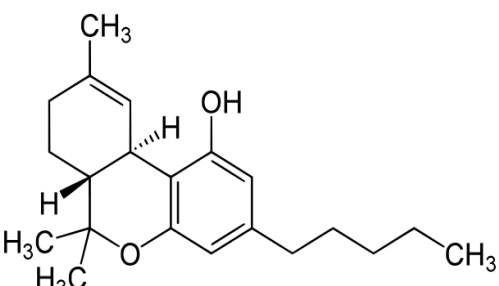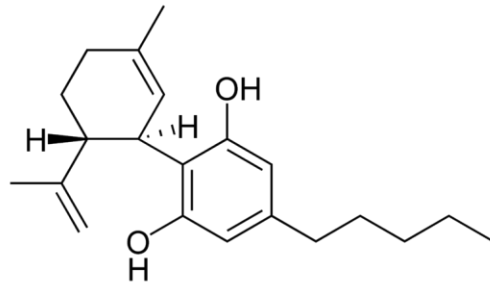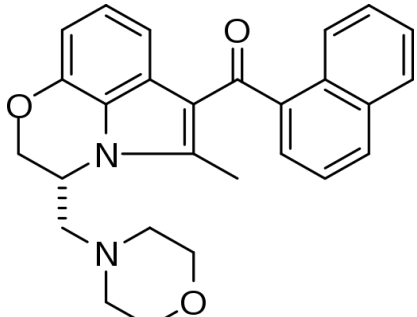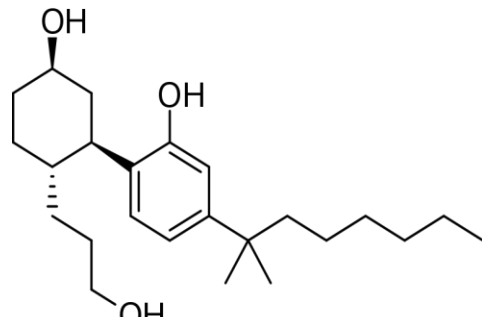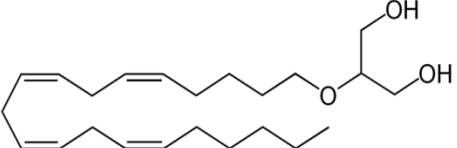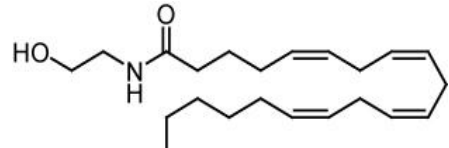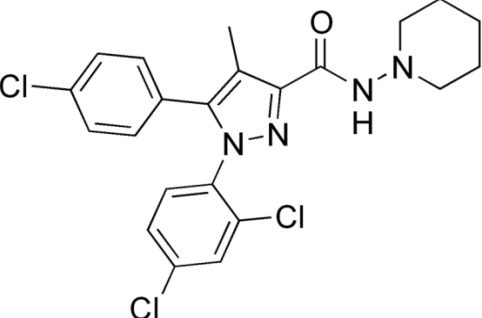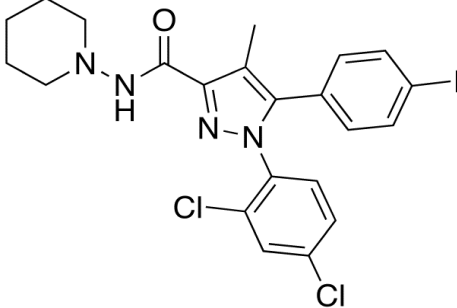
Table. 1.1. Representative phyto-, synthetic, and endogenous cannabinoid compounds.

WIN55,212-2 and AM251 were used in this studies.

Phytocannabinoids	Δ^9–tetra-hydrocannabinol	Cannabidiol (CBD)
Synthetic cannabinoids	WIN55,212-2	CP-55.940
Endogenous cannabinoids	2-Arachidonylglycrol (2-AG)	Anandamide
antagonist/inverse agonists	SR141716A (Rimonabant)	AM251
Peptide inverse agonist	Hemopressin (H-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-OH)	

1. 2. Cannabinoid Ligands

Cannabinoids are a heterogeneous group of compounds that include plant derived, naturally occurring and synthetic compounds (Table.1.1). Δ^9–THC, Cannabidiol (CBD) and cannabinol are the most abundant natural cannabinoids active at CB_1 and CB_2 receptors, present in the abused drug Marijuana (*Cannabis sativa*). Δ^9–THC has an equal affinity for both CB_1 and CB_2 receptors (Munro, Thomas et al. 1993). In 1992, an endogenous ligand for cannabinoid receptors was isolated from pig brain, arachidonoyl ethanolamide (AEA), a derivative of arachidonic acid, and was named anandamide (Devane, Hanus et al. 1992). 2-arachidonoyl glycerol (2-AG) is a more abundant endocannabinoid, but less potent than Δ^9–THC (Sugiura, Kodaka et al. 1999). Most of the endogenous cannabinoids discovered so far are high or low efficacy agonists. However, recently Pertwee et al. reported an inverse agonist called virodhamine after the Sanskrit word *virodh* meaning *oppose* (Porter, Sauer et al. 2002).

Pfizer was the first to develop high affinity non-eicosanoid cannabinoids (e.g. CP55940). Later, the aminoalkylindole cannabinoid, WIN55,212-2, extensively used in cannabinoid research, including our studies, was developed by a Sterling Winthrop. SR141716A and AM251, used in our study, are selective antagonists for the CB_1R while SR144528 is selective for the CB_2R (Rinaldi-Carmona, Barth et al. 1998). Notably, most of these compounds are highly lipophilic and water insoluble except for O-1057, which is highly water soluble and almost as potent as CP55940 (Pertwee, Gibson et al. 2000). Adding to the heterogeneous chemical composition of cannabinoids is the very recent discovery of the short peptide, hemopressin, which was identified in rat brain and acts on CB_1R as an inverse agonist (Heimann, Gomes et al. 2007; Dodd, Mancini et al. 2010).

1. 3. Brainstem Nuclei and Central Regulation of Cardiovascular Function

Many brainstem nuclei are implicated in the cardiovascular responses elicited by central CB_1R activation; in this study, attention has been focused on the rostral ventrolateral medulla (RVLM) and nucleus tractus solitarii (NTS), which play pivotal roles in central control of cardiovascular function (Strack, Sawyer et al. 1989; Dampney, Polson et al. 2003; Nassar and Abdel-Rahman 2006).

1. 3. 1. Rostral Ventrolateral Medulla (RVLM)

RVLM is the final supraspinal site within the central nervous system that integrates multitudes of influences on blood pressure (BP) from higher brain regions such as paraventricular nucleus, lateral hypothalamus, and periaqueductal gray (Guyenet, Haselton et al. 1989; Dampney, Polson et al. 2003). The RVLM is of high significance in controlling BP since bilateral lesioning leads to a profound fall in BP (Dampney, Czachurski et al. 1987).

1. 3. 1. 1. Location

The RVLM is located in the ventral part of the brainstem, lateral to the inferior olive, caudal to the facial nucleus, and ventral to the nucleus ambiguous (Farlow, Goodchild et al. 1984; Dampney, Czachurski et al. 1987). It is heterogeneous in composition containing multiple cell groups that are different in their neurochemical phenotype (e.g. rostroventrolateralis, gigantocellular nucleus and paragigantocellularis lateralis (Andrezik, Chan-Palay et al. 1981; Ruggiero DA, Cravo SL et al. 1989; Villanueva, de Pommery et al. 1991; Watanabe, Kitamura et al. 2003).

1. 3. 1. 2. Receptors and transmitters

Catecholamine containing neurons in the brainstem are divided into the noradrenergic groups (A1-A12) and adrenergic groups (C1-C3), which can be identified by tyrosine hydroxylase (TH), the enzyme that converts tyrosine into dopa (Hokfelt T., Johansson O. et al. 1984). Within the RVLM, C1 neurons, alternatively known as adrenergic neurons, are defined based on their expression of phenylethanolamine-n-methyltransferase (PNMT), the enzyme that synthesizes epinephrine from norepinephrine (Ross, Ruggiero et al. 1984; Jeske and McKenna 1992). The noradrenergic neurons are categorized into two groups: the A1 cell group located in the rostral ventromedial medulla oblongata, and the A2 group located in the dorsal vagal nucleus (Hokfelt T., Johansson O. et al. 1984). The rostral C1 subgroup contains barosensitive neurons with axonal projections to the spinal cord (Kanjhan, Lipski et al. 1995; Schreihofer and Guyenet 1997), which provides tonic excitatory inputs to the sympathetic preganglionic neurons (Guyenet, Koshiya et al. 1996; Guyenet 2006). On the other hand, caudal C1 subgroup projects to the hypothalamus and forebrain regions (Tucker, Saper et al. 1987; Verberne, Stornetta et al. 1999). Beside catecholamine-containing neurons in RVLM (Goodchild, Phillips et al. 2001), a wide variety of neurotransmitters and receptors are present in the RVLM including substance P (Pilowsky, Minson et al. 1986), neuropeptide Y (Polson, Halliday et al. 1992), enkephalin (Polson, Halliday et al. 1992; Boone and Corry 1996), adenosine receptors (A_{2A}) (Nassar and Abdel-Rahman 2008), P_2X receptors (Ralevic 2000), Angiotensin II AT_1 receptors (Potts, Allen et al. 2000), imidazoline I_1 receptors (Zhang and Abdel-Rahman 2002; Zhang and Abdel-Rahman 2005), α_{2A} adrenergic receptors (El-Mas and Abdel-Rahman 2004; Li, Wang et al. 2005), cannabinoid CB_1 receptors (Herkenham, Lynn et al. 1991; Padley, Li et al. 2003), CB_2

receptors (Van Sickle, Duncan et al. 2005) and mu-opioid receptors (Aicher, Kraus et al. 2001; Drake, Aicher et al. 2005).

1. 3. 1. 3. Function

The RVLM is a crucial brainstem nucleus for the tonic generation of sympathetic nerve activity (Ross, Ruggiero et al. 1985; Dampney, Czachurski et al. 1987). Activation of specific neurons within the RVLM causes an increase in BP by increasing peripheral resistance and cardiac output via released catecholamines (Guertzenstein 1972; Guertzenstein and Silver 1974; Feldberg and Guertzenstein 1976; McAllen and Dampney 1989). In addition to cardiovascular control, specific neurons within the RVLM are involved in nociception (Javanmardi, Parviz et al. 2005; Karlsson, Preuss et al. 2006) and breathing (Nattie and Li 1990).

1. 3. 2. Nucleus Tractus Solitarii (NTS)

The NTS is the first relay station in the baroreflex arc; upon stimulation, it elicits a reduction in the BP, HR and sympathetic outflow (Miura and Reis 1972; Aicher and Randich 1990). The NTS is located in the brainstem flanked on each side of the fourth ventricle and consists of groups of cells in a column-like structure dorsal to the RVLM. The most cardiovascularly relevant part of the NTS is located at the most caudal part of the NTS containing synapses from chemo and aortic baroreceptor processes that contact with secondary order neurons within the NTS (Nomura and Mizuno 1982; Seiders and Stuesse 1984). The latter communicate either directly or indirectly through third order neurons with other nuclei including. RVLM, hypothalamus or CVLM (Ross, Ruggiero et al. 1985; Dampney, Czachurski et al. 1987; Aicher, Kurucz et al. 1995; Aicher, Saravay et al. 1996). Functionally, activation of

cardiovascular afferents (chemo or baroreceptors) enhances the release of excitatory amino acid L-glutamate within the NTS (Miura and Reis 1972), which prompts the excitation of NTS-projections to other baroreflex arc nuclei e.g. RVLM and CVLM. Several reports have shown important roles for activation of CB_1R in the NTS in blood pressure regulation (Van Sickle, Oland et al. 2001; Rademacher, Patel et al. 2003; Seagard, Dean et al. 2004; Seagard, Hopp et al. 2005).

1. 4. CB_1R-Mediated Cardiovascular Effects

Cannabinoids elicit complex cardiovascular responses involving both peripheral and central sites. The presence of anesthesia has dramatically confounded these responses (Lake, Martin et al. 1997) and introduced additional parameters to be considered upon investigation of the CB_1R role in controlling cardiovascular functions; in particular the central part. For this reason, the cannabinoid-evoked cardiovascular effects in anesthetized animals are significantly different when compared to responses reported in studies conducted in conscious animals or in humans.

1. 4. 1. Systemic CB_1R-evoked cardiovascular effects

In anesthetized animals, systemically administered cannabinoids elicit predominantly hypotension and bradycardia. These effects are mediated peripherally through prejunctional inhibition of sympathetic outflow and vagal stimulation resulting in reduction of BP and HR, respectively (Varga, Lake et al. 1995; Varga, Lake et al. 1996; Lake, Compton et al. 1997; Niederhoffer and Szabo 1999). Systemic administration of anandamide, an endogenous cannabinoid or the synthetic cannabinoid, WIN55,212-2, elicited tri-phasic effects on BP in

anesthetized rats. An initial brief hypotensive phase, secondary to the bradycardia and was blocked by atropine pretreatment or vagotomy, followed by a transient pressor response, which was followed by more predominant depressor phase. The prolonged depressor phase was mediated via peripheral sympathoinhibition because it was attenuated by cervical spinal transection and blockade of α-adrenoceptors (Varga, Lake et al. 1995; Varga, Lake et al. 1996; Lake, Compton et al. 1997; Niederhoffer and Szabo 1999).

The cardiovascular responses of systemically administered cannabinoids in conscious animals are quite different. Systemically injected anandamide or WIN55,212-2 in conscious rats cause pressor responses along with bradycardic responses (Stein, Fuller et al. 1996; Gardiner, March et al. 2001). The elicited pressor response by systemic WIN55,212-2 in conscious animals was attenuated by ganglion blockade, providing evidence for a central site of action for the pressor response. However, in the presence of anesthesia the latter response was altered (Lake, Martin et al. 1997). Importantly, in humans, acute administration of cannabinoid is associated with tachycardia and a rise in BP (Benowitz, Rosenberg et al. 1979; Foltin, Fischman et al. 1987; Sidney 2002).

1. 4. 2. Central CB$_1$R-evoked cardiovascular effects

Centrally administered cannabinoids have been shown to predominantly elicit sympathoexcitation/pressor responses. This pressor response seems to involve sympathetic neurons in the (RVLM) because: (i) intracisternal (i.c) CB$_1$ receptor agonists (WIN55,212-2 or CP-55940) caused increases in sympathetic nerve activity, plasma norepinephrine and blood pressure, in conscious rabbits (Niederhoffer and Szabo 2000) and anesthetized rats (Pfitzer,

Niederhoffer et al. 2004) that was attenuated by pretreatment with the CB_1R antagonist SR171416A; (ii) microinjection of WIN55,212-2 into RVLM elicited a pressor response (Padley, Li et al. 2003). However, a recent study showed that in anesthetized animals, intracerebroventricular (i.c.v.) anandamide elicited pressor responses only in the presence of a CB_1R antagonist; while alone, anandamide elicited a tri-phasic blood pressure response similar to that induced by systemically administered anandamide (Malinowska, Zakrzeska et al. 2010).

1. 5. Orexin Peptides and Receptors

Orexins are a family of two peptides, the 33 amino acid orexin-A (hypocretin-1) and the shorter 28 amino acid orexin-B (hypocretin-2), derived through proteolytic cleavage from the precursor protein, prepro-orexin (PPO). Orexins were simultaneously discovered by two different groups (Sakurai, Amemiya et al. 1998) and (de Lecea, Kilduff et al. 1998) and was dubbed orexin by the former group after the Greek word 'orexis', meaning appetite and hypocretin by the latter group because of some homology with the secretin/incretin family of peptides (de Lecea, Kilduff et al. 1998). Orexins were initially reported to be exclusively located in the lateral hypothalamus (LH), yet, orexin axons were found in other brain areas including the RVLM (Machado, Bonagamba et al. 2002; Ciriello, Li et al. 2003).

Two GPCRs, Orexin 1 (OX_1R) and Orexin 2 (OX_2R) mediate the actions of orexins (Sakurai, Amemiya et al. 1998). While OX_1R has higher affinity for orexin-A, OX_2R binds orexin-A and orexin-B with similar affinities (van den Pol, Gao et al. 1998; Smart, Jerman et al. 1999). OX_1R couples to the $G_{q/11}$ G proteins, while OX_2R coupled to $G_{q/11}$ or $G_{i/o}$ in a neuronal cell line (Zhu, Miwa et al. 2003).

In addition to their role in regulation of feeding and sleep/wakefulness (Hara, Beuckmann et al. 2001; Farr, Banks et al. 2005; Verty, Boon et al. 2009), orexins have also been shown to be essential regulators of many autonomic functions, including central cardiovascular and respiratory regulation (Zhang, Fukuda et al. 2005; Zheng, Patterson et al. 2005; Shih and Chuang 2007; Huang, Dai YW et al. 2010).

1. 6. Therapeutic Potentials of Cannabinoids

Clinical assessment of existing or investigational CB_1R ligands is the focus of a worldwide research effort that affects the public health with regard to many diseases. The pharmacological modulations of cannabinoid and orexin systems are considered to be promising therapeutic strategies for many pathological conditions (e.g. obesity, metabolic syndrome, anorexia, and pain). Because of their well-documented antinociceptive action, cannabinoid receptors are being considered as therapeutic targets for pain relief. Sativex®, a mixture of Δ^9-THC and CBD developed by GW Pharmaceuticals, was approved in Canada for the treatment of neuropathic pain in multiple sclerosis. Further, due to the potent anti-emetic effects of cannabinoids, many pharmaceuticals were produced (e.g. Marinol® and Cesamet®), containing Δ^9-THC and its analogue nabilone and marketed as medicines for suppressing chemotherapy associated nausea and vomiting. Many of these drugs are also used as appetite stimulants in AIDS patients. Cannabinoids have many other potential uses in Parkinson's disease, Alzheimer's disease, inflammation, fertility, epilepsy and alcohol withdrawal (Alen, Moreno-Sanz et al. 2008; Morrish, Hill et al. 2009; de Bruin, Prickaerts et al. 2010; Dudek, Pouliot et al. 2010; Walsh, Mnich et al. 2010). SR141716A (Rimonabant) was approved under the trade name Acomplia® in the European Union, as an anti-obesity agent. However, the use of many of these drugs has

been associated with psychiatric side effects, presumably mediated by the central actions on CB$_1$R. This led to the breakthrough development of peripherally "restricted" cannabinoid ligands that possess very limited central access and centrally-mediated side effects. For instance, AZ11713908 is a new bivalent chemical entity tested for pain relief in rodents with limited central access (Xiao Hong, Chang Qing et al. 2010). In addition, the physiological role of cannabinoids and orexins in blood pressure control highlights their importance in cardiovascular disease management. The findings of the proposed research will yield neurobiological and pharmacological insight into the crosstalk between CB$_1$R and OX$_1$R *in vivo*. In an effort to understand the interaction between these two systems, Thomas, B. F. et al, at the Research Triangle Institute in North Carolina, is developing bivalent ligands that target the GPRC homodimeric (Zhang, Gilliam et al. 2010) or heterodimeric structures including the CB$_1$R and OX$_1$R heterodimer by using existing or newly developed ligands e.g. SR141716, a CB$_1$R antagonist and ACT-078573 OX$_1$R antagonist linked together by a spacer (Zhang, Perrey et al. 2010).

1.7. Aims of Study

Studies on the cardiovascular effects of cannabinoids have yielded controversial results. While CB_1R activation causes hypotension in anesthetized animals, it elicits a pressor response in conscious animals (Stein, Fuller et al. 1996; Gardiner, March et al. 2001; Randall, Harris et al. 2002; Randall, Kendall et al. 2004; Mendizabal and Adler-Graschinsky 2007). The latter findings replicate the responses reported in humans (Benowitz, Rosenberg et al. 1979; Foltin, Fischman et al. 1987; Sidney 2002). This pressor response involves central mechanisms and probably implicates sympathetic neurons in RVLM (Niederhoffer and Szabo 2000; Padley, Li et al. 2003). However, the mechanisms that underlie the central CB_1R mediated pressor response are still not clearly resolved.

It has been shown that CB_1R modulates synaptic transmission of many neurotransmitters via retrograde mechanism (Kreitzer and Regehr 2001; Ohno-Shosaku, Maejima et al. 2001; Freund, Katona et al. 2003; Piomelli 2003; Drew, Mitchell et al. 2008). Pertinent to the current studies, behavioral (feeding) and molecular findings corroborated the demonstration of an interplay between CB_1R and OX_1R signaling in a number of studies (Hilairet, Bouaboula et al. 2003; Huang, Acuna-Goycolea et al. 2007; Crespo, Heras et al. 2008; Matias, Cristino et al. 2008). Notably, studies on OX_1R signaling have demonstrated the ability of an orexin-A, endogenous OX_1R ligand, to elicit a sympathoexcitation/pressor response (Samson, Gosnell et al. 1999; Shirasaka, Nakazato et al. 1999; Chen, Hwang et al. 2000; Machado, Bonagamba et al. 2002; Ciriello, Li et al. 2003; Zhang, Fukuda et al. 2005). However, the possibility has not been considered that functional crosstalk between CB_1R and OX_1R/orexin-A in the brainstem (RVLM) might be involved in the blood pressure response elicited by the activation of either

receptor *in vivo*. Therefore, ***the present studies tested the novel hypothesis that OX$_1$R/orexin-A signaling in the RVLM is essential for the central CB$_1$R-mediated pressor response.*** Additionally*,* the current study tested the hypothesis that ***modulation of PI3K/Akt and/or ERK1/2 signaling contributes to the central CB$_1$R-mediated pressor response***. Our studies have, further elucidated the role ***of GABA$_A$R/p-nNOS/NO signaling in these responses***. Pharmacological, signal transduction and biochemical studies were undertaken in conscious normotensive rats, under the following interrelated aims:

Specific Aim 1 studies characterized the CB$_1$R-evoked sympathoexcitation/pressor response in conscious rats and tested the hypothesis that the central CB$_1$R-evoked pressor/ sympathoexcitation response involves activation of rat RVLM neurons.

Rationale: Anesthesia confounds the cannabinoid evoked hemodynamic responses (Stein, Fuller et al. 1996; Lake, Martin et al. 1997; Gardiner, March et al. 2001). To our knowledge, only one study investigated the central CB$_1$R-evoked hemodynamic responses in conscious rabbits (Niederhoffer and Szabo 2000) and no other studies have reported such findings in conscious rats (Pfitzer, Niederhoffer et al. 2004). Thus, the current studies aimed to investigate the hemodynamic responses of activation of brainstem CB$_1$R in conscious rats to circumvent the negative impacts of anesthesia on CB$_1$R-mediated hemodynamic effects (Lake, Martin et al. 1997). This pressor response is centrally mediated and possibly involves sympathetic neurons in the (RVLM) (Niederhoffer and Szabo 2000; Padley, Li et al. 2003). Therefore, a major goal of this study was to elucidate whether RVLM neurons are implicated in the CB$_1$R-evoked sympathoexcitation/pressor response. This was achieved by quantifying the expression of the immediate early gene c-Fos protein product as a marker of sympathetic neuron activity (Hirooka,

Polson et al. 1997; Horiuchi, Potts et al. 1999; Dampney, Polson et al. 2003) and addressing the following questions:

1- Is the CB_1R (protein) expressed in the RVLM?

2- What are the effects of CB_1R agonist, WIN55,212-2 (3, 10, 30 μg/rat, i.c) on BP, HR and plasma NE in conscious freely moving rats?

3-What are the effects of WIN55,212-2 (15 μg/rat, i.c) on BP, HR, plasma NE and RVLM-c-Fos expression in presence or absence of the CB_1R antagonist, AM251?

Specific Aim 2 studies tested the hypothesis that OX_1R/orexin-A signaling in the RVLM is pivotal for the central CB_1R-mediated pressor response in conscious rats.

Rationale: Recent studies have suggested a crosstalk between CB_1R and OX_1R signaling. For example, functional relevance of the central interaction between the two receptors has been shown in feeding behavioral studies where orexin-A induced-feeding was abrogated by pretreatment with sub-anorectic doses of the CB_1R antagonist SR141716A (Crespo, Heras et al. 2008). Not only CB_1R and OX_1R activation triggers similar downstream signaling targets e.g. ERK1/2 phosphorylation; intriguingly, the CB_1R antagonist SR-141716A, that does not have affinity for OX_1R, attenuated the OX_1R-evoked increase in pERK1/2 in cells co-expressing the two receptors, inferring a reciprocal physical interaction (macromolecule formation) between the two receptors (Hilairet, Bouaboula et al. 2003; Ellis, Pediani et al. 2006). Pertinent to the proposed hypothesis central administration of CB_1R or OX_1R agonists, elicits pressor response (Niederhoffer and Szabo 1999; Chen, Hwang et al. 2000; Niederhoffer and Szabo 2000; Zhang,

Fukuda et al. 2005). Although, orexin-A-containing neurons are exclusive to the hypothalamus, orexin-A immunoreactive (orexin-A-ir) fibers, were found in close proximity to catecholamine immunoreactive (CAir) neurons in the RVLM (Machado, Bonagamba et al. 2002; Ciriello, Li et al. 2003). Expression of the CB_1R exhibits similar patterns in RVLM (Herkenham, Lynn et al. 1991; Padley, Li et al. 2003). Collectively, these reported findings highlight the possibility that functional crosstalk between CB_1R and OX_1R/orexin-A in the RVLM might be involved in the blood pressure response elicited by the activation of either receptor *in vivo*. Studies under this aim addressed the following specific questions:

1- Does central CB_1R activation (WIN55,212-2) elevate RVLM-orexin-A levels?

2- What are the BP and HR effects of graded doses of the OX_1R agonist, orexin-A (0.3, 1, 3 nmol/rat, i.c) in conscious freely moving rats?

3- Does central OX_1R blockade (SB-408124) attenuate orexin-A- and WIN55,212-2-centrally-mediated cardiovascular effects?

4- Does central CB_1R blockade (AM251) attenuate orexin-A-mediated cardiovascular effects?

Specific Aim 3 studies tested the novel hypothesis that PI3K/Akt-ERK1/2 signaling is pivotal for the central CB_1R-mediated sympathoexcitation/pressor response.

Rationale: The cellular mechanism(s) underlying the sympathoexcitatory/pressor effects of central CB_1R activation are understudied. Cellular and behavioral (feeding) studies identified a crucial role for the PI3K/Akt and ERK1/2 pathway in CB_1R-evoked pharmacological responses.

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For instance, it was demonstrated that CB_1R activation inhibits both PI3K/Akt and ERK1/2 signaling in some *in vitro* studies (Ellert-Miklaszewska, Kaminska et al. 2005; Greenhough, Patsos et al. 2007). In contrast, CB_1R activation caused rapid ERK1/2 phosphorylation via PI3K pathway in some cell lines, e.g. U373 MG human astrocytoma and prostate cells (Galve-Roperh, Rueda et al. 2002; Sanchez, Ruiz-Llorente et al. 2003). Further, systemic THC activated the PI3K/Akt pathway in mouse hippocampus, striatum, and cerebellum via a mechanism that does not involve activation of ERK1/2. Brainstem PI3K/Akt and ERK1/2 signaling play a tonic role in central control of blood pressure. It has been shown that prolonged inhibition of ERK1/2 phosphorylation in the RVLM gradually lowered blood pressure in both normotensive and hypertensive rats (Seyedabadi, Goodchild et al. 2001). Moreover, activation of ERK1/2 signaling in the RVLM underlies Angiotensin II-mediated pressor responses (Chan, Hsu et al. 2005; Chan, Wang et al. 2007). Notably, PI3K/Akt signaling evokes a depressor response and its activation in NTS mediated insulin-evoked hypotensive and bradycardic responses (Huang, Lu et al. 2004). Importantly, it was shown that brainstem (RVLM and NTS) and hypothalamic (PVN) PI3K pathway is elevated in spontaneous hypertensive rats (SHR) compared to the normotensive counterparts, Wister-Kyoto rats (WKY) (Veerasingham, Yamazato et al. 2005; Zubcevic, Waki et al. 2009). In the latter study, chronic blockade of PI3K in the NTS resulted in exacerbation of hypertension in SHR but not WKY, suggesting a restraining role (depressor) for the PI3K pathway on BP in SHR. Collectively, the above discussed cellular and behavioral studies raised the interesting possibility that modulation of PI3K/Akt and or ERK1/2 signaling might underlie central CB_1 receptor-mediated pressor response. Therefore, studies under this aim addressed the following specific questions:

1- Does i.c. WIN55,212-2 (15 µg/rat, i.c) enhance ERK1/2 and Akt phosphorylation in the rat RVLM and NTS?

2- What are the effects of PI3K inhibition (wortmannin) on the CB_1R-evoked (WIN55,212-2) dose-related pressor response?

3- What are the effects of inhibition of ERK1/2 phosphorylation on the CB_1R-evoked (WIN55,212-2) pressor effect?

4- What are the effects of i.c. WIN55,212-2 on ERK1/2 and Akt phosphorylation in the RVLM and NTS of rats pretreated with the CB_1R antagonist, AM251, the PI3K inhibitor, wortmannin or the ERK1/2 inhibitor, PD98059?

Specific Aim 4 elucidated the novel hypothesis that the central CB_1R-evoked pressor response is mediated via modulation of GABAergic neurotransmission in the brainstem. Studies under this aim were further expanded to investigate whether RVLM-p-nNOS/NO signaling is implicated in central CB_1R-evoked pressor response.

Rationale: Multiple lines of evidence has shown that that postsynaptically-released endocannabinoids act as retrograde secondary messengers at both inhibitory, as well as, excitatory synapses, to inhibit the release of GABA and glutamate, respectively (Vaughan, McGregor et al. 1999; Kreitzer and Regehr 2001; Ohno-Shosaku, Maejima et al. 2001). Therefore, Padley et al. and others have proposed that RVLM CB_1R-evoked sympathoexcitation is, mediated via disinhibition of the tonic inhibitory (GABAergic) neurotransmission in the RVLM, which consequently results in increased central sympathetic outflow (Randall, Harris et

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al. 2002; Padley, Li et al. 2003). To our knowledge, this was only tested *in vitro*, by Vaughan et al. in cultured RVLM neurons, where CB_1R activation inhibited the release of GABA (Vaughan, McGregor et al. 1999). Whether modulation of GABAergic transmission mediated by CB_1R activation, has a significant role in the central CB_1R-evoked pressor response had not been investigated.

CB_1 receptor-mediated effects have been shown to involve activation of the neuronal nitric oxide synthase- nitric oxide (nNOS-NO) signaling pathway (Azad, Marsicano et al. 2001; Jones, Carney et al. 2008). Furthermore, studies on hippocampus suggest that NO is involved in depolarization-induced CB_1R-mediated suppression of IPSCs as a retrograde signal molecule and that operation of this cascade is conditional on cholinergic receptor activation (Makara, Katona et al. 2007). Pertinent to the current investigation, nNOS-NO signaling in the RVLM has been shown to be critically implicated in the central regulation of autonomic functions (Martins-Pinge, Araujo et al. 1999; Martins-Pinge, Garcia et al. 2007; Mayorov 2007; Nassar and Abdel-Rahman 2008). Yet, it remains unclear whether p-nNOS/NO signaling is implicated in the CB_1R-evoked pressor response. Studies under this aim investigated the hypothesis that $GABA_A$ receptor/p-nNOS/NO signaling is crucial to cardiovascular effects mediated via central CB_1 receptor activation by addressing the following questions:

1- What are the effects of inra-RVLM microinjection of WIN55,212-2 on BP, HR, RVLM p-nNOS and NO?

2- What are the effects of $GABA_AR$ stimulation (muscimol) on the central CB_1R-mediated effects on BP, HR and activity of catecholaminergic and nitroxidergic neurons?

CHAPTER TWO- MATERIALS AND METHODS

2. 1. Preparation of the Rats

Male Sprague-Dawley rats, weighing 300-350 g (Charles River, Raleigh, NC) were received and housed two per cage in a room with controlled environment at a constant temperature of $23 \pm 1°C$, humidity of $50\% \pm 10\%$ and a 12:12-h light/dark cycle. Food (Prolab Rodent Chow, Prolab RMH 3000; Granville Milling, Creedmoor, NC) and water were provided ad libitum. All surgical and animal care procedures were performed in accordance with and approved by the Institutional Animal Care and use committee and in accordance with the Institute of Laboratory Animal Resources, (2010) Guide for the Care and Use of Laboratory Animals 8th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, D.C.

2. 1. 1. Intra-arterial catheterization and intracisternal cannulation

Intra-arterial catheterization and intracisternal cannulation (i.c) were performed as in reported studies by Abdel-Rahman et al., (Zhang and Abdel-Rahman 2005; Nassar and Abdel-Rahman 2008). Five days before the experiment, rats were anesthetized with i.p ketamine (90 mg/kg) and xylazine (10 mg/kg). A polyethylene catheter (PE50 connected to PE10) was placed in the abdominal aorta via the femoral artery for BP measurement. The catheters were advanced 5 cm into the femoral vessels and secured with sutures. Finally, the catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. The catheters were flushed with heparin in saline (200 U/ml) and plugged by stainless steel pins. For (i.c) the head was placed in a David Kopf stereotaxic frame and a stainless steel guide cannula (23G; Small

Parts, Miami, FL) was implanted into the cisterna magna. The dura matter covering the foramen magnum was exposed. The guide cannula was passed between the occipital lobe and the cerebellum through a hole drilled 1 to 1.5 mm distal to the caudal edge of the occipital bone so that the guide cannula tip protruded into the cisterna magna. The cannula was secured in place with small metal screws and dental acrylic cement (Durelon; Thompson Dental Supply, Raleigh, NC). The patency of the guide cannula was verified when a spontaneous flow of cerebrospinal fluid was observed and by gross post-mortem histological verification after routine injection of 2 μL of fast green dye (EM Sciences, Cherry Hill, NJ) at the end of the experiment. Incisions were closed by surgical staples and swabbed with povidone-iodine solution. Each rat received an intramuscular injection of 30,000U of penicillin G benzathine and penicillin G procaine in aqueous suspension (Durapen) and a subcutaneous injection of buprenorphine hydrochloride (Buprenex, 30 μg/kg) and was housed in a separate cage.

2. 1. 2. Blood pressure (BP) and heart rate (HR) measurements

On the day of experiment, arterial catheter was flushed with heparin 100 IU/ml saline and connected to a Gould-Statham pressure transducer (Oxnard-CA). BP was recorded by ML870 (PowerLab 8/30), and analyzed using LabChart (v. 6) pro software (ADInstruments, Colorado Spring, CO). Data were continuously recorded and stored for offline analysis. Heart rate readings were extracted from BP recordings using the LabChart (v.6) blood pressure analysis module. Under these settings, four animals were connected simultaneously, each receiving different treatment for optimal comparisons. BP and HR were allowed to stabilize for at least 60 min;data collected during the last 30 min represented basal MAP and HR.

2. 2. Immunofluorescence

2. 2. 1. Preparation of tissues

A modified protocol similar to that previously reported (Wang and Abdel-Rahman 2005; Nassar and Abdel-Rahman 2006; Matias, Cristino et al. 2008) was used for brain tissues preparation before being processed for immunohistochemistry (IHC) or immunofluorescence (IF). At the conclusion of hemodynamic measurements, unless otherwise indicated, the rats were deeply anesthetized by sodium pentobarbital (i.p.), transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min with approximately 400 mL after flushing with ice-cold saline (100 mL). Brains were removed, and placed in the same fixative buffer for 24 h, then transferred to 30% sucrose in PBS solution, pH 7.4 and kept until they sank. Brains were then either directly sectioned or embedded in OCT and then flash frozen in 2-methylbutane (cooled on dry ice for at least 30 min). Serial coronal frozen sections of 30 μm thickness of brainstem containing RVLM, rostrally from -12.8 to -11.8 mm, relative to bregma (Paxinos and Watson 2005) were cut at -24°C with a microtome cryostat (HM 505 E; Microm International GmbH, Walldorf, Germany) and collected in each well of a cell culture plate containing ice-cold PBS. Free-floating sections were then processed for (IF) or (IHC) whereas, for long-term storage, sections were kept at (–20°C) in cryoprotectant solution (50% PBS, 30% ethylene glycol, 20% glycerol).

2.2. 2. Staining and image acquisition

Free floating sections, prepared as above, were then washed 3X in TBS for 15 min, and incubated for 3 h in blocking buffer (1% bovine serum albumin, 5% normal donkey serum in

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TBS containing 0.1% Triton X-100; TBST) at RT with continuous shaking, before they were incubated 48-72 h at 4ºC with shaking in specific antibody or antibodies mixture diluted in blocking buffer as indicated for each experiment. After being washed 3X in TBST, immunofluorescence was revealed by incubation for 2 h in fluorescent (e.g. FITC, Cy3 or Cy5) conjugated donkey anti IgG .

Fig. 2.1. Schematic of coronal section in rat brainstem showing sites of rostral ventrolateral medulla (RVLM), nucleus tractus solitarii (NTS) and adjacent anatomical landmarks used to identify each nucleus at -12.80 mm from the bregma. 4V: fourth ventricle; AmbC: ambiguus nucleus, combact part; Bo; Botzinger complex; C1: adrenaline cells; IOPr: Inferior Olive, principle nucleus; py: Pyramidal tract; solDL and solV: solitary tract dorsolateral and ventral part, respectively. Adapted from (Paxinos and Watson 2005) with

Bregma -12.8
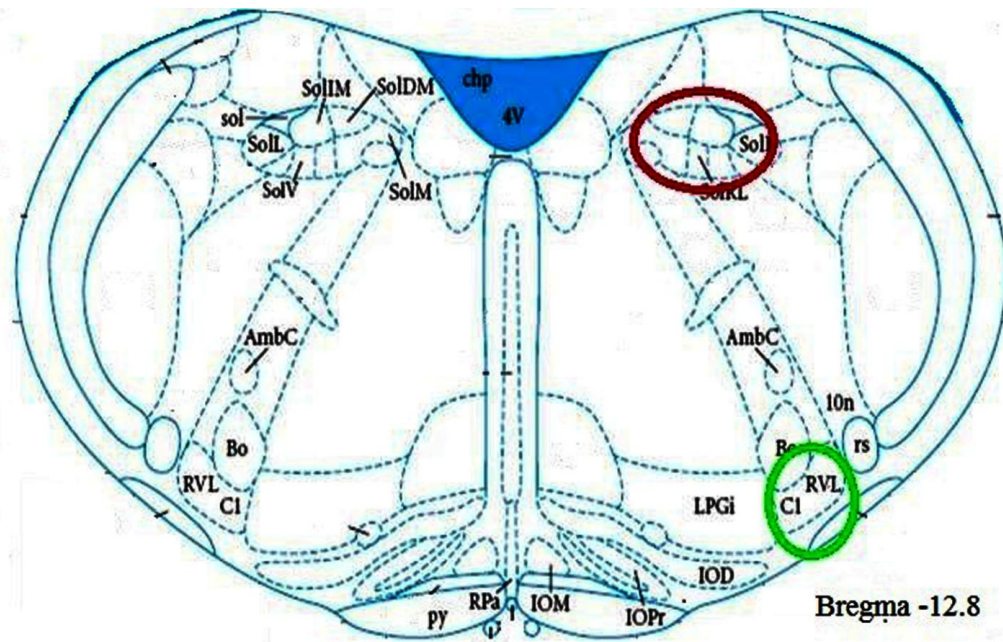
Sections were washed twice in TBS in dark and mounted on slides and coverslipped with Vectashield mounting medium containing DAPI as counterstain (Vector Laboratories, Burlingame, CA) and left in dark overnight to harden. Images were acquired by multi-track acquisition mode to eliminate channels-cross talk, if needed, using confocal laser microscopy (Carl Zeiss LSM 510, Thornwood, New York). Representative images were edited by the Zeiss LSM Image Browser software (v 4.2) and Adobe Photoshop (v. CS4, Adobe Systems, San Jose, CA), where only image brightness and contrast only were adjusted for clarity.

2. 3. Western Blot Analysis.

A modified protocol from recent study (Bender and Abdel-Rahman 2009) was employed. Generally, at the conclusion of hemodynamic measurements, animals received a lethal dose of sodium pentobarbital (i.p.), brains were removed, flash frozen in 2-methylbutane (cooled on dry ice for at least 30 min), and stored at -80ºC until use. Brains were equilibrated to -20ºC and sectioned with a cryostat (HM505E; Microm International GmbH, Waldorf, Germany) rostrally to the NTS and RVLM according to atlas coordinates. Tissue from both nuclei was collected bilaterally using a 0.75 mm punch instrument (Stoelting Co., Wood Dale, IL), approximately -12.8 to -11.8 or -13.92 to -12.84 mm, respectively, relative to bregma according to atlas coordinates (Paxinos and Watson 2005). Tissue was homogenized on ice by sonication in cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM activated sodium orthovanadate) containing protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). After centrifugation at 4ºC at 10,600g for 20 min, protein in the supernatant was quantified using a standard Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA). Equal

amounts of protein were denatured at 97ºC for 5 to10 min, separated on NuPAGE Novex Bis-Tris 4 to 12% SDS-PAGE gels (Invitrogen, Carlsbad, CA) using MOPS NuPAGE running buffer, and electroblotted to nitrocellulose membranes in semi-dry transfer cell TRANS-BLOT SD (Bio-Rad Laboratories, Hercules, CA) using transfer buffer (containing 230 mM glycine, 25 mM Tris, 0.7 mM SDS, 20% methanol) at 26 volts for 1 hr. Nonspecific binding sites on the membranes were blocked at room temperature using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hr. Membranes were then incubated with a selected specific antibody according to experimental design (see methods under individual experiments).

CHAPTER THREE-CHARACTERIZATION OF CANNABINOID RECEPTOR AGONIST WIN55,212-2 CENTRALLY EVOKED SYMPATHOEXCITATION/PRESSOR RESPONSE IN CONSCIOUS RATS; INVOLVEMENT OF ROSTRAL VENTROLATERAL MEDULLA NEURONS

3. 1. Abstract

Previous studies on the cardiovascular effects of cannabinoids, mainly via cannabinoid receptor 1 (CB$_1$R), have yielded controversial results perhaps due to the use of anesthesia. Therefore, it was important to investigate the central cardiovascular effects of the cannabinoid agonist WIN55,212-2 in conscious Sprague-Dawley rats to circumvent the confounding effects of anesthesia on these responses. Intracisternal (i.c) administration of WIN55,212-2 (3, 10, 30 μg/rat) in conscious freely moving rats (n=4) dose-dependently elevated mean arterial pressure (MAP), reduced heart rate (HR) and increased plasma norepinephrine (NE) levels; the latter reflects central sympathoexcitation. Further, the responses evoked by a median dose of WIN55,212-2 (15 μg/rat, i.c) were abolished by the selective CB$_1$R antagonist AM251 (30 μg/rat, i.c) indicating CB$_1$R-mediated effects. Further, we measured the catecholaminergic (tyrosine hydroxylase immunoreactivity, TH-ir) neuronal activity, indirectly via a marker of neuronal activity, c-Fos, within the RVLM following activation of CB$_1$R in the presence or absence of AM251. WIN55,212-2 (15 μg/rat, i.c) significantly ($P < 0.05$) increased the percentage of TH-ir and nNOS-ir neurons expressing c-Fos; AM251 pretreatment mitigated the increase in RVLM-catecholaminergic neuronal activity. Taken together, these findings, highlight a pivotal role for the RVLM in the central WIN55,212-2 evoked pressor response in conscious rats.

3. 2. Introduction

Cannabinoids elicit complex cardiovascular responses involving both peripheral and central sites of action via cannabinoid receptors; CB_1 and CB_2 (Gardiner, March et al. 2002; Niederhoffer, Schmid et al. 2003; Pfitzer, Niederhoffer et al. 2004; Seagard, Dean et al. 2004; O'Sullivan, Randall et al. 2007; Wheal, Bennett et al. 2007; Malinowska, Zakrzeska et al. 2010). The presence of anesthesia has dramatically confounded these responses (Stein, Fuller et al. 1996; Lake, Martin et al. 1997; Gardiner, March et al. 2001) and introduced additional parameters to be considered upon investigation of the CB_1R role in controlling cardiovascular functions; in particular the central part. For this reason, the cannabinoid-evoked cardiovascular effects in anesthetized animals are significantly different when compared to responses reported in studies conducted in conscious animals or in humans. Whereas, systemically administered cannabinoids caused hypotension in anesthetized animals, studies on conscious animals showed that CB_1R activation via systemically administered cannabinoids elicits pressor response (Stein, Fuller et al. 1996; Gardiner, March et al. 2001; Randall, Harris et al. 2002; Randall, Kendall et al. 2004; Mendizabal and Adler-Graschinsky 2007). The latter findings are relevant because they replicate the responses reported in humans (Benowitz, Rosenberg et al. 1979; Foltin, Fischman et al. 1987; Sidney 2002).

To our knowledge, only one study investigated the hemodynamic responses of centrally administered cannabinoids in conscious rabbits (Niederhoffer and Szabo 2000) and no other studies have reported such findings in conscious rats (Pfitzer, Niederhoffer et al. 2004). Our studies focus on cardiovascular responses elicited by activation of central CB_1R, which are

expressed in various brainstem nuclei that are implicated in the central regulation of cardiovascular function (Tsou, Brown et al. 1997; Padley, Li et al. 2003).

Many brainstem nuclei might be implicated in the cardiovascular responses elicited by central CB_1R activation; in this study, attention has been focused on the RVLM, which plays pivotal role in controlling sympathetic tone (Strack, Sawyer et al. 1989; Dampney, Polson et al. 2003; Nassar and Abdel-Rahman 2006). The RVLM is the final supraspinal site within the central nervous system that contains tonically active pre-sympathetic neurons, which are regulated by an array of tonic excitatory and inhibitory inputs (Ross, Ruggiero et al. 1984; Guyenet, Filtz et al. 1987; Guyenet, Haselton et al. 1989). The mechanism by which the central CB_1R activation induces sympathoexcitation is not fully delineated. The notion that presynaptic stimulation of CB_1R modulates neurotransmitter release (Freund, Katona et al. 2003; Padley, Li et al. 2003; Piomelli 2003; Drew, Mitchell et al. 2008), and the *in vitro* findings that CB_1R activation inhibited GABAergic transmission in cultured RVLM neurons (Vaughan, McGregor et al. 1999) have led to the suggestion that central CB_1R-evoked sympathoexcitation could be mediated via indirect activation of pre-sympathetic neurons within the RVLM. Therefore, we tested the hypothesis that the RVLM neurons are implicated in CB_1R-evoked sympathoexcitation/pressor responses. This was achieved by exploiting the rapid response of the immediate early gene c-Fos protein product, which has been extensively used as a marker of neuronal activity (Hirooka, Polson et al. 1997; Horiuchi, Potts et al. 1999; Dampney, Polson et al. 2003).

In the current study, we investigated the effects of the CB_1R agonist WIN55,212-2, administered into the cisterna magna, on blood pressure, heart rate and plasma norepinephrine

(NE) (an index of sympathetic activity)(Micalizzi and Pals 1979; Weinstock, Zavadil et al. 1979; Hubbard, Buchholz et al. 1986; Pfitzer, Niederhoffer et al. 2004) in conscious freely moving rats, in presence or absence of the selective CB_1R antagonist, AM251. In a parallel experiment, c-Fos expression was evaluated to delineate whether RVLM neurons are implicated in the CB_1R-elicited pressor response. We have additionally examined the expression of CB_1R (protein) in the RVLM as only one study reported the expression of CB_1R mRNA without investigating its protein product (Padley, Li et al. 2003).

3. 3. Material and Methods

3. 3. 1. Measurement of plasma norepinephrine (NE)

Plasma norepinephrine was measured using a commercially available ELISA kit (cat. # 17-NORHU-E01-RES; ALPCO Diagnostics, Windham, NH) following the manufacturer's instructions. Data were represented as percentage change from baseline plasma NE levels prior to vehicle or WIN55,212-2 injection.

3. 3. 2. Western blotting

RVLM protein samples from naïve non-treated rats were prepared as detailed under general methods, Chapter 2, for detection of CB_1R. Membranes were incubated overnight at 4ºC with either polyclonal rabbit anti-CB_1R antibody (Ab) diluted 1:500 (Abcam, Cambridge, MA) or the same Ab preincubated with blocking peptide (CB_1R-antigen) overnight at 4°C.

3. 3. 3. Immunofluorescence

3.3. 3. 1. Tissue and slide preparation

At the conclusion of the experiment, free-floating sections were prepared as detailed under general methods (see Chapter 2). For co-labeling and quantification of TH-ir neurons expressing c-Fos, brainstem sections containing the RVLM were incubated with either mixture of primary antibodies outlined in Table 3.1 for 48 h at 4 ºC. Sections were then washed and incubated with one of the mixtures of secondary antibodies for 2 h as indicated in Table 3.1.

3. 3. 3. 2. Quantification of labeled neurons

Images were acquired and projected simultaneously on the attached monitor for quantification of co-localization. For immunohistochemical analysis of brainstem sections, the RVLM of 4-6 sections per animal was examined in rostral–caudal sections -12.8 to -11.8 mm relative to the bregma. Criteria used to identify positively labeled cells were: c-Fos-ir labeling was identified as nuclear staining (red) with a visible nucleolus. TH-ir cells were identified by cytosolic labeling (green) with visible processes and a blank nuclear region. TH-ir neuron containing c-Fos were counted manually using 20 X objective. Cells were considered to be co-labeled if *the location of nuclear c-Fos staining corresponded to the blank region in cytosolic labeling of nNOS or TH-ir in the same focal plane*. All slides were coded and the examiner was blinded to the treatment at all time.

3. 3. 3. 3. Image acquisition and processing:

Representative images were acquired and processed following procedures outlined under general methods (Chapter 2).

3. 3. 4. Drugs

WIN55,212-2 and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). AM251 was purchased from Cayman Chemical (Ann Arbor, MI). Alkamus was purchased from Rhone-Poulenc (Cranbury, NJ). WIN55,212-2 and AM251 (30 μg/rat) were dissolved in (1:1:18) mixture of DMSO/ Alkamus/ sterile saline. All drugs were delivered i.c in a volume of 5 μl/ rat.

3. 4. Protocols and Experimental Groups

3. 4. 1. Experiment 1: WIN55,212-2 dose-related centrally-elicited hemodynamic effects

A pilot study showed that the use of previously reported doses of WIN55,212-2, in conscious rabbit (Niederhoffer and Szabo 2000) or anesthetized rats (Pfitzer, Niederhoffer et al. 2004) failed in to elicit similar hemodynamic responses (n=3; data not shown). Therefore we have investigated the changes in BP, HR and plasma NE in freely moving rats in response to i.c. administration of WIN55,212-2 in a group of rats (n=4) that received 5 μl vehicle and 3 successive doses of WIN55,212-2 (3, 10, 30 μg/rat, i.c). Injections were made into the cisterna magnum via a 30-guage stainless-steel injector extended 2.0 mm beyond the tip of the previously implanted guide cannula and connected to a 20 μl Hamilton syringe through PE-10 tubing. WIN55,212-2 or vehicle doses were loaded together and separated by air bubbles to avoid any potential problems caused by frequent removal and reinsertion of the injector. Each dose or the vehicle was delivered in a total volume of 5 μl by hand over one min and at least one hr lapsed between injections to allow BP and HR to return to baseline.

3. 4. 2. Experiment 2: Hemodynamic and neurochemical effects of i.c. WIN55,212-2 in presence or absence of central CB$_1$R blockade (AM251)

Four groups of rats (n=6-8) received WIN55,212-2 (15 μg/rat, i.c); a dose selected based on the results of experiment 1 above or an equal volume of its vehicle 30 min after i.c. administration of the selective CB$_1$R antagonist AM251 (30 μg/rat) or an equal volume of vehicle. The dose of AM251 was chosen based on preliminary study where 3 doses were tested (10, 30, 100 μg/rat, i.c). This dose range was based on a previous study (O'Sullivan, Randall et al. 2007; Wheal, Bennett et al. 2007). In all experimental groups, BP and HR were recorded as detailed under methods. Experiments were terminated 30 min after WIN55,212-2 or its vehicle administration.

Brain tissues were processed for detection and quantification of c-Fos in C1 area of the RVLM, identified by positive labeling for tyrosine hydroxylase (TH-ir) as detailed under methods to delineate whether RVLM neurons are implicated in central cardiovascular effects evoked by WIN55,212-2.

3.5. Statistical Analysis

Mean arterial pressure (MAP) was calculated as: diastolic pressure + one third (systolic pressure - diastolic pressure). MAP and HR values are expressed as mean ± S.E.M. change from their respective baseline before AM251 treatment. Hemodynamic data were analyzed by repeated measures ANOVA using SPSS 16.0 statistical package for Windows®, for differences in time and treatment trends followed by a one way ANOVA to assess individual differences at different time points among different groups. Tukey's (equal variance) and Games Howell

(unequal variance) tests were used for post hoc analysis. Contrasts based on the t-test and the ANOVA error terms were used to compare pre-treatment-to-post-treatment values in each group. These contrasts examined whether there were drug-evoked changes from baseline. $P < 0.05$ was considered significant. A one-way ANOVA followed by Bonferroni comparison test was used to evaluate the effect of various treatments on c-Fos. $P < 0.05$ was considered significant.

Fig. 3.1. Time line for surgical procedures and experimental protocols for experiment 1.
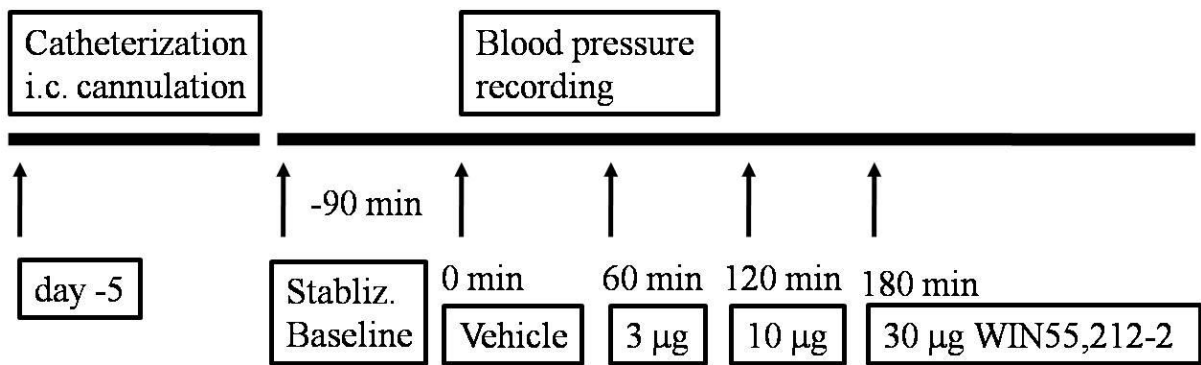
Fig. 3.2. Time line for surgical procedures and experimental protocols for experiment 2.
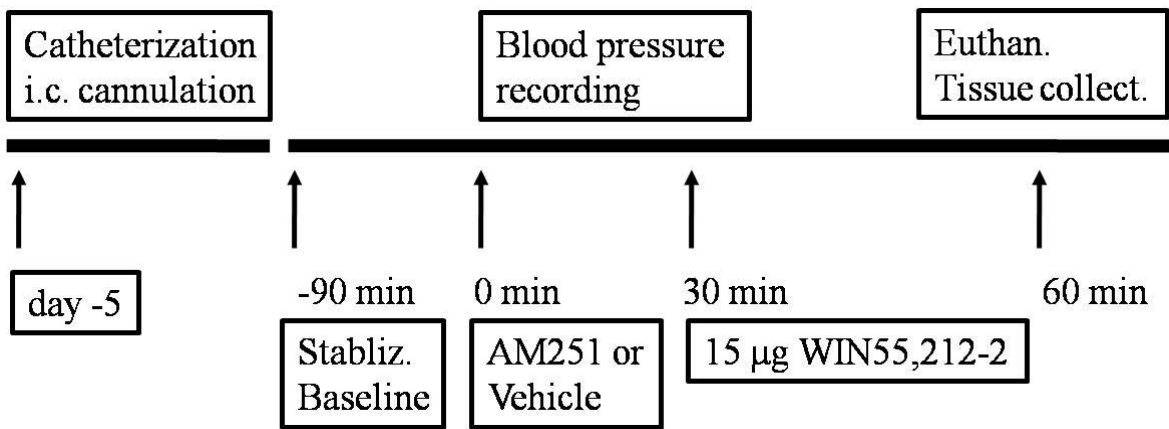
Table. 3.1. Combination of primary and secondary antibodies used in colocalization studies of c-Fos and TH-ir in RVLM neurons. All secondary conjugated antibodies were purchased from Jackson Immunoresearch, CA. TH: tyrosine hydroxylase; Ab: antibody.

	c-Fos/TH
1st primary Ab	Mouse anti-TH (1:500; Chemicon., Temecula, CA)
1st secondary Ab	FITC conjugated donkey anti-mouse; 1:200
2nd primary Ab	Rabbit anti-c-Fos (1:2000; Calbiochem, San Diego, CA)
2nd secondary Ab	Cy3 conjugated donkey anti-rabbit, 1:200

3. 6. Results

3. 6.1. Identification of cannabinoid receptor 1 in the rat RVLM

Fig. 3.3 shows representative Western blots depicting RVLM CB_1R with a predominant band at approximately 64 kDa. and two more bands at 53 and 37 kDa corresponding to different glycosylated and non-glycosylated forms of CB_1R (Song C and Howlett 1995). The detected bands were undetectable in membrane (Fig. 3.3) that was prepared similarly and the anti-CB_1R antibody was pre-incubated with its specific blocking peptide (antigen) recommended by the manufacturer (Abcam, Cambridge, MA) confirming the specificity of the utilized antibody.

3. 6. 2. WIN55,212-2-evoked dose-related increases in MAP and plasma NE and bradycardia in conscious rats

Baseline parameters (MAP, HR) were not significantly different in all groups used in the study (Table 3.1). Further, MAP and HR and plasma NE were similar following the different pretreatments (vehicle or AM251) at the time of WIN55,212-2 or vehicle injection. The mean Plasma NE value prior to vehicle or WIN55,212-2 injection was 1050 ± 80 pg/ml (n=26). The effects of the cannabinoid receptor agonist WIN55,212-2 on MAP, HR and percentage change of plasma NE are shown in Figs. 3.4 and 3.5. Three increasing doses of WIN55,212-2 (3, 10 and 30 µg/rat, i.c) dose-dependently increased MAP and reduced HR; plasma NE was also increased in a dose-dependent manner. The pressor effect of WIN55,212-2 peaked at approximately 7-10 min post-injection, and subsided by 30 min. Similarly, a significant ($P < 0.05$) increase in plasma NE paralleled the pressor response as it peaked at 10 min and subsided 30 min post-injection. On the

other hand, the bradycardic response was immediate and lasted longer than the increases in blood pressure and plasma NE (Fig. 3.4).

3. 6. 3. Hemodynamic, biochemical and neurochemical effects of i.c WIN55,212-2 in the presence or absence of central CB$_1$R blockade (AM251)

The objective of this study was to verify the involvement of central CB$_1$R in the WIN55,212-2-evoked pressor response and the potentially underlying causes of this response, the elevation in plasma NE and RVLM neuronal activation. We selected a dose of WIN55, 212-2 (15 μg/rat, i.c), which produced responses (MAP, HR and plasma NE) lower than those produced by the 30 μg but higher than those produced by the 10 μg WIN55,212-2 (Fig. 3.5). The CB$_1$R antagonist AM251 (30 μg/rat, i.c) or its vehicle was injected 30 min prior to WIN55 212-2 or its vehicle. AM251 did not significantly alter the baseline of the measured parameters (Table 3.1 and Fig. 3.5); however, it virtually abolished the pressor and bradycardic responses as well as the elevation of plasma NE elicited by subsequent WIN55,212-2 (15 μg/rat, i.c) administration. WIN55,212-2 significantly ($P < 0.05$) enhanced c-Fos expression within the RVLM neurons, compared to vehicle treated animals (Figs. 3.6 and 3.7). AM251 (30 μg/rat, i.c) had no significant effect on the basal c-Fos ($P > 0.05$). However, in animals pretreated with AM251, the WIN55,212-2-evoked increase in c-Fos expression in RVLM neurons was abrogated (Fig. 3.6 and 3.7).

Fig. 3.3. Identification of CB$_1$R in the RVLM by Western blotting. Rabbit anti-CB$_1$R antibody (1:500, Abcam, left lane), or antibody blocked by cprresponding CB$_1$R antigen (right panel) incubated overnight with the membrane. The multiple bands that represented the CB$_1$R (left lane) were virtually undetectable when the same Ab was pre-incubated with CB$_1$R antigen.
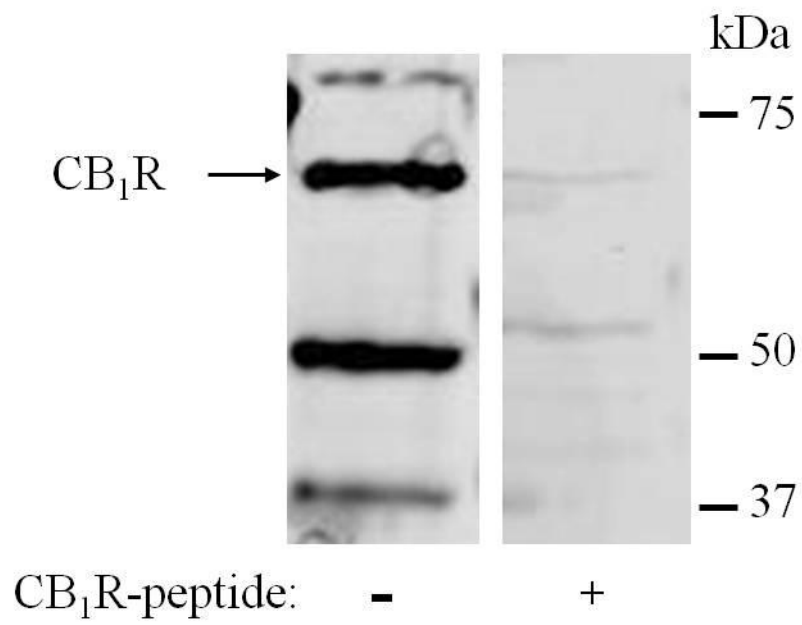
Table. 3.2. MAP (mmHg) and HR (bpm) values before WIN55,212-2 and before and after pretreatment with the CB_1R antagonist, AM251or corresponding vehicle, (when applicable). Values are means ± S.E.M

Treatment	n	Before		After	
		MAP	HR	MAP	HR
Vehicle	4	115.0±6.0	395±12	------	-----
3 μgWIN55,212-2	4	111.3±3.0	375±10	------	-----
10 μgWIN55,212-2	4	108 ± 6.0	360± 11	------	-----
30 μgWIN55,212-2	4	112.0±6.0	356±15	------	-----
Vehicle + 15 μg WIN55,212-2	6	111.3±3.0	375±10	112.0±3.0	363±13
AM251 + Vehicle	5	113.0 ±4.0	398±16	110.0 ±4.0	390±20
Vehicle	4	117.0±6.0	400±12	115.0±4.0	410±12
AM251 + 15 μg WIN55,212-2	8	110.0±4.0	392±16	111.0±2.0	388±16

Fig. 3.4. WIN55,212-2 dose-related cardiovascular and plasma NE responses in conscious rats. Typical tracings depicting the dose-dependent changes in arterial pressure (AP) (top panel) elicited by WIN55,212-2. Changes in mean arterial pressure (ΔMAP, top panel), heart rate (ΔHR, middle panel) and percentage change of plasma NE from baseline (bottom panel) evoked by intracisternal WIN55,212-2 (WIN55; 3, 10 and 30 μg/rat) or an equal volume of vehicle (veh) in conscious rats (n=4) (bottom panel). One hour was allowed between consequetive doses to allow BP and HR to return to baseline values. Arrow marks WIN55,212-2 injection. Values are mean ± S.E.M. of 4 observations. * or # $P<0.05$ significantly different compared to vehicle (veh) or low dose WIN55,212-2 (3 μg WIN55) values, respectively.

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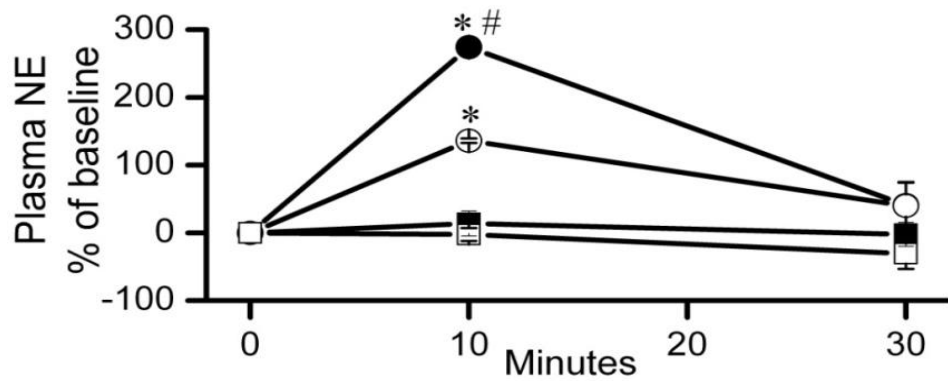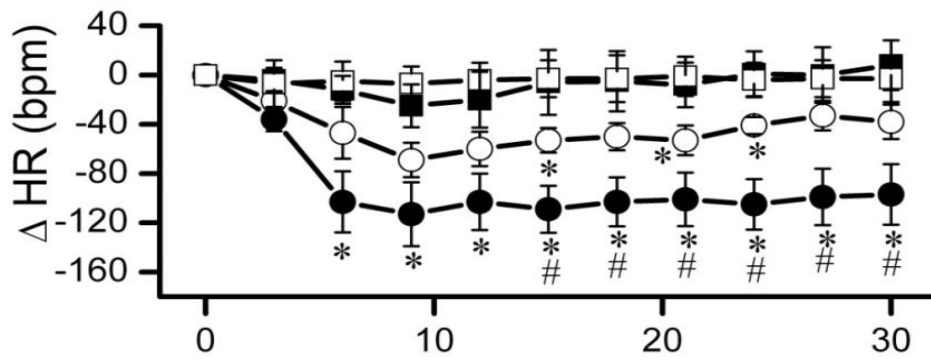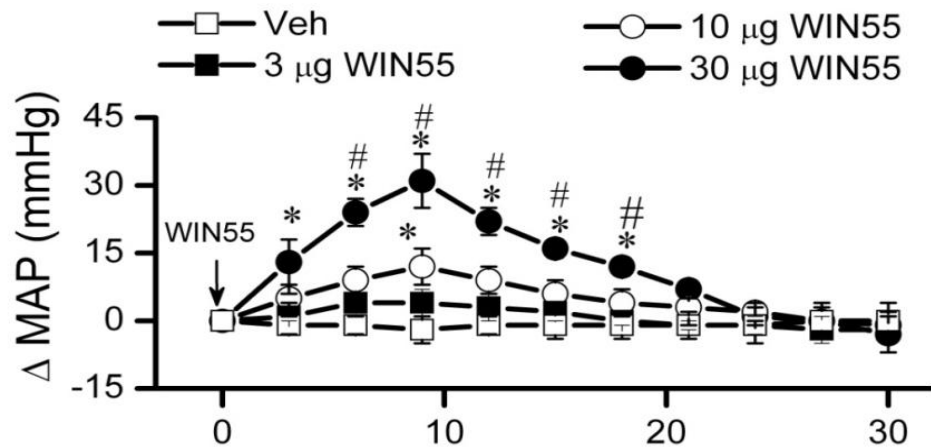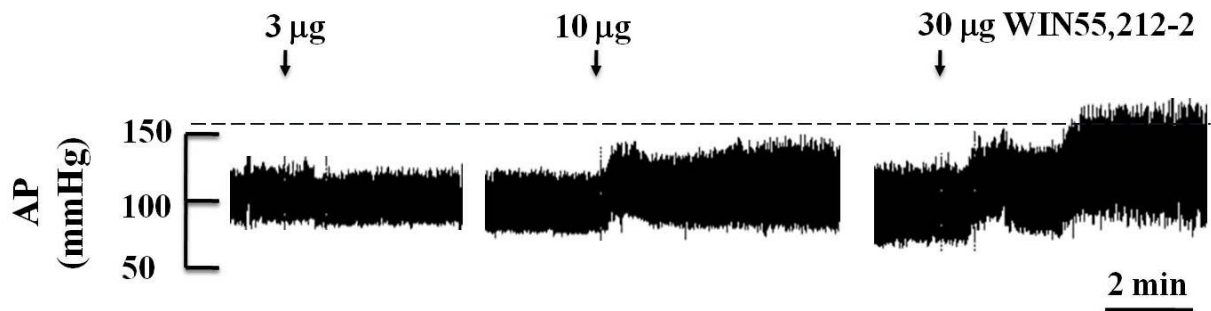
Fig. 3.5. Cardiovascular and plasma NE responses elicited by i.c WIN55,212-2 in absence or presence of central CB$_1$R blockade (AM251). Time course of changes in mean arterial pressure (ΔMAP, top panel), heart rate (ΔHR, middle panel) and percentage change of plasma NE from baseline (bottom panel) evoked by intracisternal WIN55,212-2 (Veh+15 µg WIN55) or an equal volume of its vehicle, indicated by the arrow (top panel), in conscious rats intracisternally pretreated, 30 minutes earlier, with the selective CB$_1$R antagonist AM251 (30 µg/rat) or equal volume of vehicle. Values are mean ± S.E.M. of 4 to 8 observations. * or # $P < 0.05$ versus respective "AM251+Veh" or "AM251+15 µg WIN" values, respectively

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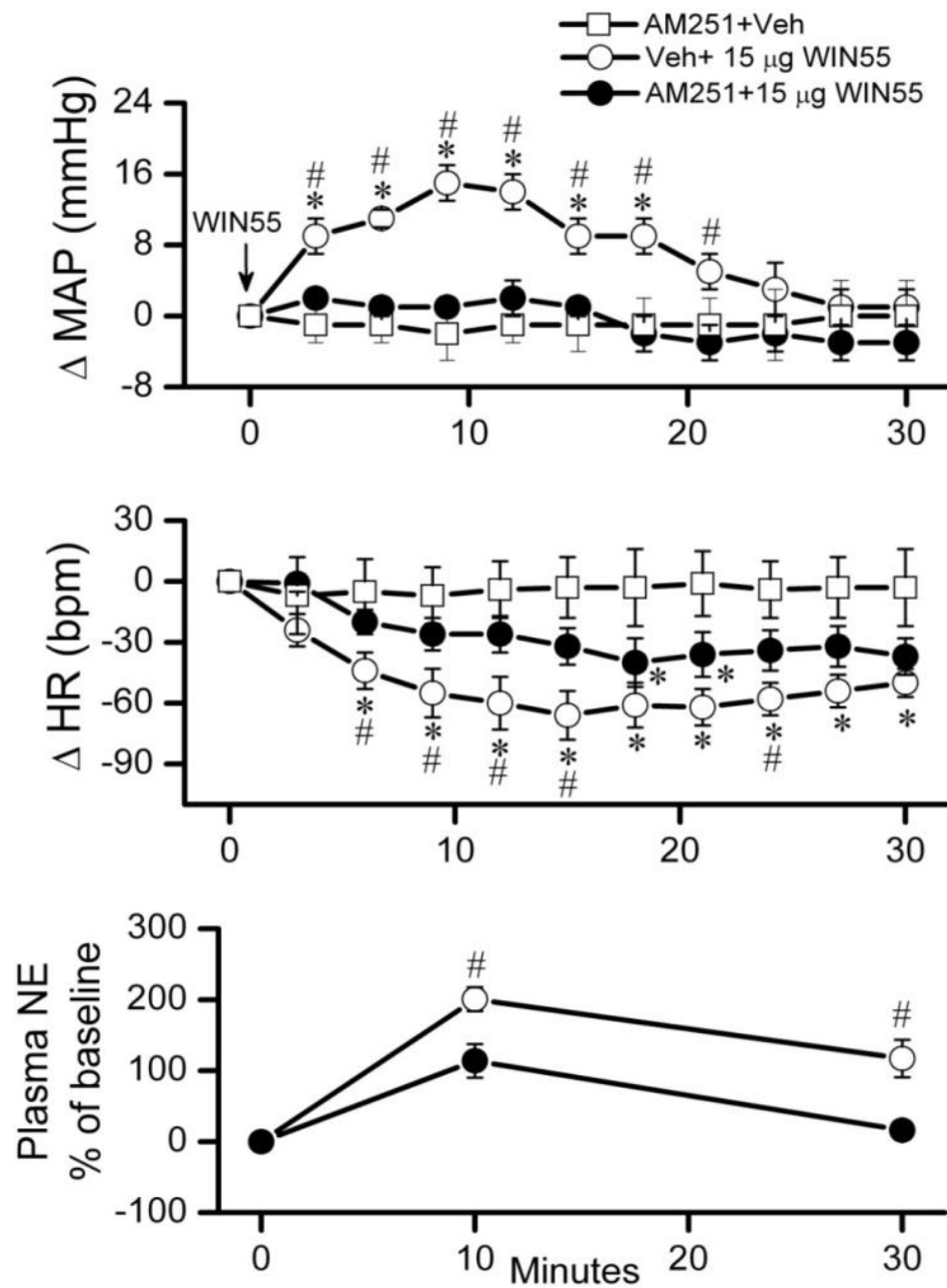
Fig. 3.6. Photomicrographs depicting CB₁R-induced c-Fos expression in catecholaminergic neurons in the RVLM. Confocal dual-channel images showing tyrosine hydroxylase immunoreactive (TH-ir) neurons (green) and c-Fos immunoreactive (Fos-ir) cell nuclei (red) in RVLM of rats treated as described under methods section with (A) vehicle, (B) 15 μg WIN55,212-2, (C) AM251 + vehicle and (D) AM251 + 15 μg WIN55,212-2. White or yellow arrowheads indicate single labeled TH-ir neurons or c-Fos-ir cell nuclei, respectively. White arrows denote c-Fos/TH colabeled cells. Scale bar, 20 μm.
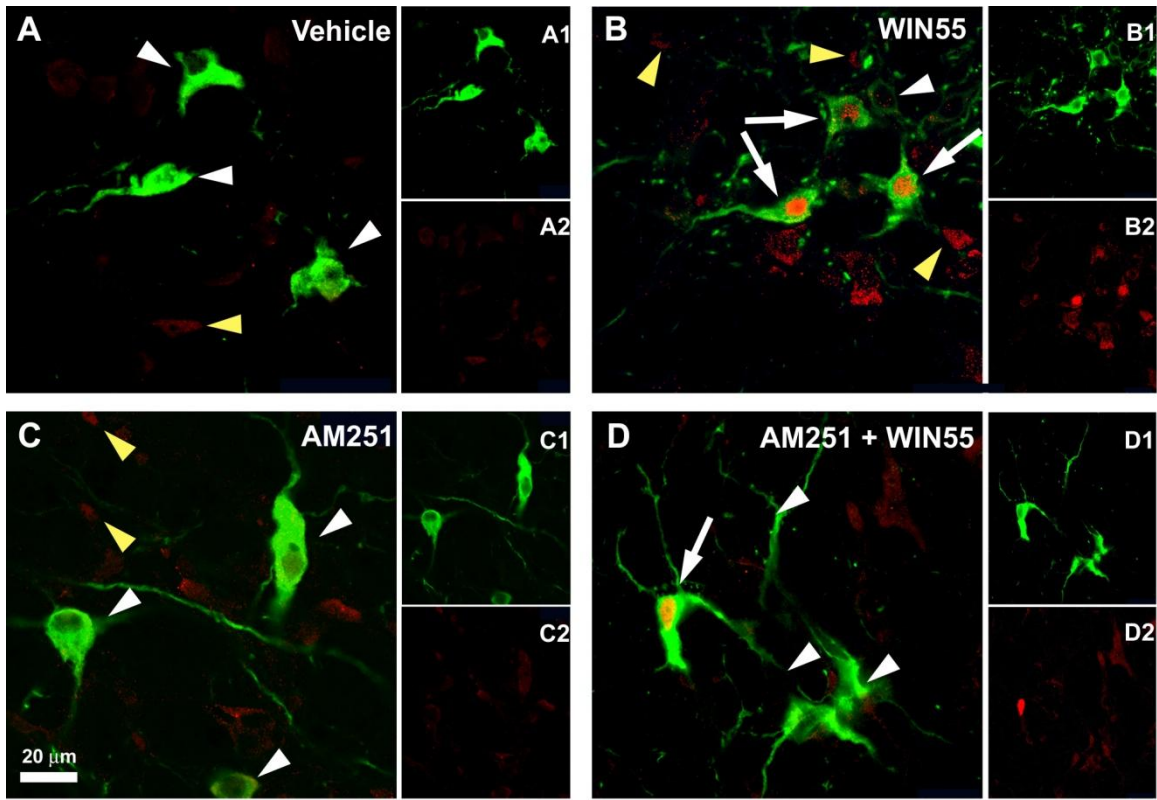
Fig. 3.7. Quantitative analysis of CB$_1$R-induced c-Fos expression in RVLM catecholaminergic neurons. The number of TH-ir neurons, the number c-Fos/TH-ir double-labeled neurons and the percentage of TH-ir neurons co-labeled with c-Fos-ir in the RVLM of rats treated as described under methods with either vehicle, WIN55,212-2 (.15 µg/rat, i.c), AM251 (30 µg/rat, i.c), AM251 prior to WIN55,212-2, are shown. Bar graphs represent mean ± S.E.M. counts in coronal brainstem sections from 4-6/animal, (n=3-5)/group using one-way ANOVA followed by Bonferroni comparison test. * or # P <0.05 vs respective vehicle or all other treatments values, respectively.

Number of TH-ir neurons

Number of double-labeled neurons

Percent of TH-neurons with c-Fos

Vehicle
WIN55
AM251
AM251+WIN55

*#

*#

Number of neurons / section

Number of neurons / section

Percent

65
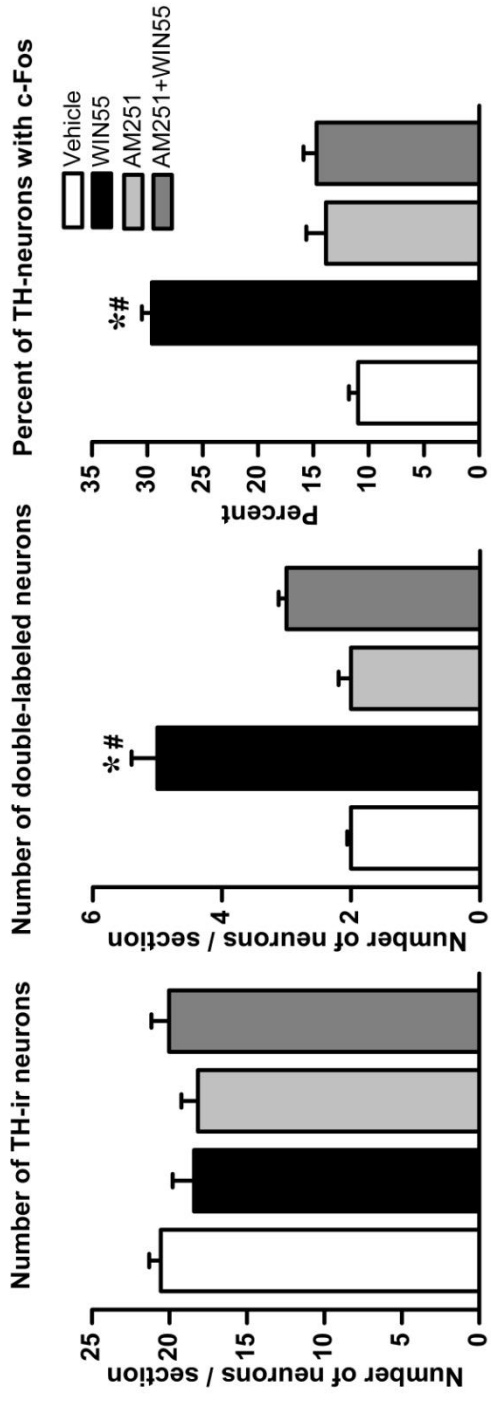
3. 7. Discussion

The most important findings of this study are: (i) i.c. WIN55,212-2, a non-selective CB_1-CB_2 receptor agonist, elicited dose-dependent increases in blood pressure and plasma NE along with bradycardic response in conscious rats; (ii) these responses were virtually abolished by prior treatment with selective CB_1R antagonist (AM251); (iii) activation of central CB_1R (WIN55,212-2) enhanced RVLM-catecholaminergic activity (c-Fos) and this response was attenuated by prior blockade of central CB_1R (AM251). Together, the present findings yield insight into the pivotal role of the enhanced sympathetic nervous system, at least in part at the RVLM level, in the pressor response elicited by central CB_1R activation.

The aim of this study was to investigate the centrally elicited hemodynamic effects of CB_1R in a conscious animal model that will be used in the subsequent molecular studies and to determine if the RVLM is implicated in CB_1R-evoked sympathoexcitation/pressor response. As a first step, we confirmed the expression of CB_1R (protein) in the RVLM by detecting the calculated bands at 64 and 53 kDa of the N-glycosylated and non-glycosylated forms of CB_1R, respectively (Song C and Howlett 1995). Although Padley et al. have reported the expression of CB_1R gene in the RVLM (Padley, Li et al. 2003), to our knowledge, this is the first study to detect CB_1R protein expression in the RVLM.

In the present study, i.c administration of WIN55,212-2, dose-dependently increased MAP and NE plasma levels, denoting an increase in central sympathetic tone in conscious rats. This finding contrasts with the reduction in plasma NE levels caused by peripheral CB_1R activation (systemic cannabinoids delivery) (Niederhoffer, Schmid et al. 2003). Additionally, i.c.

WIN55,212-2 elicited a dose-dependent reduction in HR, which seems to be vagally-mediated because such bradycardic responses were absent in presence of atropine or vagotomy (Niederhoffer and Szabo 1999; Niederhoffer and Szabo 2000). The pressor response peaked at 5-10 min after WIN55,2122 administration and lasted for additional an 15 min. However, the bradycardic response was immediate and lasted beyond the 30 min recording time. Our studies were conducted in conscious rats, to circumvent the negative impact of anesthesia that was shown to dramatically compromise cannabinoid evoked hemodynamic responses; in particular the central part (Stein, Fuller et al. 1996; Lake, Martin et al. 1997; Gardiner, March et al. 2001). Notably, the pressor response elicited by central WIN55,212-2 administration fully agrees with reported findings in experimental animals (Niederhoffer and Szabo 1999; Niederhoffer and Szabo 2000; Pfitzer, Niederhoffer et al. 2004), and reflects a similar response n humans (Foltin, Fischman et al. 1987; Sidney 2002). WIN55,212-2 doses used were relatively higher than those reported in previous studies (Pfitzer, Niederhoffer et al. 2004). This may be attributed to: (i) the use of conscious animals, which exhibit a higher sympathetic tone compared to anesthetized animals utilized; (ii) the difference in experimental set up; enough time was given, in our hand, between successive WIN55,212-2 doses to allow BP and HR return to baseline, whereas Pfitzer et al. allowed only 20 minutes interval between doses that resulted in cumulative responses.

Because there are no currently available selective CB_1R agonists, it was important that we confirm the involvement of CB_1R in the pressor response elicited by WIN55,212-2. The latter is a mixed CB_1R/CB_2R agonist (Showalter, Compton et al. 1996; Griffin, Atkinson et al. 1998) and both receptors are expressed in the brainstem. The ability of the selective CB_1R antagonist AM251 to virtually abolish the pressor response and the elevation of plasma NE levels elicited

by i.c. WIN55,212-2, agrees with reported findings (Niederhoffer and Szabo 1999; Niederhoffer and Szabo 2000; Pfitzer, Niederhoffer et al. 2004), and suggests that the pressor response was mediated via activation of central CB_1R in our model system. It is important to note, however, that the lack of a change in blood pressure following AM251 administration argues against the involvement of central CB_1R signaling in tonic control of blood pressure.

Another main objective of the current study was to directly elucidate the role of the RVLM neurons in the central CB_1R-evoked pressor response. We present the first *in vivo* evidence that central CB_1R-evoked activation of RVLM neurons underlies, at least partly, the sympathoexcitation/pressor response elicited by central CB_1R activation as demonstrated by the significant increase in catecholaminergic neurons (TH-ir) expressing c-Fos, a marker of neuronal activity, following i.c WIN55,212-2 versus vehicle treated animals. While the CB_1R antagonist, AM251, has not altered the number of RVLM-TH-ir neurons expressing c-Fos compared to vehicle treated rats, it abrogated the WIN55,212-2-evoked increase in c-Fos expressing neurons. These neurochemical responses parallel and further confirm the blood pressure responses elicited by WIN55,212-2 and AM251 discussed above.

In summary, the present study highlights the importance of the sympathoexcitation in the pressor response elicited by brainstem-CB_1R activation. This new evidence in conscious rats yields insight into the RVLM as an important neuroanatomical substrate for CB_1R agonists. On the other hand, the activation of RVLM-catecholaminergic neurons could have resulted from input from other neuronal pools that send projections to the RVLM because WIN55,212-2 was administered intracisternally in the present study. This important issue will be addressed in Chapter 4.

CHAPTER FOUR-A PIVOTAL ROLE FOR BRAINSTEM OREXIN IN THE CENTRAL PRESSOR RESPONSE ELICITED BY THE CANNABINOID RECEPTOR AGONIST WIN55,212-2 IN CONSCIOUS RATS

4. 1. Abstract

Despite the well-established evidence for crosstalk between the cannabinoid receptor 1 (CB_1R) and the orexin receptor 1 (OX_1R), a potential role for orexin in the pressor response mediated by central CB_1R activation has not been explored. In the current study, we tested the novel hypothesis that brainstem OX_1R/orexin-A signaling is implicated in the pressor response induced by central CB_1R activation. We demonstrated, for the first time, a significant increase in orexin-A levels in the RVLM along with the pressor response elicited by intracisternal (i.c) injection of the CB_1R agonist WIN55,212-2, (15 µg/rat), in conscious rats. Additional studies in conscious rats established a causal role for orexin-A in WIN55,212-2 evoked pressor response because: (i) the WIN55,212-2-evoked pressor response and the elevation of RVLM orexin-A level were abolished by selective blockade of brainstem CB_1R (AM251, 30 µg/rat, i.c); (ii) the selective OX_1R antagonist SB-408124 (10 nmoles/rat, i.c) abrogated the pressor response elicited by either orexin-A (3 nmoles/rat, i.c) or WIN55,212-2(15 µg/rat, i.c). On the other hand, central blockade of CB_1R (AM251) had no effect on the pressor response elicited by i.c. orexin-A. Equally important, multiple labeling immunofluorescence revealed co-localization of CB_1R, OX_1R and orexin-A within the C1 area of the RVLM. Our findings yield new insight into a functional crosstalk between central CB_1R and OX_1R signaling and identify brainstem orexin-A as a possible neuronal mediator of CB_1R-evoked pressor response in conscious rats.

4. 2. Introduction

Recent *in vivo* evidence supports the demonstration of an interplay between CB_1 and OX_1 receptors signaling (Hilairet, Bouaboula et al. 2003; Ellis, Pediani et al. 2006). For example, in feeding regulation studies, CB_1R activation in lateral hypothalamic neurons results in modulation of orexin signaling (Huang, Acuna-Goycolea et al. 2007; Matias, Cristino et al. 2008). The functional relevance of the central interaction between the two receptors has been demonstrated in feeding behavioral studies where orexin-A induced-feeding was abrogated by pretreatment with sub-anorectic doses of the CB_1R antagonist SR141716A (Crespo, Heras et al. 2008). This interaction is possible because CB_1R modulates synaptic transmission of many neurotransmitters (Freund, Katona et al. 2003; Piomelli 2003; Drew, Mitchell et al. 2008) including orexins (Huang, Acuna-Goycolea et al. 2007; Matias, Cristino et al. 2008; Verty, Boon et al. 2009).

Although, orexin-A neurons are exclusive to the hypothalamus, orexin-A immunoreactive (orexin-A-ir) fibers, were found in close proximity to catecholamine reactive (CAir) neurons in the RVLM (Machado, Bonagamba et al. 2002; Ciriello, Li et al. 2003). Importantly, the expression of CB_1R exhibits similar patterns in RVLM (Herkenham, Lynn et al. 1991; Padley, Li et al. 2003). While most of the *in vivo* studies on OX_1R signaling dealt with feeding behavior, a few studies have demonstrated the ability of orexin-A to elicit sympathoexcitation/ pressor responses following intracisternal (Chen, Hwang et al. 2000; Zhang, Fukuda et al. 2005), intracerebroventricular (Samson, Gosnell et al. 1999; Shirasaka, Nakazato et al. 1999) or microinjection into the RVLM (Chen, Hwang et al. 2000; Machado, Bonagamba et al. 2002; Ciriello, Li et al. 2003). Notably, the hypothalamic peptide orexin-A possesses 10-fold higher affinity than orexin-B, at the OX_1R (van den Pol, Gao et al. 1998; Smart, Jerman et al. 1999).

Collectively, these reported findings highlight the possibility that functional crosstalk between CB_1R and OX_1R/orexin-A in the RVLM might be involved in the blood pressure response elicited by activation of either receptor *in vivo*.

The aim of the present study was to test the hypothesis that OX_1R/orexin-A signaling in the RVLM is essential for the central CB_1R-mediated pressor response. To test this hypothesis, we conducted integrative cardiovascular studies on the effects of i.c. CB_1R (WIN55,212-2) or OX_1R (orexin-A) agonists on blood pressure and heart rate in conscious unrestrained rats. The experimental design permitted investigation of the effect of selectively blocking either receptor on the cardiovascular responses elicited by both agonists as well as the orexin-A levels (following CB_1R activation) in the RVLM. Finally, we investigated whether the OX_1R/orexin-A and CB_1R are co-localized in catecholamine expressing neurons in the RVLM.

4. 3. Materials and Methods

4. 3. 1. Immunofluorescence

The IF protocol detailed under general methods (Chapter 2) was utilized for colocalization studies. Sections were incubated for 48 h at 4ºC in the presence of Ab mixtures shown in Table. 4.1. After washing 3X in TBST, multiple immunofluorescence was revealed by incubation for 2 h in a mixture of fluorescent conjugated donkey anti IgG as indicated in Table 4.1. Specificity of the rabbit anti-orexin-A antibody (Novus Biologicals, Littleton, CO) was tested by Western blotting, which detected a single band at ~ 3.5 kDa corresponding to synthetic orexin-A (Phoenix Pharmaceutical; Burlingame, CA).

4. 3. 2. Orexin-A detection

RVLM orexin-A levels were quantified using a standard ELISA kit (EK-003-30; Phoenix Pharmaceutical, Burlingame, CA) in accordance with procedures provided by the manufacturer and reported elsewhere (Feng, Vurbic et al. 2007). Briefly, at the conclusion of experiment 1 (see below), animals were euthanized, brains were removed, flash frozen in 2-methylbutane (cooled on dry ice for at least 30 min), and stored at -80ºC until use. Tissues from the bilateral RVLM were collected and pooled from each animal using a 0.75-mm punch tool (Stoelting Co., Wood Dale, IL), approximately -12.8 to -11.8 mm relative to bregma (Paxinos and Watson 2005). Samples were then acid-extracted, protein was quantified using a standard Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA) and assayed in duplicate wells for orexin-A. Values, for each sample, were averaged and expressed as ng/mg total protein.

4. 3. 3. Drugs

WIN55,212-2, SB-408124 and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). AM251 was purchased from Cayman Chemical (Ann Arbor, MI). Alkamus was purchased from Rhone-Poulenc (Cranbury, NJ). WIN55,212-2 and AM251 were dissolved in (1:1:18) mixture of DMSO/Alkamus/sterile saline. Orexin-A was purchased from Phoenix Pharmaceutical (Burlingame, CA), freshly prepared in sterile saline and kept on ice during the experiment. SB-408124 was dissolved in 10% DMSO in saline. All drugs were delivered i.c in volume of 5 μl/ rat over a period of 1-3 min. Each vehicle was tested on at least three animals prior its utilization. As none of these vehicles significantly changed the basal MAP and HR, we refer to all of them as (vehicle).

4. 4. Protocols and Experimental groups

Drug or vehicle injections were made into the cisterna magna of conscious unrestrained rats as detailed in Chapters 2 and 3 following procedures in previous studies (Zhang and Abdel-Rahman 2005; Nassar and Abdel-Rahman 2006; Zhang and Abdel-Rahman 2008).

4. 4. 1 Experiment 1: Effect of central CB_1R activation (WIN55,212-2) on blood pressure and RVLM-orexin-A levels

A total of 17 conscious rats, divided into 4 groups (n=3-5, each) were used in this part of the study. In all rats, blood pressure and heart were allowed to stabilize at baseline before drug/vehicle administration and were continuously monitored for 10-15 min after i.c. WIN55,212-2 or its vehicle. Two groups received WIN55,212-2 (15 μg/rat, i.c) 30 min after i.c injection of the CB_1R antagonist AM251 (30 μg/rat) or an equal volume of vehicle. The other two groups served as controls and received only vehicle (i.c) or AM251 (30 μg/rat, i.c) followed by vehicle. The brains were collected at a time that coincided with the peak pressor response (10-15 min) for subsequent measurement of RVLM orexin-A levels as described under methods. Additional non-treated animals were prepared for multiple co-localization studies of CB_1R/orexin-A or OX_1R within the C1 area of RVLM. C1 area of the brainstem was identified anatomically as described under general methods (Chapter 2) and by positive labeling for tyrosine hydroxylase immunoreactive neurons.

4. 4. 2 Experiment 2: Effect of central OX_1R blockade (SB-408124) on i.c. orexin-A-mediated cardiovascular effects

There are no reported studies on the dose range of the selective OX_1R antagonist (SB-408124) needed to block central OX_1R in rats. Therefore, as a first step, it was important to replicate in our model system the dose-pressor response curve elicited by i.c. orexin-A (0.1, 1 and 3 nmoles; n=4) based on reported dose-range for the peptide (Samson, Gosnell et al. 1999; Chen, Hwang et al. 2000). In a parallel preliminary study, we identified a dose of the selective OX_1R antagonist (SB-408124) and verified its ability to block the central OX_1R. Two additional groups of rats received i.c. orexin-A (3 nmoles; n=8) or an equal volume of sterile saline (n=5) 10 min after i.c. of the OX_1R receptor antagonist SB-408124 (10 nmoles/rat). Notably, the 3 nmoles of orexin-A produced a comparable pressor response to that caused by i.c. WIN55,212-2 (15 μg) in the previous experiment.

Table. 4.1. Combination of primary and secondary antibodies used in colocalization studies of CB$_1$R and orexin-A/OX$_1$R. All secondary conjugated antibodies were purchased from Jackson Immunoresearch, CA. TH: tyrosine hydroxylase; Ab: antibody.

Targets	CB$_1$R/OX$_1$R/n-tubulin
1st primary Ab	Mouse anti-GAD65 (1:400; Chemicon., Temecula, CA)
1st secondary Ab	FITC conjugated donkey anti-mouse, 1:200
2nd primary Ab	Mouse anti-n-tubulin (1:1000; Abcam, Cambridge, MA)
2nd secondary Ab	FITC conjugated donkey anti-mouse; 1:200
3rd primary Ab	Goat anti-CB$_1$R (1:200; Santa Cruz Biotech, Santa Cruz, CA)
3rd secondary Ab	Cy5 conjugated donkey anti-goat;1:200

Targets	CB$_1$R/orexin-A/GAD65
1st primary Ab	Rabbit anti-orexin-A (1:500; Novus Biologicals, Littleton, CO)
1st secondary Ab	Cy3 conjugated donkey anti-rabbit;1:200
2nd primary Ab	Rabbit anti-OX$_1$R (1:200; Alpha diagnostic, San Antonio, TX)
2nd secondary Ab	Cy3 conjugated donkey anti-rabbit; 1:200
3rd primary Ab	Goat anti-CB$_1$R (1:200; Santa Cruz Biotech, Santa Cruz, CA)
3rd secondary Ab	Cy5 conjugated donkey anti-goat;1:200

Targets	CB$_1$R/TH/n-tubulin
1st primary Ab	Rabbit anti-TH (1:500; Chemicon., Temecula, CA)
1st secondary Ab	Cy3 conjugated donkey anti-rabbit, 1:200
2nd primary Ab	Mouse anti-n-tubulin (1:1000; Abcam, Cambridge, MA)
2nd secondary Ab	FITC conjugated donkey anti-mouse; 1:200
3rd primary Ab	Goat anti-CB$_1$R (1:200; Santa Cruz Biotech, Santa Cruz, CA)
3rd secondary Ab	Cy5 conjugated donkey anti-goat;1:200

Fig. 4.1. Time line for surgical procedures and experimental protocols for experiment 4. 4. 3. * In the AM251 pretreated group animals received orexin-A, 30 min later.

Catheterization i.c. cannulation		Blood pressure recording		Euthan. Tissue collect.	

day -5

-90 min   0 min   10 min*   40 min

Stabliz. Baseline

SB-408124 → WIN55,212-2

AM251 → Orexin-A
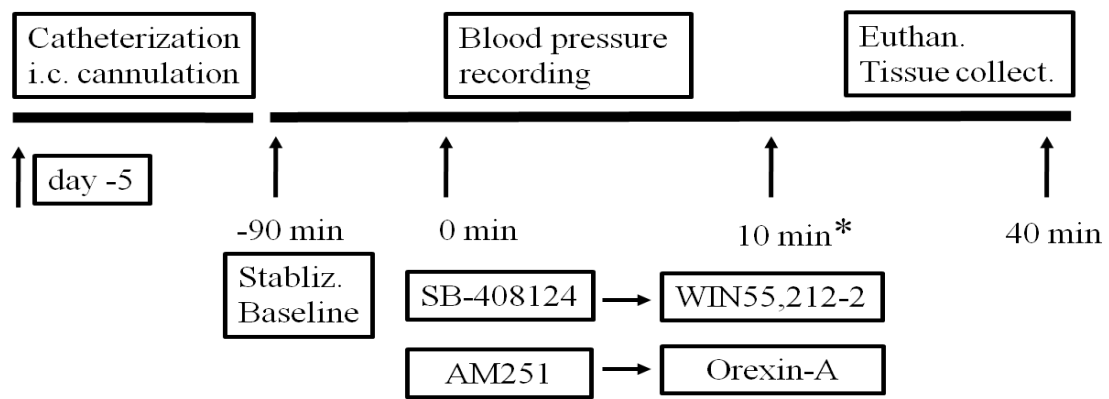
4. 4. 3. Experiment 3: Effect of central OX_1R blockade on the pressor response elicited by i.c WIN55,212-2

The objective of this experiment was to determine whether the increased release in orexin-A in the RVLM (experiment 4. 4. 1) plays a causal role in the pressor response elicited by i.c. WIN55,212-2. To achieve this goal, one group of animals (n=8) received WIN55,212-2 (15 μg, i.c) 10 min after central OX_1R blockade (SB-408124; 10 nmoles, i.c). WIN55,212-2 evoked responses in experiment 3. 4. 2, Chapter 3, were re-blotted and compared to responses after SB-408124. We also determined in another group (n=7) whether central CB_1R blockade (AM251; 30 μg/rat), 30 min earlier, influenced the pressor effect elicited by orexin-A (3 nmoles, i.c). The cardiovascular responses elicited by WIN55,212-2 or orexin-A in this experiment were compared to the corresponding pressor response elicited by either agonist in the absence of the antagonist.

4. 5. Statistical Analysis

Analysis of hemodynamic data was performed as detailed in Chapter 3. A one way ANOVA was used to evaluate the effect of various treatments on RVLM-orexin-A levels in the RVLM (experiment 4. 3. 1.), compared to the baseline (vehicle alone) followed by Bonferroni post-hoc test. $P<0.05$ was considered significant. All values are expressed as means ± S.E.M.

4.6. Results

4. 6. 1. Colocalization of orexin-A/OX₁R and CB₁R in rat RVLM

Multiple labeling findings in brainstem coronal sections revealed numerous orexin-A immunoreactive (orexin-A ir) fibers with characteristic varicose like structures as well as OX_1R immunoreactive neurons (OX_1R-ir) in close proximity to CB_1R (CB_1R-ir) labeled neurons in the RVLM (Fig. 4.2). Many of these neuronal structures displayed tyrosine-hydroxylase immunoreactivity which was used as marker of catecholaminergic neurons (TH-ir).

4. 6. 2. Activation of brainstem CB₁R elicits increase in RVLM-orexin-A levels

Baseline MAP and HR were not significantly different in all groups of rats used in the study (Table 4.2.). Further, MAP and HR were similar following the different pretreatments (vehicle, AM251 or SB-408124) at the time of WIN55,212-2 or orexin-A injection. During the 10-15 min of hemodynamic measurements, i.c. WIN55,212-2 (15 µg/rat) elicited similar responses to those previously shown in experiment 3. 4. 2, (Chapter 3), where it increased MAP and induced bradycardia in conscious rats. These cardiovascular responses were mediated via activation of central CB_1R because pretreatment with the selective CB_1R antagonist AM251 (30 µg/rat, i.c) virtually abolished the pressor and bradycardic responses elicited by WIN55,212-2 (15 µg/rat, i.c). Measurement of orexin-A levels in the RVLM of WIN55,212-2 (15 µg/rat, i.c) or control (vehicle)-treated rats revealed significantly higher ($P < 0.05$) levels of the peptide in the treatment group (Fig. 4.3). The WIN55,212-2 (15 µg/rat)-evoked increase in RVLM orexin-A levels were abrogated by prior treatment with the selective CB_1R antagonist AM251 (30 µg/rat). AM251 alone had no effect on RVLM orexin-A levels ($P > 0.05$) (Fig. 4.3).

Table. 4.2. MAP (mmHg) and HR (bpm) values before and after pretreatment (preceding i.c. vehicle, WIN55,212-2 or orexin-A). Values are means ± S.E.M.

Treatment	n	Before		After	
		MAP	**HR**	**MAP**	**HR**
Vehicle	3	115.0±6.0	395±12	110.0±6.0	386±11
Vehicle + 15 μg WIN55,212-2	5	111.3±3.0	375±10	108.0±5.0	350± 11
AM251 + vehicle	4	108 ± 6.0	360± 11	114.3±3.0	390±10
AM251 + 15 μg WIN55,212-2	5	112.0±6.0	356±15	117.0±6.0	376±15
Vehicle + orexin-A	6	111.3±3.0	375±10	112.0±3.0	363±13
SB-408124 + Vehicle	5	113.0 ±4.0	398±16	110.0 ±4.0	390±20
SB-408124 + orexin-A	8	117.0±6.0	400±12	115.0±4.0	410±12
SB-408124 + WIN55,212-2	8	110.0±4.0	392±16	111.0±2.0	388±16
AM251 + orexin-A	7	109.0±3.0	360±18	113± 3.0	375±18

Fig. 4.2. Photomicrographs depicting anatomical colocalization of orexin-A/OX$_1$R along with CB$_1$R in the rats RVLM by multiple labeling immunofluorescence. confocal images depict: (A) CB$_1$R-ir (A1, blue), OX$_1$R immunoreactive cells (OX$_1$R-Air) (A2, red) and n-tubulin (A3, green). Merged image (A4) shows colocalization of CB$_1$R and OX$_1$R (purple) in rat RVLM. (B) CB$_1$R-ir (B1, blue) colocalization with orexin-A immunoreactive fibers (orexin-Air) (B2, red) and GABA presynaptic maker (GAD65-ir) (B3, green) punctuated structures in rats RVLM. Merged image (B4) shows close anatomical localization of CB$_1$R-ir, orexin-A-ir fibers, and GAD65-ir cells and punctate-like structures indicative of pre-and postsynaptic localization. (C) CB$_1$R immunereactivity (CB$_1$R-ir) (C1, blue) within the ventrolateral medulla (RVLM) along with tyrosine hydroxylase immunoreactive neurons (TH-ir) (C2, red) and neuronal marker n-tubulin (A3, green). Merged image (C4) showed that CB$_1$R-ir is localized on TH-ir (arrow) and non-TH-ir (arrowhead and asterisk) neuronal structures.
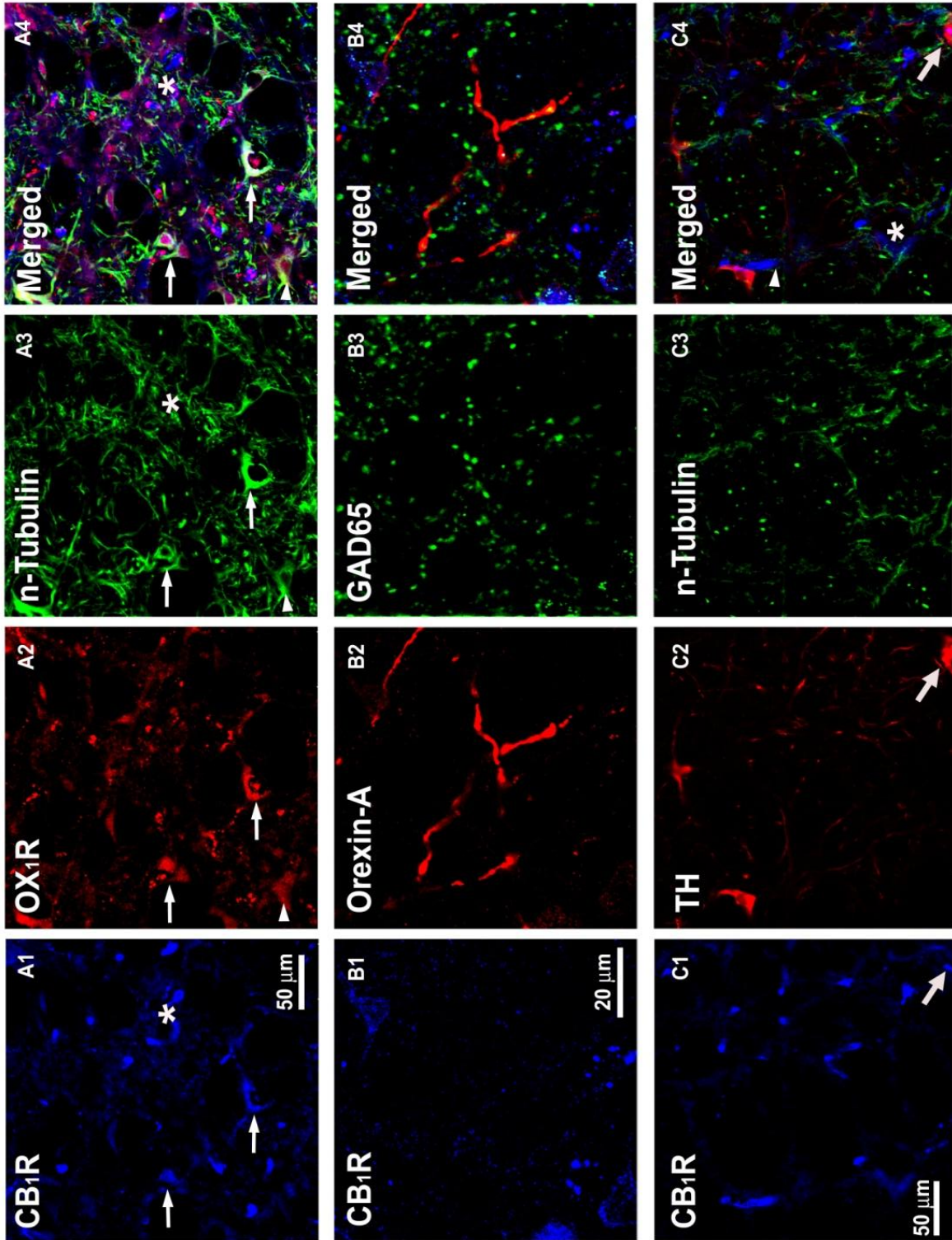
Fig. 4.3. Brainstem CB$_1$R stimulation evoked elevation of RVLM-orexin-A levels.
Changes in RVLM-orexin-A levels determined by ELISA as detailed under methods
in four groups of animals (n=3-5 each) that received one of the following i.c.
treatments: (Vehicle; n=3), (Vehicle + WIN55,212-2; n=5), (AM251 + vehicle; n=4)
or (AM251 + WIN55,212-2; n=5). Animals were sacrificed at a time that preceded the
WIN55,212-2-elicited pressor response. Bar graphs represent mean ± S.E.M. (n=3-5 in
each group) using one-way ANOVA followed by Bonferroni comparison test. * or # *P*
<0.05 compared to either "vehicle" or "AM251 + WIN55,212-2" values, respectively.
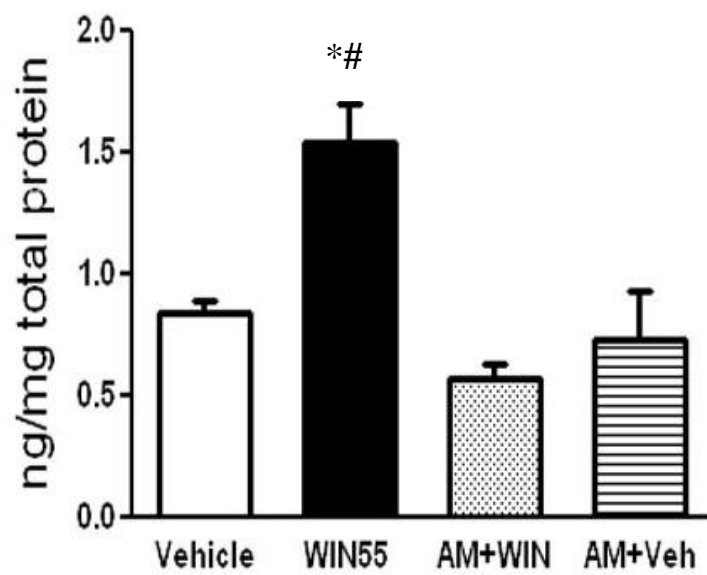
Fig. 4.4. Orexin-A-evoked dose-related hemodynamic effects in conscious rats. Dose-related changes in mean arterial pressure (ΔMAP, top panel) and heart rate (ΔHR, bottom panel) evoked by intracisternal (i.c) orexin-A (0.1, 1, 3 nmoles/rat) (n=4) in conscious unrestrained rats. Values are mean ± S.E.M. of. * or # $P < 0.05$ versus respective "0.1 nmole orexin-A" or "1 nmole orexin-A" values, respectively.
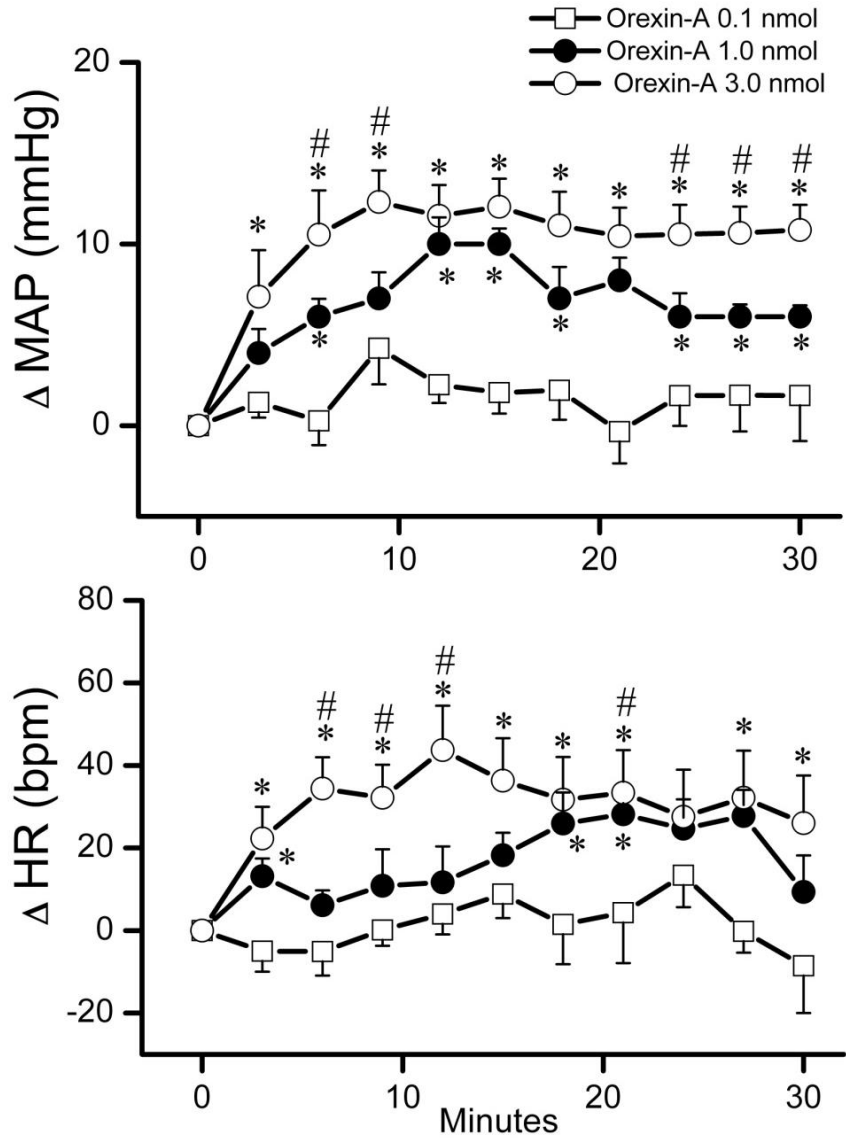
Fig. 4.5. Orexin-A-evoked hemodynamic effects in conscious rats is mediated via OX$_1$R. Time course of changes in mean arterial pressure ((ΔMAP, top panel) and heart rate (ΔHR, bottom panel) evoked by i.c. 3 nmoles of orexin-A (Vehicle + orexin-A) (n=6) or an equal volume of its vehicle, indicated by the arrow (top panel), in conscious rats pretreated, 10 min earlier, with the selective OX$_1$R receptor agonist SB-408124 (10 nmole/rat, i.c) or equal volume of vehicle (SB-408124 + orexin-A) (n=8) and (SB-408124 + Veh) (n=5). Values are mean $\pm$ S.E.M.. * or # $P < 0.05$ versus respective "SB-408124+ Veh" or "SB-408124 + orexin-A" values, respectively.
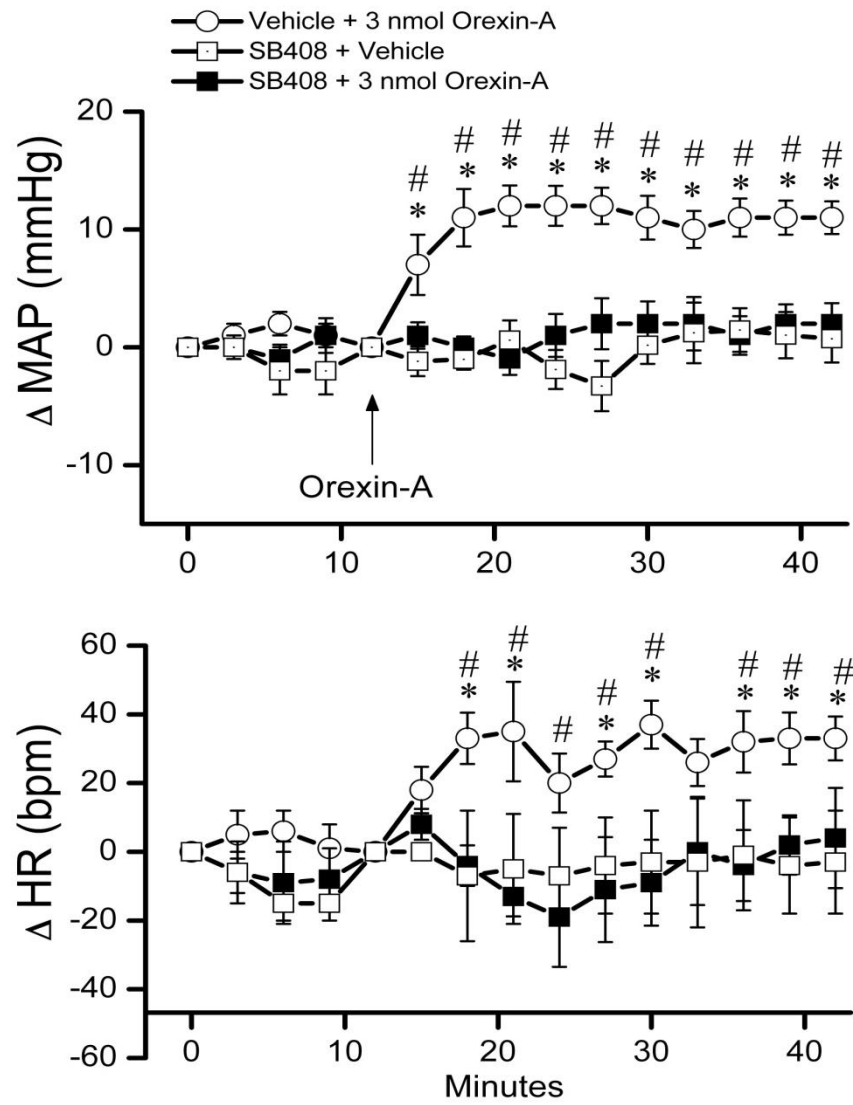
Fig. 4.6. Brainstem OX$_1$R signaling is pivotal to i.c. WIN55,212-2-evoked sympathoexcitation in conscious rats. Time course of changes in mean arterial pressure (ΔMAP, top panel), heart rate (ΔHR, bottom panel) evoked by i.c. WIN55,212-2 (15 µg/rat) (Veh + WIN55) or an equal volume of its vehicle, indicated by the arrow (top panel), in conscious rats pretreated, 10 min earlier, with the selective OX$_1$R antagonist SB-408124 (10 nmoles /rat, i.c) or equal volume of vehicle (SB-408124 + WIN55) and (SB-408124 + Veh). Values are mean $\pm$ S.E.M. of 5 to 8 observations. * or # $P < 0.05$ versus respective "SB-408124 +Veh" or "SB-408124 + WIN55" values, respectively.

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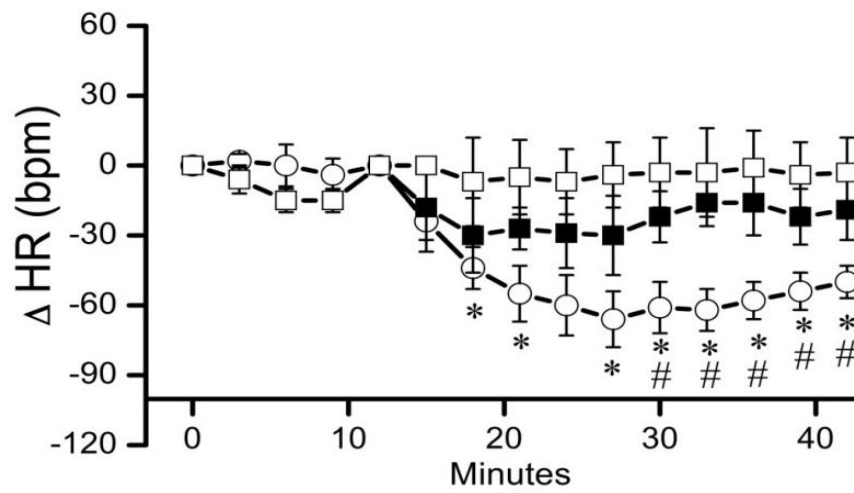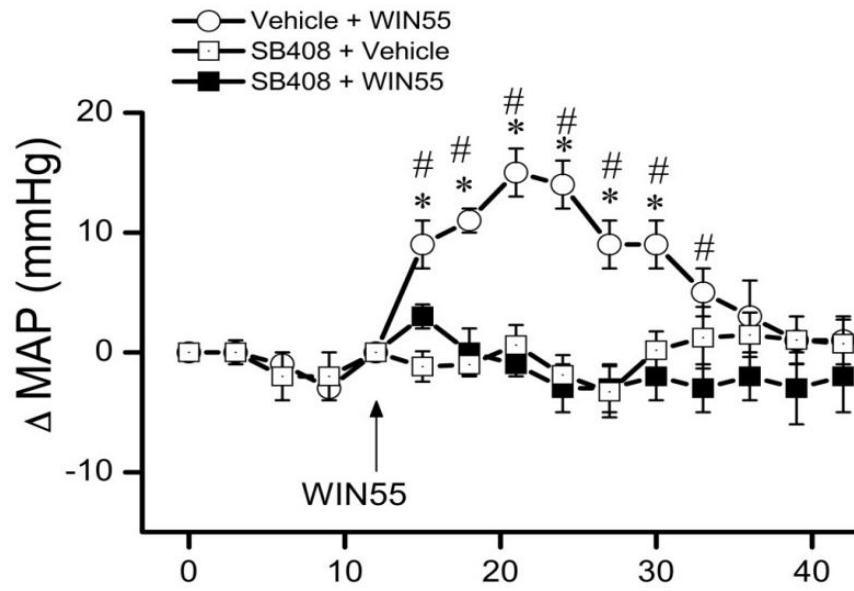
Fig. 4.7. Blockade of brainstem CB₁R signaling has no effect on i.c. orexin-A-evoked sympathoexcitation in conscious rats. Time course of changes in mean arterial pressure (ΔMAP, top panel), heart rate (ΔHR, bottom panel) evoked by i.c. orexin-A (3 nmoles/rat) (Veh + orexin-A), (reblotted from Fig. 4.5.) or an equal volume of its vehicle, indicated by the arrow (top panel), in conscious rats pretreated 30 min earlier with selective CB₁R antagonist, AM251 (30 μg /rat, i.c) (n= 7) or equal volume of vehicle (AM251 + orexin-A) and (AM251 + Vehicle) the later was reblotted from Fig. 3.5. Values are mean ± S.E.M. of 5 to 8 observations. * $P < 0.05$ vs respective (AM251+vehicle) values.
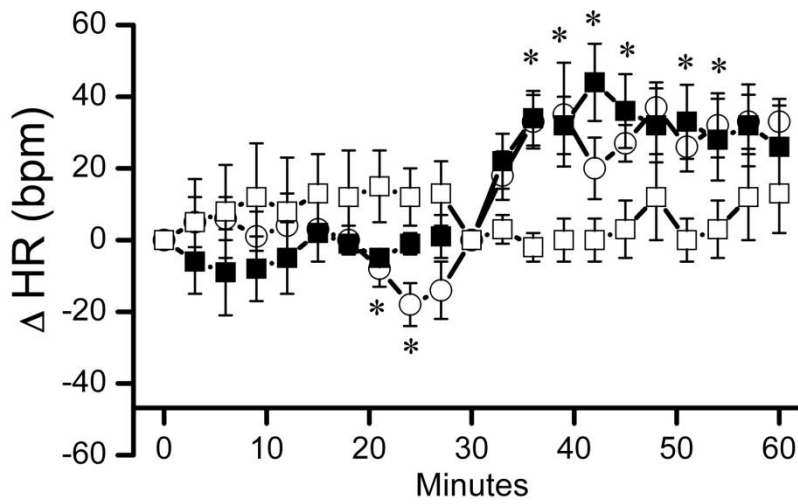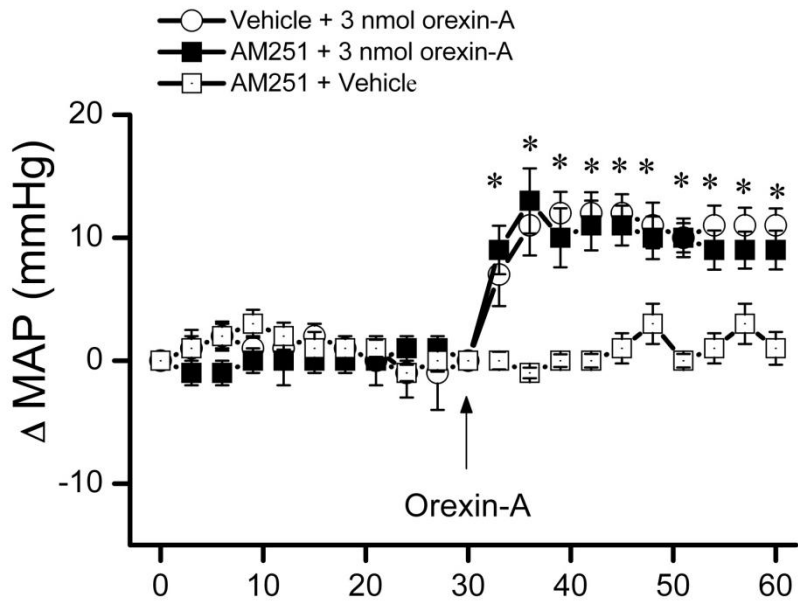
4. 6. 3. Central OX_1R activation underlies orexin-A-evoked pressor response in conscious rats

Intracisternal orexin-A (0.1, 1, 3 nmoles/rat) elicited dose-related increases in MAP in conscious unrestrained rats (Fig. 4.4). This pressor response was mediated via activation of OX_1R because i.c. pretreatment with the selective OX_1R antagonist SB-408124 (10 nmoles/rat) virtually abolished the orexin-A-evoked pressor response (Fig. 4.5).

4. 6. 4. Evidence for the involvement of orexin-A/ OX_1R signaling in WIN55,212-2-evoked pressor response

In this experiment, we employed a dose of the selective CB_1R and OX_1R antagonists that adequately blocked the pressor response elicited by i.c. administration of the respective agonist (see above). Pretreatment with the selective OX_1R antagonist SB-408124 (10 nmoles/rat, i.c) attenuated the WIN55,212-2 (15 μg/rat, i.c)-evoked pressor response (Fig. 4.6). On the other hand, pretreatment with the selective CB_1R antagonist AM251 (30 μg/rat, i.c) had no significant effect on the pressor response elicited by orexin-A (3 nmoles/rat, i.c) (Fig. 4.7).

4. 7. Discussion

In this study we tested the hypothesis that orexin-A/OX$_1$R signaling in the brainstem contributes to the central CB$_1$R-evoked pressor response. The most important findings of the current study are: (i) centrally administered WIN55,212-2 (CB$_1$R agonist) elicited significant increases in blood pressure and orexin-A levels in the RVLM; (ii) the pressor response and elevation of RVLM orexin-A levels elicited by WIN55,212-2 were virtually abolished by prior blockade of central CB$_1$R (AM251); (iii) prior blockade of central OX$_1$R (SB-408124) abrogated the elevations in blood pressure caused by CB$_1$R activation; (iii) by contrast, prior blockade of CB$_1$R (AM251) had no effect on the pressor response elicited by central OX$_1$R activation by orexin-A; (iv) CB$_1$R and OX$_1$R are colocalized in tyrosine hydroxylase expressing (presympathetic) neurons along with orexin-A fibers in the RVLM. These findings implicate brainstem orexin-A/OX$_1$R in the central CB$_1$R-evoked pressor response in conscious rats.

The CB$_1$R exists mostly presynaptically and modulates synaptic transmission of both inhibitory (GABA) and excitatory (glutamate) neurotransmitters (Freund, Katona et al. 2003; Piomelli 2003; Drew, Mitchell et al. 2008). For this reason, it has been suggested that central CB$_1$R-evoked sympathoexcitation is probably mediated via indirect modulation of the sympathetic neuronal activity in the RVLM, by converging excitatory and inhibitory inputs (Pilowsky and Goodchild 2002; Padley, Li et al. 2003). In support of this notion, findings in cultured RVLM neurons showed that CB$_1$R activation inhibited GABAergic transmission (Vaughan, McGregor et al. 1999). Whether this mechanism results in activation of excitatory mediators, e.g. orexins, within the brainstem has not been investigated. Therefore, we reasoned that orexin and its receptors in the RVLM might play important role in the CB$_1$R-mediated

pressor response because *in vitro* evidence suggests crosstalk between the CB_1R and orexin systems (Hilairet, Bouaboula et al. 2003; Ellis, Pediani et al. 2006). Further, such crosstalk is functionally relevant as indicated by *in vivo* (feeding) studies (Crespo, Heras et al. 2008; Verty, Boon et al. 2009). Perhaps a potential crosstalk between these two systems in a neuronal pool that controls sympathetic flow (RVLM) has remained unexplored because orexins-containing neurons are restricted to the lateral hypothalamic area (de Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998). Nonetheless, the axons of these neurons innervate different areas of the CNS, including the medullary cardiovascular regulatory nuclei RVLM and NTS (Machado, Bonagamba et al. 2002; Zheng, Patterson et al. 2005). Further, locally injected orexins directly excite RVLM presympathetic neurons (Chen, Hwang et al. 2000; Machado, Bonagamba et al. 2002; Huang, Dai YW et al. 2010). In support of our hypothesis, we demonstrate, for the first time, that central CB_1R activation significantly elevated orexin-A levels in the RVLM along with the pressor response. It was important, however, to determine if the increase in RVLM-orexin-A levels caused by WIN55-212-2 was CB_1R-mediated and plays a causal role in the pressor response. Both assumptions were confirmed because selective blockade of central CB_1R (AM251, i.c) abrogated both the neurochemical and blood pressure responses elicited by WIN55,212-2.

In agreement with reported findings (Chen, Hwang et al. 2000), we show that i.c orexin-A elicited dose-dependent increases in MAP and HR in conscious rats. Because orexin-A has 10-fold higher affinity for OX_1R, unlike OX_2R that possesses equal sensitivity to both orexins-A and B (Sakurai, Amemiya et al. 1998), we reasoned that orexin-A-evoked hemodynamic responses are mediated via OX_1R. Contrary to this view, however, recent findings, which may

have been confounded by the use of anesthesia, suggest that i.c. orexin-A elicits cardiovascular responses, mostly via OX_2R activation, with little contribution from OX_1R (Huang, Dai YW et al. 2010). Nonetheless, the present finding that blockade of central OX_1R (SB-408124, i.c) abrogated the orexin-A-evoked pressor response along with the following reported findings support our conclusion that the central pressor effect elicited by orexin-A is mediated via OX_1R activation: (i) OX_1R antagonist (SB-334867), but not OX_2R-antiserum, totally abolished the cardiovascular responses elicited by intra-NTS (Shih and Chuang 2007) or i.c. (Huang, Dai YW et al. 2010) orexin-A; (ii) orexin-A produced approximately 2 fold increases in MAP/sympathetic activity compared to the effects produced by same dose of orexin-B (Samson, Gosnell et al. 1999; Shirasaka, Nakazato et al. 1999; Chen, Hwang et al. 2000; Shih and Chuang 2007). Together, we concluded that the pressor response elicited by i.c. orexin-A in our model system is mediated via central OX_1R activation. We, then, utilized a dose of the OX_1R selective antagonist SB-408124, which appropriately blocked the pressor response elicited by i.c. orexin-A, to support our hypothesis that orexin-A signaling in the brainstem plays a pivotal role in the CB_1R-mediated pressor response. .

We present the first *in vivo* evidence that prior central blockade of OX_1R (SB-408124) abolished the central CB_1R (WIN55,212-2)-evoked pressor response. This finding is in line with the molecular and behavioral (feeding) studies that suggest reciprocal interactions between CB_1R and OX_1R (Hilairet, Bouaboula et al. 2003; Ellis, Pediani et al. 2006; Crespo, Heras et al. 2008). Indeed, it is not clear at what level these two receptors interact in central cardiovascular regulating areas. Nevertheless, our study has established a link between CB_1R-evoked release of orexin-A and activation of OX_1R in the RVLM in the pressor response elicited by central CB_1R

activation. Importantly, blockade of brainstem CB_1R (AM251) did not alter the hemodynamic effects elicited by i.c. orexin-A. This latter observation strongly suggests that the i.c. orexin-A-mediated pressor response is not dependent on CB_1R signaling. This finding might be organ/tissue dependent because the CB_1R antagonist SR-141716A attenuated the OX_1R-evoked increase in p-ERK1/2 in cells co-expressing the two receptors (Hilairet, Bouaboula et al. 2003).

In summary, we provide the first evidence for a functional crosstalk between cannabinoid and orexin systems within the medullary cardiovascular controlling centers. CB_1R activation in the brainstem elicited a pressor response along with an elevation of orexin-A levels in the RVLM. Importantly, blockade of orexin-A/OX_1R signaling abrogated the CB_1R-evoked pressor response. On the other hand, the orexin-A/OX_1R-evoked pressor response was not affected by blockade of CB_1R. Colocalization studies have unraveled close proximity of orexin-A/OX_1R to CB_1R labeled neurons and punctuated structures, which support the pharmacological findings. It is important to highlight that characterizing the interaction between orexins and cannabinoids is clinically relevant given the established link between hypertension and obesity.

CHAPTER FIVE-DIFFERENTIAL MODULATION OF BRAINSTEM PI3K/Akt AND ERK1/2 SIGNALING UNDERLIES CENTRALCB$_1$ RECEPTOR-MEDIATED PRESSOR RESPONSE IN CONSCIOUS RATS

5. 1. Abstract

We have previously shown that central activation of CB$_1$R (WIN55,212-2) elicits pressor response in conscious rats. The cellular mechanisms that underlie the WIN55,212-2 pressor effect are not known. Because cellular and behavioral studies have identified a role for the PI3K/Akt and ERK1/2 pathway in CB$_1$R signaling, we tested the hypothesis that brainstem CB$_1$R-mediated modulation of PI3K/Akt and ERK1/2 signaling underlies the WIN55,212-2-mediated pressor response. Intracisternal injection (i.c) of 15 μg WIN55,212-2 significantly increased ERK1/2 and decreased Akt phosphorylation in the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarii (NTS). The cellular responses elicited by central CB$_1$R activation were abrogated by pretreatment with the selective CB$_1$R antagonist AM251 (30 μg/rat, i.c). Further, pretreatment with PD98059 (ERK/MEK inhibitor, 10 μg/rat, i.c) attenuated the WIN55,212-2-mediated pressor response and the increase in neuronal pERK phosphorylation. On the other hand, i.c. wortmannin (PI3K inhibitor, 0.428 μg/rat) pretreatment augmented the dose-dependent pressor response and the elevation in neuronal pERK phosphorylation elicited by WIN55,212-2 (7.5 or 15 μg/rat, i.c). The present study yields new insight into the role of the brainstem ERK1/2 and PI3K/Akt pathway in the pressor response elicited by central CB$_1$R activation.

5. 2. Introduction

The cellular mechanism(s) that underlie(s) the pressor effects of central CB_1R activation are understudied. Cellular and behavioral studies have identified a crucial role for the PI3K/Akt and ERK1/2 signaling in many CB_1R-evoked actions. For example, CB_1R activation causes rapid ERK1/2 phosphorylation that is PI3K-dependent in some neuronal cell lines, e.g. U373 MG human astrocytoma cells (Galve-Roperh, Rueda et al. 2002), but not in hippocampal neurons (Derkinderen, Valjent et al. 2003). This difference might be accounted for, at least partly, by the dependence of PI3K/Akt signaling on ERK1/2 activation in some tissues but not in others. This notion is supported by the *in vitro* study showing that the $\Delta^{(9)}$-THC-mediated anti-cancer effect in human prostate cells involved PI3K/Akt and ERK1/2 signaling pathway activation (Sanchez, Ruiz-Llorente et al. 2003). By contrast, systemic THC activated the PI3K/Akt pathway in mouse hippocampus, striatum, and cerebellum via a mechanism that does not involve activation of ERK1/2 (Ozaita, Puighermanal et al. 2007). On the other hand, it was demonstrated that CB_1R activation inhibits both PI3K/Akt and ERK1/2 signaling in some *in vitro* studies (Ellert-Miklaszewska, Kaminska et al. 2005; Greenhough, Patsos et al. 2007).

Notably, brainstem ERK1/2 signaling plays an important role in central control of blood pressure. Prolonged inhibition of ERK1/2 phosphorylation in the RVLM gradually lowered blood pressure in both normotensive and hypertensive rats (Seyedabadi, Goodchild et al. 2001). Furthermore, both short and long-term activation of ERK1/2 underlies the angiotensin II-mediated pressor response in the RVLM (Chan, Hsu et al. 2005; Chan, Wang et al. 2007). However, previous reports from the advisor's laboratory have shown that ERK1/2 phosphorylation in the RVLM plays a causal role in the acute hypotensive response elicited by

activation of central α_{2A} adrenergic and imidazoline receptors (Zhang and Abdel-Rahman 2005; Nassar and Abdel-Rahman 2008). Notably, PI3K/Akt signaling evokes a depressor response and its activation in the NTS mediates insulin-evoked hypotensive and bradycardic responses (Huang, Lu et al. 2004). Importantly, it was shown that both the brainstem (RVLM and NTS) and the hypothalamic (PVN) PI3K pathway is elevated in spontaneous hypertensive rats (SHR) compared to the normotensive counterparts, Wister-Kyoto rats (WKY) (Veerasingham, Yamazato et al. 2005; Zubcevic, Waki et al. 2009). In the latter study, chronic blockade of PI3K in the NTS resulted in exacerbation of hypertension in SHR but not WKY, suggesting a restraining (depressor) role for PI3K pathway on BP in SHR. Importantly, indirect evidence based on studies in cultured neurons implicates ERK1/2 and PI3K/Akt signaling in CB_1R modulation of neuronal signaling (Vaughan, McGregor et al. 1999; Wang, Liu et al. 2003); (Huang, Lu et al. 2004; Wang, Ou et al. 2005). Whether PI3K/Akt and or ERK1/2 signaling are involved in the pressor response elicited by central CB_1R activation has not been investigated.

The aim of the present study was to test the novel hypothesis that PI3K/Akt and/or ERK1/2 signaling contributes to the central CB_1R-mediated sympathoexcitation/pressor response. To test this hypothesis, we investigated the effect of i.c WIN55,212-2 on MAP and HR and the associated changes in phosphorylation of Akt, a PI3K downstream effector, and ERK1/2 in the RVLM and NTS. To ascertain a causal involvement of the observed changes in these signaling molecules in the central CB_1R-mediated pressor response, we investigated the effects of pretreatment with CB_1R antagonist (AM251), PI3K inhibitor (wortmannin) and ERK1/2 inhibitor (PD98059) on the neurochemical (pAkt and pERK1/2) and blood pressure responses elicited by CB_1R activation.

5. 3. Materials and Methods

5. 3. 1. Western Blotting

Samples were obtained at the conclusion of the hemodynamic measurement and prepared for western blotting to measure changes in Akt and ERK1/2 phosphorylation in the RVLM and NTS. Nitrocellulose membranes were prepared as described under general methods (Chapter 2) and then incubated for 48 hr at 4ºC with mixture of mouse monoclonal anti-phospho-Akt antibody (pSer473) (1:1000; cat. # 4051S) and rabbit polyclonal anti-Akt antibody (1:1000; cat. # 9272) or mouse monoclonal anti-phospho-ERK1/2 (T202/Y204) antibody (1:000; cat.# 9106S) and rabbit polyclonal anti-ERK antibody (1:1000; cat.# 9102) in Odyssey blocking buffer. All four antibodies were purchased from Cell Signaling Technology (Danvers, MA). Membranes were washed 4x with PBST then incubated for 60 min at room temperature with mixture containing IRDye680-conjugated goat anti-rabbit and IRDye800-conjugated goat anti-mouse (1:1500; LI-COR Biosceinces, Lincoln, NE). Membranes were washed 4x with PBST and once in PBS before being scanned. Bands representing phosphorylated and total protein were detected simultaneously using the Odyssey dual infrared fluorescence scanner (LI-COR Biosciences, Lincoln, NE). All data were averaged values of integrated density ratio of phosphorylated protein (pAkt or pERK1/2) normalized to corresponding total protein (tAkt or ERK1/2) expressed as percentage of control (vehicle), analyzed with Odyssey application software *v.*3 (LI-COR Biosciences, Lincoln, NE).

5. 3. 2. Drugs

WIN55,212-2, wortmannin, PD98059 and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). AM251 was purchased from Cayman Chemical (Ann Arbor, MI). Alkamus was purchased from Rhone-Poulenc (Cranbury, NJ). WIN55,212-2 and AM251 (30 μg/rat) were dissolved in (1:1:18) mixture of DMSO/Alkamus/sterile saline. Wortmannin was dissolved in 10% DMSO in saline (1nmole ~ 0.4283 μg/rat). Wortmannin dose and vehicle were based on previous report (Narita, Ohnishi et al. 2002). PD98059 was dissolved in DMSO (10 μg/rat) as in a previous study (Zhang and Abdel-Rahman 2005). Each vehicle was tested on at least three animals prior to its utilization. As none of these vehicles significantly changed the basal MAP and HR, we refer to all of them as (vehicle).

5. 4. Protocols and experimental groups

5. 4. 1. Experiment 1: Effects of i.c. WIN55,212-2 on RVLM and NTS Akt and ERK1/2 phosphorylation

We investigated the effects of i.c WIN55,212-2 on Akt/ERK1/2 phosphorylation in the brainstem at a time that preceded the pressor response as was previously discussed in Chapter 3. Brain tissues were collected from animals sacrificed 5 min after i.c. WIN55,212-2 (15 μg/rat) or vehicle (n=3-5) and prepared for Western blotting as detailed under methods to detect and quantify phosphorylated Akt (pAKt) and ERK1/2 (pERK1/2) phosphorylation in the RVLM and NTS.

Fig. 5.1. Time line for surgical procedures and experimental protocols for studies under this aim. * In the PD98059 pretreated group animals received WIN55,212-2, 20 min later.

Catheterization
i.c. cannulation

Blood pressure
recording

Euthan.
Tissue collect.

day -5

-90 min

Stabliz.
Baseline

0 min

Vehicle,
AM251,
Wortmannin,
*PD98095

30 min

15 µg WIN55,212-2
or Vehicle

60 min
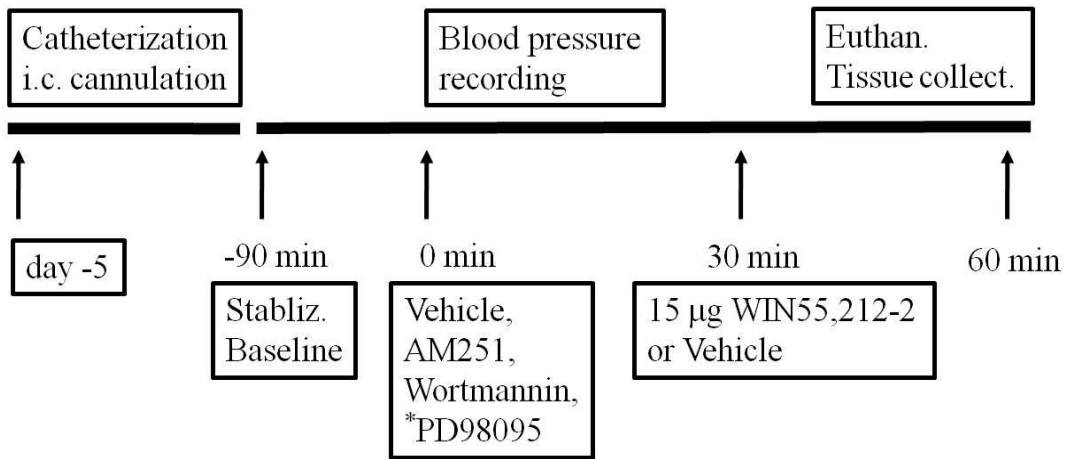
5. 4. 2. Experiment 2: Effect of PI3K inhibition on the dose-related pressor response

    elicited by central WIN55,212-2 administration

This experiment aimed to elucidate the effect of PI3K/Akt inhibition (wortmannin) on the WIN55,212-2-evoked pressor response. Four groups (n=5-8) of rats received wortmannin (0.428 µg) 30 min prior to WIN55,212-2 (7.5 or 15 µg/rat, i.c) or its vehicle. Hemodynamic measurements were continued for 30 min after WIN55,212-2 or vehicle. The data were compared to the hemodynamic effects of WIN55,212-2 given alone or its vehicle as reported in Chapter 3, experiment 3. 4. 2.

5. 4. 3. Experiment 3: Effect of ERK1/2 inhibition on WIN55,212-2 centrally-elicited

    hemodynamic effects

In this experiment we sought to delineate the effect of ERK1/2 inhibition on WIN55,212-2-mediated central pressor and bradycardia responses. BP and HR were recorded in two groups of rats (n=6-8) that received the pharmacological ERK1/2 inhibitor, PD98059 (10 µg/rat, i.c) 20 min before WIN55,212-2 (15 µg/rat, i.c) or its vehicle.

Furthermore, we investigated the effect of i.c. WIN55,212-2 on phosphorylation levels of NTS and RVLM-Akt and ERK1/2 following blockade of CB_1R (AM251), PI3K (wortmannin) or ERK1/2 (PD98059) signaling, by collecting brain tissues (NTS and RVLM) at the completion of the above experiments (5. 4. 2) and (5. 4. 3) and from animals in experiment (3. 4. 2) Chapter 3. Phosphorylation levels of Akt and ERK1/2 in NTS and RVLM neurons were measured by Western blotting and compared to corresponding total protein as detailed under methods.

5. 5. Statistical Analysis

Analysis of hemodynamic data was performed as detailed in Chapter 3. A one-way ANOVA was used to evaluate the effect of various treatments on pAkt and pERK1/2 in the NTS or RVLM in experiments 5. 4. 2 and 5. 4. 3. $P < 0.05$ was considered significant.

5. 6. Results

Baseline MAP and HR were not significantly different in all groups of rats used in the study (Table. 5.1). Further, MAP and HR were similar following different pretreatments (vehicle, wortmannin or PD98059) at the time of WIN55,212-2 or vehicle injection (Table. 5.1).

5. 6. 1. WIN55,212-2 enhances pERK1/2 and reduces pAkt in NTS and RVLM neurons

WIN55,212-2 (15 μg/rat, i.c) increased MAP and reduced HR as previously shown in Chapter 3; however, animals were sacrificed 5 min after treatment, at a time that preceded the peak pressor response. pERK1/2 phosphorylation was significantly ($P < 0.05$) elevated in NTS and RVLM of animals treated with WIN55,212-2 compared to vehicle treated animals as shown in (Fig. 5.2). Further, i.c. WIN55,212-2 significantly lowered NTS and RVLM pAkt phosphorylation compared to vehicle treated animals ($P < 0.05$). Fig. 5.2 shows representative pERK1/2 and pAkt bands along with total protein bands obtained as described under methods from representing animals for each WIN55,212-2 (n=5) or vehicle (n=3) treated groups.

Table. 5.1. Baseline MAP (mmHg) and HR (bpm) values before and after pretreatment (preceding i.c. vehicle or WIN55,212-2). Values are means ± S.E.M.

Treatment	*n*	Before		After	
		MAP	**HR**	**MAP**	**HR**
Vehicle + 7.5 μg WIN55,212-2	6	111.3±3.0	375±10	108.0±5.0	350± 11
Wortmannin + Vehicle	5	112.0±6.0	356±15	117.0±6.0	376±15
Wortmannin + 7.5 μg WIN55,212-2	8	112 ± 6.0	360± 11	111.0 ±5.0	380±10
Wortmannin + 15 μg WIN55,212-2	8	111.3±3.0	375±10	112.0±3.0	363±13
PD98059 + Vehicle	5	113.0 ±4.0	398±16	110.0 ±4.0	390±20
PD98059 + 15 μg WIN55,212-2	8	117.0±6.0	400±12	115.0±4.0	410±12

Fig. 5.2. Intracisternal WIN55,212-2 reduces Akt and enhances ERK1/2 phosphorylation in NTS and RVLM neurons.
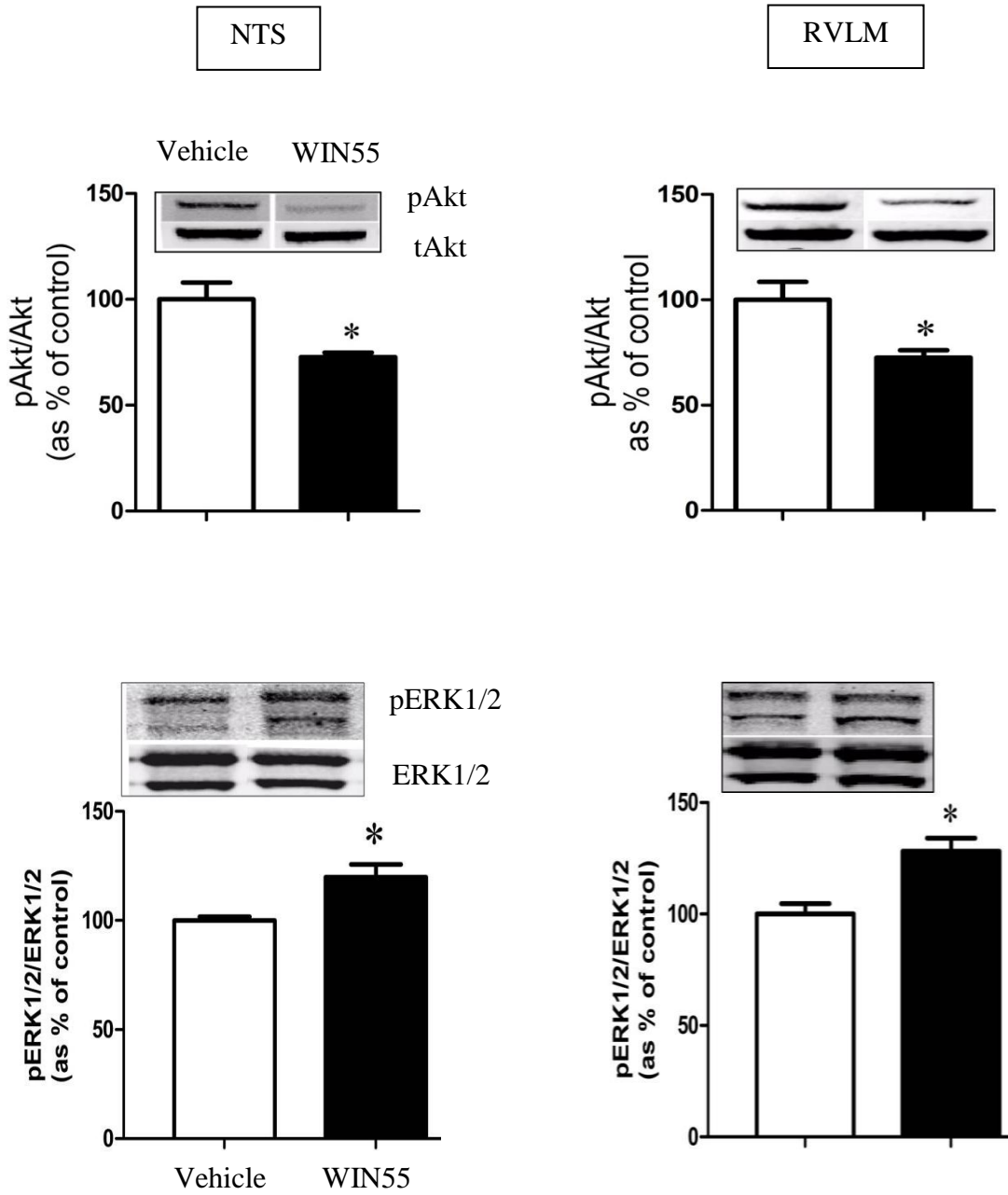
5. 6. 2. PI3K inhibition exacerbates the dose-dependent pressor response elicited by central CB_1R activation.

Pretreatment with the PI3K inhibitor, wortmannin (0.42 µg/rat, i.c) significantly enhanced the WIN55,212-2 (15 µg i.c)-evoked pressor and bradycardic responses (Fig. 5.3.B, re-blotted from Fig. 3.5, Chapter 3). To further confirm the involvement of PI3K phosphorylation in the WIN55,212-2-evoked pressor response, we investigated the effect of wortmannin pretreatment on a modest pressor response elicited by a lower dose of WIN55,212-2 (7.5 µg/rat, i.c) in an additional group of rats. As shown in Fig. 5.3.A, pretreatment with wortmannin significantly enhanced the pressor response elicited by WIN55,212-2 (7.5 µg/rat, i.c). However, wortmannin did not significantly alter the bradycardic effect of WIN55,212-2 (7.5 µg) at this dose. Wortmannin pretreatment did not significantly alter baseline MAP or HR, Table. 5.1.

5. 6. 3. ERK1/2 inhibition (PD98059) attenuates the pressor response elicited by central CB_1R activation

To investigate whether brainstem pERK1/2 signaling is implicated in the central CB_1R-mediated pressor response, conscious rats were pretreated with the MEK/ERK/1/2 inhibitor, PD98059, before WIN55,212-2 (15 µg, i.c). PD98059 (10 µg/rat, i.c) had no significant effects on baseline MAP and HR during the observation period (Table. 5.2). However, PD98059 pretreatment significantly attenuated the pressor response elicited by central CB_1R activation (Fig. 5.4).

5. 6. 4. Neurochemical effects of WIN55,212-2 (pAkt and pERK1/2) in the presence or absence of AM251, wortmannin or PD98059 in rat NTS and RVLM

These molecular studies were undertaken to complement the findings with the pharmacological interventions described above and to more directly elucidate the role of the pAkt-ERK1/2 pathway in the pressor response elicited by central CB_1R activation. Compared to vehicle treated rats, WIN55,212-2 elicited a significant ($P < 0.05$) elevation in ERK1/2 phosphorylation and produced a dose-related reduction in Akt phosphorylation in the RVLM and NTS (Fig. 5.5 A and D) and (Fig. 5.6 A and D). Further, these WIN55,212-2-evoked neurochemical responses were abrogated by AM251 pretreatment (Fig. 5.5 A and D, and Fig. 5.6 A and D). Further, PD98059 abrogated the WIN55,212-2-induced reduction in Akt phosphorylation (Fig. 5.5 C and F). Finally, PD98059 abrogated ($P < 0.05$), while wortmannin slightly enhanced ($P > 0.05$) the WIN55,212-2-evoked upregulation of ERK1/2 phosphorylation in the RVLM and NTS (Fig. 5.6 B, C, E and F).

Fig. 5.3. PI3K inhibition exacerbates the dose-dependent pressor response elicited by central CB₁R activation. Time course of changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) evoked by intracisternal injection of WIN55,212-2 in presence or absence of PI3K inhibition. Effects of WIN55,212-2 (WIN; 7.5 μg/rat) in **A)** or (WIN; 15 μg/rat) in **B)** or an equal volume of its vehicle (Veh1) in conscious rats pretreated, 30 minutes earlier, with wortmannin (WORT) [phosphatidylinositol 3-kinase (PI3K) inhibitor, 0.428 μg/rat, i.c.] or an equal volume of its vehicle (Veh2). **A)** * or # $P < 0.05$ versus respective "Veh2 + Veh1" and "WORT + Veh1" or "Veh2 + 7.5 μg WIN" values. **B)** * or # $P < 0.05$ versus respective "Veh2 + Veh1" and "WORT + Veh1" or "Veh2 + 15 μg WIN" values.

A)
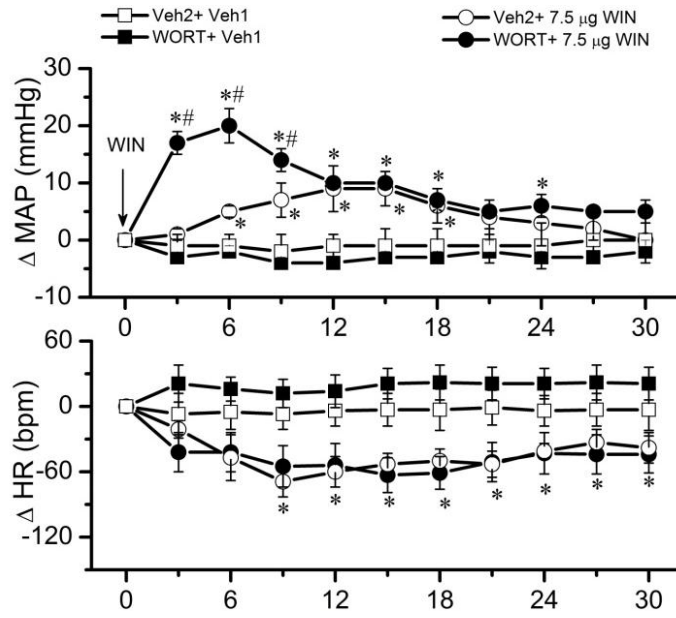
B)
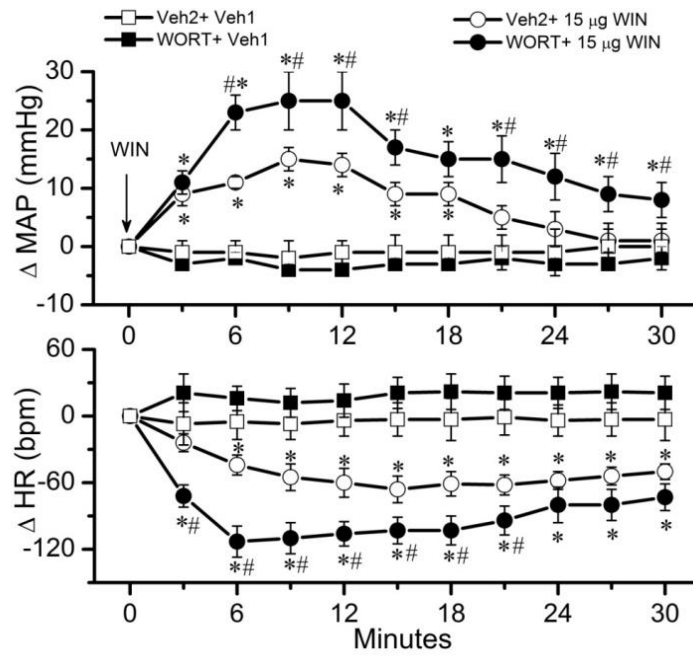
Fig. 5.4. pERK1/2 inhibition (PD98059) attenuates the pressor response elicited by central CB$_1$R activation. Time course of changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) evoked by intracisternal injection of WIN55,212-2 (WIN; 15 μg/rat) or an equal volume of its vehicle (Veh1) in conscious rats pretreated, 20 minutes earlier, with PD98059 (PD098) [MEK/ERK1/2 phosphorylation inhibitor, 10 μg/rat, i.c.] or equal volume of its vehicle (Veh3). Values are mean $\pm$ S.E.M. of 5 to 8 observations. * or # $P < 0.05$ versus respective "Veh3 + Veh1" and "PD98059 + Veh1" or "Veh3 + 15 μg WIN" values.

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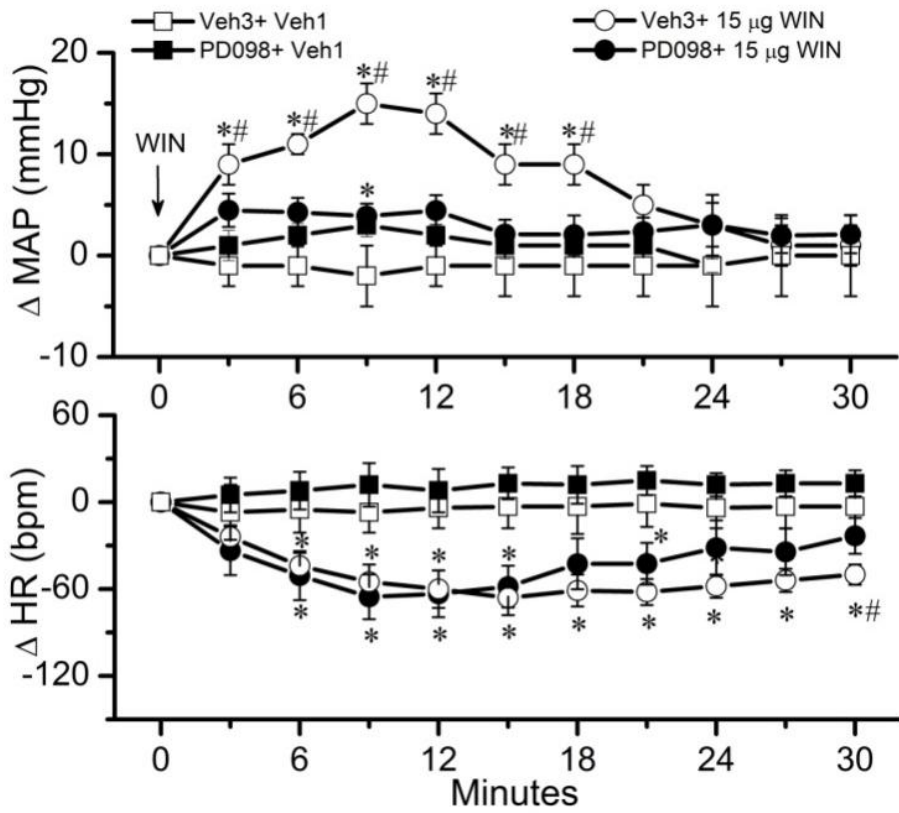
Fig. 5.5. Effects of WIN55,212-2 on pAkt in the presence or absence of AM251, wortmannin or PD98059 in rat NTS and RVLM. Changes in pAkt in rat NTS (top panel; A, B and C) and RVLM (bottom panel; D, E and F) neurons elicited by WIN55,212-2 (i.c) in animals pretreated with vehicle, AM251, (30 μg/rat, i.c) (A and D), wortmannin (Wort; 0.42 μg/rat, i.c) (B and E) or PD98059 (PD098; 10 μg/rat, i.c) (C and F). Data are presented as integrated density ratio of phosphorylated Akt (pAkt) and total Akt (tAkt), expressed as percentage of control (vehicle). Values are mean ± S.E.M. of 4 to 5 observations. * or # $P < 0.05$ versus respective (vehicle) or (vehicle+ 15 μg WIN) values, respectively.
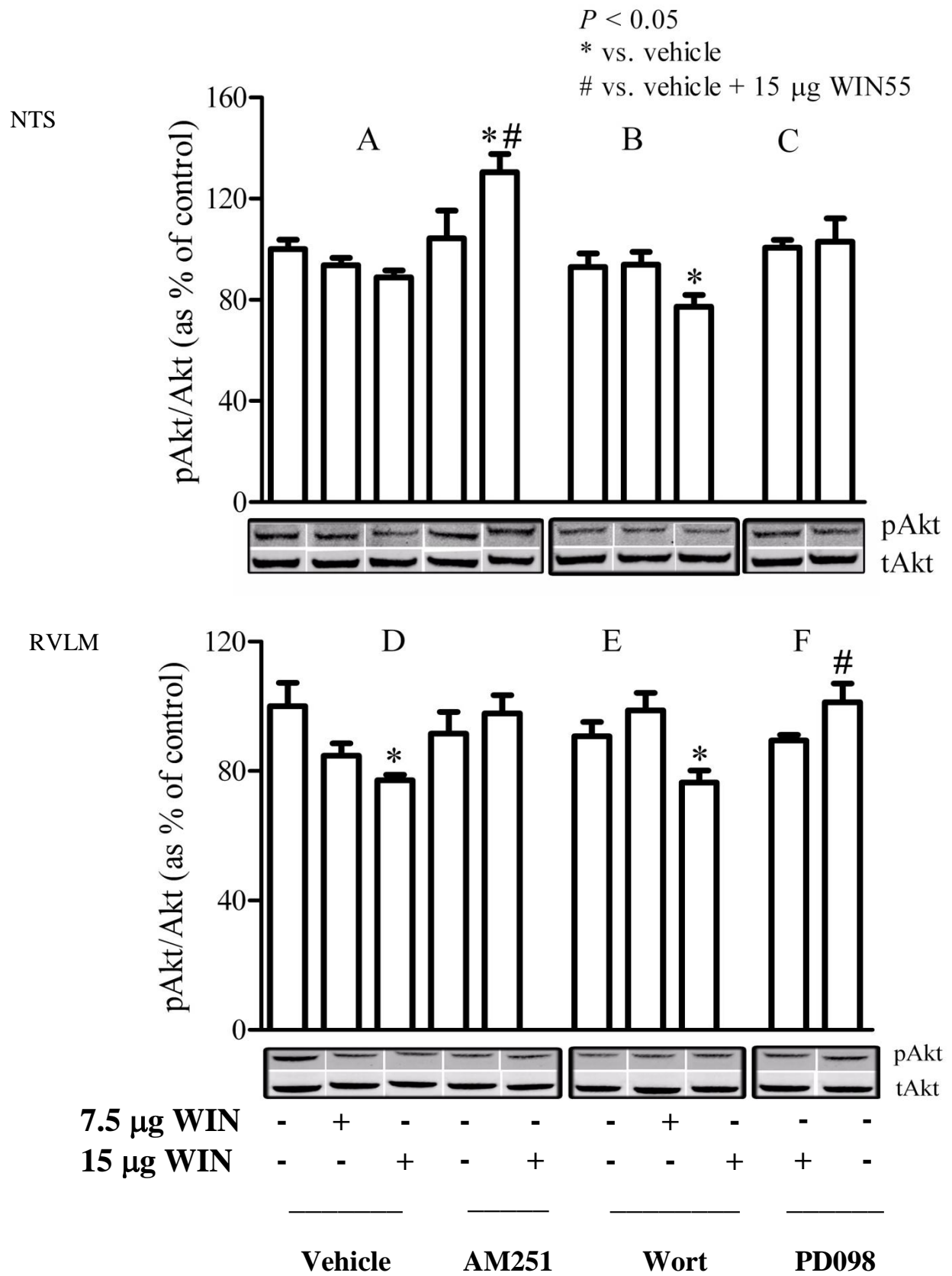
Fig. 5.6. Effects of WIN55,212-2 on pERK1/2 in the presence or absence of AM251, wortmannin or PD98059 in rat NTS and RVLM. Changes in pERK levels in rat NTS (top panel; A, B and C) and RVLM (bottom panel; D, E and F) neurons elicited by WIN55,212-2 (i.c) in animals pretreated with vehicle, AM251, (30 μg/rat, i.c) (A and D), wortmannin (Wort; 0.42 μg/rat, i.c) (B and E) or PD98059 (PD098; 10 μg/rat, i.c) (C and F). Data are presented as integrated density ratio of phosphorylated ERK1/2 (pERK1/2) and total ERK, expressed as percentage of control (vehicle). Values are mean ± S.E.M. of 4 to 5 observations. *, # or & $P < 0.05$ versus respective (vehicle), (vehicle+ 15 μg WIN) or (vehicle + 7.5 μg WIN55,212-2) values, respectivly.

NTS

A   B   C

$P < 0.05$
* vs. vehicle
# vs. vehicle+15 µg WIN55
& vs. vehicle +7.5 µg WIN55

RVLM

D   E   F

7.5 µg WIN	-	+	-	-	-	-	+	-	-	-
15 µg WIN	-	-	+	-	+	-	-	+	+	-

Vehicle	**AM251**	**Wort**	**PD098**

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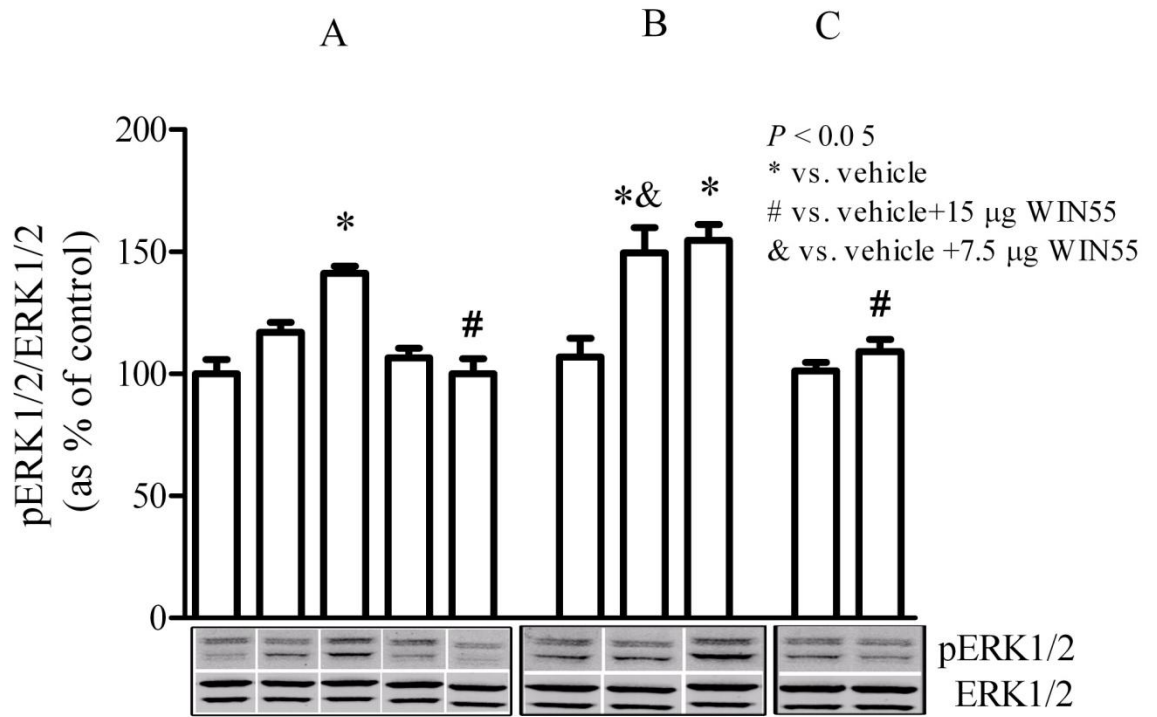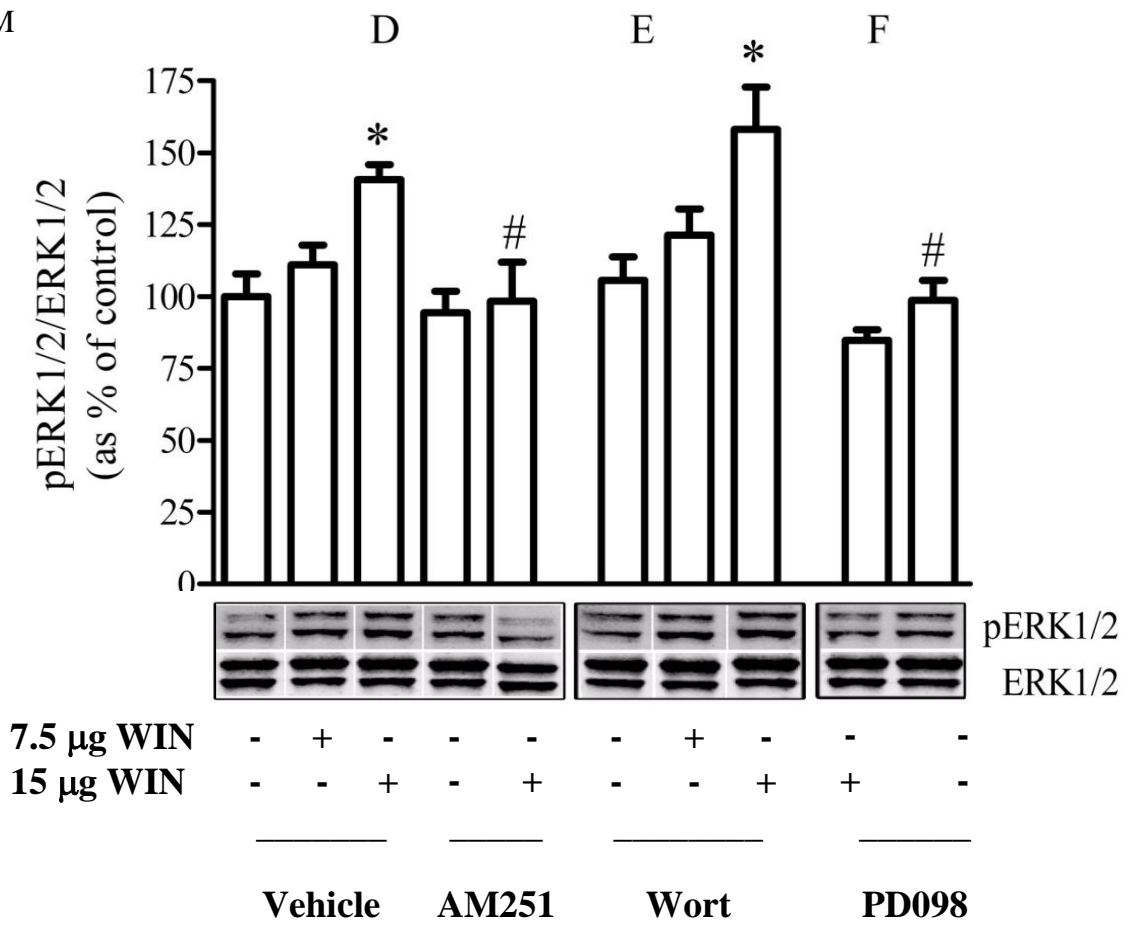
5.7. Discussion

In this study, we tested the hypothesis that alteration in brainstem PI3K/Akt and/or ERK1/2 signaling contributes to the central CB_1R-mediated pressor response. The most important findings of this study are: (i) centrally administered WIN55,212-2, a non-selective CB_1-CB_2 receptor agonist, elicited pressor and bradycardic responses along with significant enhancement of ERK1/2 and reduction of Akt phosphorylation, respectively, in the NTS and RVLM; (ii) the pressor and neurochemical responses elicited by WIN55,212-2 were virtually abolished by central CB_1R blockade (AM251); (iii) prior inhibition of PI3K (wortmannin) enhanced the elevations in blood pressure and pERK1/2 phosphorylation and augmented the reductions in Akt phosphorylation caused by WIN55,212-2, (iv) by contrast, prior inhibition of brainstem ERK1/2 phosphorylation (PD98059) abrogated the elevations in blood pressure and brainstem ERK1/2 phosphorylation without significantly affecting the reduction in brainstem pAkt phosphorylation caused by WIN55,212-2. These findings implicate differential alteration of brainstem PI3K/Akt (reduced) and ERK1/2 (enhanced) signaling as potential cellular mechanisms for the central CB_1R-evoked sympathoexcitation/pressor response in conscious rats.

The sympathoexcitatory effect of central CB_1R activation is well-documented (findings of Chapter 3); however, the underlying mechanisms are not well understood. Herein, we hypothesized that brainstem PI3K/Akt and/or ERK1/2 signaling contributes to the central CB_1R-mediated pressor response. In the current study, we provide the first evidence in support of our hypothesis. We showed that i.c. WIN55,212-2-evoked pressor response is paralleled with a reduction in pAkt phosphorylation in the NTS and RVLM neurons. The findings suggest that the reduction in brainstem Akt phosphorylation plays a causal role in the central CB_1R-mediated

pressor response. In support of this conclusion are the findings that the dose-related inhibition of Akt phosphorylation in the NTS and RVLM elicited by WIN55,212-2 occurs within minutes and precedes the peak pressor response. Our Western blot findings are consistent with findings that CB_1R activation resulted in down-regulation of the PI3K/Akt signaling (Ellert-Miklaszewska, Kaminska et al. 2005; Greenhough, Patsos et al. 2007). However, others have shown that CB_1R activation up-regulated PI3K/Akt signaling in U373 MG human astrocytoma cells (Galve-Roperh, Rueda et al. 2002), hippocampal slices (Derkinderen, Valjent et al. 2003), and *in vivo* (Ozaita, Puighermanal et al. 2007). The differences between the present and reported findings might be due to differences in the model system used or differences in PI3K-Akt signaling response to CB_1R activation in different brain areas. For example, in the latter study, CB_1R activation enhanced PI3K/Akt signaling in mouse hippocampus, striatum and cerebellum but not in the cortex. We are not aware of any reports on the effect of CB_1R activation on PI3K-Akt signaling in the brainstem. Nonetheless, in further support of a causal role for the observed inhibition in brainstem Akt phosphorylation in the central CB_1R-mediated pressor response are the findings that pharmacological inhibition of brainstem PI3K/Akt signaling (wortmannin) significantly enhanced the WIN55,212-2 evoked pressor response. Importantly, the selected wortmannin dose had no significant impact on neuronal pAkt phosphorylation in the NTS or RVLM or on basal blood pressure.

The cellular mechanism that underlies the mediation of the pressor response following inhibition of brainstem PI3K-Akt signaling is not fully understood. There is evidence to suggest that activation of brainstem PI3K/Akt signaling in NTS mediates insulin-evoked hypotensive and bradycardic responses (Huang, Lu et al. 2004). Furthermore, it was shown that brainstem

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(RVLM and NTS) and hypothalamic (PVN) PI3K signaling pathway is elevated in spontaneous hypertensive rats (SHR), compared with the normotensive counterparts, Wister-Kyoto rats (WKY) (Veerasingham, Yamazato et al. 2005; Zubcevic, Waki et al. 2009). In the latter study, chronic blockade of PI3K in the NTS increased blood pressure in SHR but not WKY, suggesting a restraining role (depressor) for PI3K pathway on BP in SHR. Accordingly; we reasoned that CB_1R-evoked pressor response could be mediated, at least partially, via inhibition of brainstem PI3K/Akt signaling. As discussed above, the neurochemical data support this notion (Fig. 5.5). Equally important, the CB_1R-evoked pressor response and brainstem ERK1/2 phosphorylation were both exacerbated in presence of wortmannin. Notably, in the RVLM, chronic inhibition of PI3K/Akt by wortmannin resulted in hypotension, and attenuation of the angiotensin II-evoked pressor response in SHR but not WKY (Seyedabadi, Goodchild et al. 2001). Taken together, these findings suggest different roles for brainstem PI3K/Akt in modulating BP taking into account the type of receptor activated, basal sympathetic tone and BP, and type of the targeted neuronal pool. Nonetheless, the ability of central CB_1R activation to inhibit the restraining influence of brainstem PI3K/Akt pathway in our normotensive rats may unleash endogenous neuronal activators or cause an imbalance between excitatory and inhibitory neurotransmitters in the brainstem.

Multiple lines of evidence suggest that the ERK1/2 pathway underlies diverse physiological functions evoked by CB_1R. Herein, we provide the first evidence that brainstem-ERK1/2 signaling is involved in central CB_1R-mediated pressor response. i.c. WIN55,212-2 significantly elevated pERK1/2 in the NTS and RVLM, two nuclei that were shown in many studies to be involved in the central CB_1R-evoked pressor response (Padley, Li et al. 2003;

Rademacher, Patel et al. 2003). Abrogation of the WIN55,212-2-mediated hemodynamic responses and attenuation of the concomitant activation of ERK1/2 pathway by pretreatment with AM251(i.c), a selective CB_1R antagonist, precluded the involvement of CB_2R. Brainstem pERK1/2 signaling plays a tonic role in the central control of blood pressure because; (i) blunting ERK1/2 (3-7 hours) activity in the RVLM lowered blood pressure in both normotensive and hypertensive rats (Seyedabadi, Goodchild et al. 2001); (ii) PD98059, a MEK/ERK inhibitor, attenuated i.c.v short-term urotensin-mediated pressor response but not the associated tachycardia (Lin, Matsumura et al. 2004); (iii) RVLM-ERK1/2 is pivotal for long-term angiotensin II-mediated central control of cardiovascular functions (Chan, Wang et al. 2007). However, studies from our laboratory suggested that ERK1/2 phosphorylation in the RVLM leads to hypotension in conscious animals (Zhang and Abdel-Rahman 2005; Nassar and Abdel-Rahman 2008). These contradictory observations may suggest a bi-directional role for brainstem ERK1/2 pathway in the central regulation of blood pressure. In the current study, blockade of the brainstem ERK1/2 signaling by i.c. PD98059 abrogated the elevation of pERK1/2 phosphorylation and the pressor response produced by WIN55,212-2; however it did not affect the bradycardic effect.

Careful analysis of the neurochemical changes in pAkt and pERK1/2 detected by Western blot revealed that: whereas wortmannin had no effect on pERK1/2 phosphorylation, it substantially enhanced WIN55,212-2-induced ERK1/2 phosphorylation (more evident in the NTS than RVLM). Additionally, changes in pAkt were not significantly different in animals that received PD98059 alone or followed by WIN55,212-2 compared to vehicle treated animals. Together, we conclude that the effects of WIN55,212-2 on PI3K/Akt are upstream to ERK1/2

signaling because of the significant increase in ERK1/2 phosphorylation by WIN55,212-2 in presence of PI3K/Akt inhibitor, wortmannin.

In summary, the present study highlights for the first time a novel PI3K/Akt/ERK1/2 signaling system in the brainstem (RVLM and NTS) that seems to contribute, at least in part, to the sympathoexcitation caused by brainstem-CB_1R activation in conscious rats. The findings of this study demonstrate that central CB_1R activation elicits down-regulation of PI3K/Akt pathway in the RVLM and NTS along with the pressor response. This notion is further supported by the exacerbation of the WIN55,212-2 evoked pressor response when PI3K/Akt was inhibited by wortmannin. We speculate that inhibition of PI3K/Akt signaling in the brainstem might underlie the CB_1R-mediated modulation of inhibitory (GABA) neurotransmission (addressed in chapter 6). In contrast, CB_1R-elicited sympathoexcitation was associated with an elevation in the pERK1/2 phosphorylation in the brainstem. Further, suppressing ERK1/2 signaling by PD98059 (i.c) abolished CB_1R-pressor response. We conclude that PI3K/Akt signaling is upstream to ERK1/2 signaling because of the significant increase in ERK1/2 phosphorylation by WIN55,212-2 in presence of PI3K/Akt inhibition (wortmannin). Further studies are needed to elucidate the signaling pathways between PI3K/Akt and ERK1/2, which seem to influence the balance between sympathoexcitatory and sympathoinhibitory modulators.

CHAPTER SIX-A CAUSAL LINK BETWEEN CENTRAL CB_1R-EVOKED INHIBITION OF BRAINSTEM GABAERGIC NEUROTRANSMISSION AND THE PRESSOR RESPONSE; IMPLICATION FOR RVLM-nNOS-NO SIGNALING

6. 1. Abstract

Activation of neuronal nitric oxide synthase (nNOS), which leads to NO release, underlies a wide array of CB_1R-evoked pharmacological actions. As discussed earlier, central CB_1R, activation enhances brainstem ERK1/2 phosphorylation and the latter enhances nNOS activity. Therefore, we elucidated the role of p-nNOS-NO in the central CB_1R-evoked pressor response. We simultaneously measured blood pressure, heart rate and real time rostral ventrolateral medulla (RVLM)-NO levels following unilateral intra-RVLM microinjection of the CB_1R agonist WIN55212-2 (100, 200 or 300 pmol/80nL, n=4) in conscious rats. WIN55,212-2-elicited increases in blood pressure were associated with a slight increase in heart rat. Interestingly, RVLM NO levels significantly increased in parallel to the WIN55212-2-evoked hemodynamic effects (n=4, $P < 0.05$). The vehicle for WIN55212-2 had no effect on the measured variables. Additionally, nNOS phosphorylation was significantly higher in the injected RVLM compared to the contralateral (control) RVLM (n=4, $P < 0.05$). Finally, given the well-established link between nNOS-NO signaling and GABAergic transmission in cardiovascular regulatory area, we explored the possibility that enhanced NO generation might inhibit GABAergic transmission and subsequently account for the central CB_1R-mediated pressor response. Activation of central $GABA_A$ receptor by muscimol (0.1 μg/rat, i.c) virtually abolished the WIN55,212-2 (15 μg/rat, i.c)-evoked hemodynamic responses. Additionally, we measured the catecholaminergic (tyrosine hydroxylase immunoreactivity, TH-ir) and nitroxidergic (nNOS-ir) neuronal activity, indirectly

via a marker of neuronal activity, c-Fos, within the RVLM following activation of CB_1R in the presence or absence of AM251 or muscimol. WIN55,212-2 (15 μg/rat, i.c) significantly ($P < 0.05$) increased the percentage of TH-ir and nNOS-ir neurons expressing c-Fos; AM251 or muscimol pretreatment mitigated the increase in RVLM-catecholaminergic and nitroxidergic neuronal activity. Together, these findings suggest a pivotal role for p-nNOS-NO/GABAergic transmission in central CB_1R-evoked pressor response.

6. 2. Introduction

nNOS-NO signaling in the RVLM has been shown to be critically implicated in the central regulation of autonomic functions (Martins-Pinge, Araujo et al. 1999; Martins-Pinge, Garcia et al. 2007; Mayorov 2007; Nassar and Abdel-Rahman 2008). Interestingly, multiple studies have demonstrated a link between nNOS-NO signaling and GABAergic transmission in the cardiovascular regulatory area, e.g. nNOS-generated NO reduced GABA release in the dorsal periaqueductal gray matter and RVLM and was associated with pressor responses (Samuel H H Chan, Ling-Lin Wang et al. 2003; Karlsson, Chaitoff et al. 2007; Martins-Pinge, Garcia et al. 2007). Pertinent to the current investigation, many of CB_1R mediated effects involve activation of nNOS-NO signaling pathway including locomotor activity and thermoregulation (Azad, Marsicano et al. 2001; Jones, Carney et al. 2008). Moreover, presynaptic CB_1R activation inhibits the release of GABA and glutamate at both inhibitory as well as excitatory synapses (Vaughan, McGregor et al. 1999; Kreitzer and Regehr 2001; Ohno-Shosaku, Maejima et al. 2001; Neu, Foldy et al. 2007). Interestingly, studies on hippocampus suggest that nNOS-generated NO is involved in CB_1R-mediated suppression of GABA release (see Chapter 1) (Makara, Katona et al. 2007). Because GABA is the primary inhibitory transmitter which exerts

tonic inhibition on RVLM-presympathetic neurons, Padley et al. and others have proposed that RVLM CB_1R-evoked sympathoexcitation is, presumably, mediated via disinhibition of GABAergic neurotransmission within the RVLM (Randall, Harris et al. 2002; Padley, Li et al. 2003). To our current knowledge, this was only tested *in vitro* by Vaughan et al. in cultured RVLM neurons, where CB_1R ligands inhibited the release of GABA (Vaughan, McGregor et al. 1999) and has not addressed the nNOS-NO implication. Notably, we have discussed in Chapter 4 that CB_1R-evoked pressor response is mediated via OX_1R/orexin-A signaling in the brainstem. Intriguingly, a recent study has demonstrated a causal link between orexins-evoked cardiovascular effects and nNOS-NO-GABA signaling in the NTS (Shih and Chuang 2007). Moreover, molecular and pharmacological evidence in Chapter 5 has indicated that inhibition and activation of PI3K/pAkt or pERK1/2 signaling, respectively, underlies the CB_1R-evoked pressor response. There is evidence to suggest that GABAergic transmission is enhanced or ameliorated, respectively, following Akt or ERK1/2 phosphorylation (Wang, Liu et al. 2003; Bell-Horner, Dohi et al. 2006). Taken together we hypothesize that nNOS-NO suppression of GABAergic neurotransmission in the RVLM mediates, at least partly, the central CB_1R-evoked pressor response.

6. 3. Material and Methods

6. 3. 1. Intra-arterial catheterization, and intracisternal (i.c) and intra-RVLM cannulation

Male SD rats (Charles River, NC) were prepared for intra-arterial catheterization and intra-RVLM cannulation. Under sterile conditions and anesthesia (ketamine [9 mg/100g] and xylazine

[1mg/100 g], i.p.), an arterial catheter for BP measurement and a guide cannula for i.c. injections, as applicable, were placed into the abdominal aorta via the femoral artery as detailed under general methods, Chapter 2. For intra-RVLM cannulation, the method described in several studies from the advisor's laboratory was followed (Li, Wang et al. 2005; Zhang and Abdel-Rahman 2005; Guichu Li and Abdel-Rahman AA 2007). A stainless steel guide cannula (21.5 gauge, 14mm in length) was implanted 2 mm above the RVLM level at coordinate of -2.8 posterior, ± 2 lateral and -0.5 mm dorsoventral with the interaural line as the reference according to rat brain atlas (Paxinos and Watson 2005). The guide cannula was then secured in place with small metal screws and dental acrylic cement. At least five days were allowed for recovery.

6. 3. 2. Preparation and calibration of the carbon fiber electrodes

The carbon fiber electrodes were prepared according to procedure detailed in a recent publication from the laboratory and elsewhere (Friedemann MN, Robinson SW et al. 1996; Guichu Li and Abdel A. Abdel-Rahman 2009) and then coated with a 2-step coating process prior to use; first with nafion and then with o-phenylenediamine (o-PD). Nafion coating performed by dip-coating in 5% nafion (Aldrich, Milwaukee, WI), drying at 85 ºC for 5 minutes repeated 7 times, to enhance its selectivity for monoamine neurotransmitters over anionic species such as ascorbic acid and DOPAC. Then the probes were electrochemically coated in 5 mM o-phenylenediamine dihydrochloride (1,2-benzenediamine, Sigma Chemical Co., St Louis, MO). A constant voltage (+0.9 V vs. Ag∕AgCl reference) was applied to the electrode for 20 minutes. The solution was continually stirred during the coating process. Coated electrodes were calibrated before use by a response to an increasing concentration of NO, by adding increasing volume of standard 2 mM NO solution while the selectivity was measured against ascorbic acid.

Electrodes whose sensitivity was >50,000 and displayed a NO: ascorbic acid sensitivity ratio >500:1, and linearly responded to increasing conc. of NO (2 to 10 μM) (r > 0.997) were used in the subsequent experiment.

6. 3. 3. Real time measurement of RVLM NO- and drug microinjections

A probe that combines a stainless steel injector (30-gauge, 21.5 mm in length) and the NO-carbon fiber electrode that permits intra-RVLM microinjections was inserted directly into the RVLM of unrestrained rats through the previously implanted guide cannula (Guichu Li and Abdel A. Abdel-Rahman 2009). *In vivo* NO monitoring was performed using the IVEC-10 system that is computer-controlled (Medical SystemsCorp., Greenvale, NY). An oxidation potential of + 0.9 V, with respect to the Ag∕AgCl reference electrode, was applied to the carbon fiber electrode for 0.1 second at a rate of 5 Hz. Each data point represented the average of 5 measurements. The system can measure 1-second NO concentration change, and its detection limit is 35 ± 7 nM NO (Friedemann MN, Robinson SW et al. 1996)

The injector was connected to PE-10 tubing via PE-50 tubing attached to a Hamilton microsyringe (1μl). Chemical identification of the RVLM was based on obtaining a pressor response elicited by injecting 1 nmol L-glutamate at the beginning of the experiment, as reported in previous studies (Limin Mao 1998; Zhang and Abdel-Rahman 2002; Li, Wang et al. 2005; Guichu Li and Abdel-Rahman AA 2007). To avoid potential problems because of microinjecting the drugs following the removal and reinsertion of the injector, microinjections of different drugs was accomplished by separating drugs or vehicle with a small air bubble as reported (Limin Mao 1998; Li, Wang et al. 2005; Guichu Li and Abdel-Rahman AA 2007). At the end of the

experiment, fast green dye 40 nL (minimal volume is used so as not to affect the tissue integrity and the biochemical studies) was injected for histological verification. Coronal sections containing the RVLM region- before being micro-punched for protein extraction and measuring p-nNOS (see below)- as shown in Fig. 6. B (references are shown on the diagram) were examined under light microscopy (4X) for the correct placement of the injector tip. Animals that failed the positive verification were excluded from data interpretation.

6. 3. 4. Blood pressure and heart rate measurements

Hemodynamic measurements were performed as detailed under general methods (Chapter 2) for experiments included i.c. injections. For experiments with intra-RVLM microinjections, 90 min were allowed following the chemical identification of the intra-RVLM injection site to permit full recovery from the L-glutamate elicited pressor and bradycardic responses.

6. 3. 5. Western Blotting

RVLM micropunches were prepared as detailed under general methods (see Chapter 2) from rats used in experiment 1 at the conclusion of hemodynamic measurements to detect changes in phosphorylation of nNOS. Briefly, prepared membranes were incubated for 48 h at 4ºC in mix of rabbit anti-p-nNOS and mouse anti-nNOS diluted 1:500 and 1:200 (BD Biosciences, San Diego, CA) in Odyssey blocking buffer, respectively. Procedure of image acquisition and band quantification detailed under general methods (see Chapter 2) was followed to detect changes in RVLM-p-nNOS/t-NOS in the injected site and compared to those of the contra-lateral site (served as control).

6. 3. 6. Immunofluorescence

6. 3. 6. 1. Tissue and slide preparation

At the conclusion of the experiment, free-floating sections were prepared as detailed under general methods (see Chapter 2). For co-labeling and quantification of TH and nNOS-ir neurons expressing c-Fos, brainstem sections containing the RVLM were incubated with either mixture of primary antibodies outlined in Table 6.1 for 48 h at 4 ºC. Sections were then washed and incubated with one of the mixtures of secondary antibodies for 2 h as indicated in Table 6.1.

6. 3. 6. 2. Quantification of labeled neurons

Images were acquired and projected simultaneously on the attached monitor for quantification of co-localization. For immunohistochemical analysis of brainstem sections, the RVLM of 4-6 sections per animal was examined in rostral–caudal sections -12.8 to -11.8 mm relative to the bregma. Criteria used to identify positively labeled cells were: c-Fos-ir labeling was identified as nuclear staining (red) with a visible nucleolus. TH-ir or nNOS-ir cells were identified by cytosolic labeling (green) with visible processes and a blank nuclear region. c-Fos, TH or nNOS positive cells were counted manually using 20 X objective. Cells were considered to be co-labeled if *the location of nuclear c-Fos staining corresponded to the blank region in cytosolic labeling of nNOS or TH-ir in the same focal plane*. All slides were coded and the examiner was blinded to the treatment at all time.

6. 3. 6. 3. Image acquisition and processing:

Representative images were acquired and processed following procedures outlined under general methods (Chapter 2).

Fig. 6.1. Chemical and histological verification of the site of intra-RVLM microinjections. A) Typical tracing showing effect of injection of 1nmol L-glutamate on arterial pressure (AP) and heart rate (HR). B) Typical photomicrograph (left panel) and schematic diagrams (right panel) of coronal sections of rat brainstem identifying intra-RVLM injection site produced by injecting 40 nL fast green dye at the conclusion of the experiment. Numbers on upper right corner reflect the rostro-caudal coordinates relative to bregma according to rat brain atlas (Paxinos and Watson 2005). NA: Nucleus Ambiguous

A)
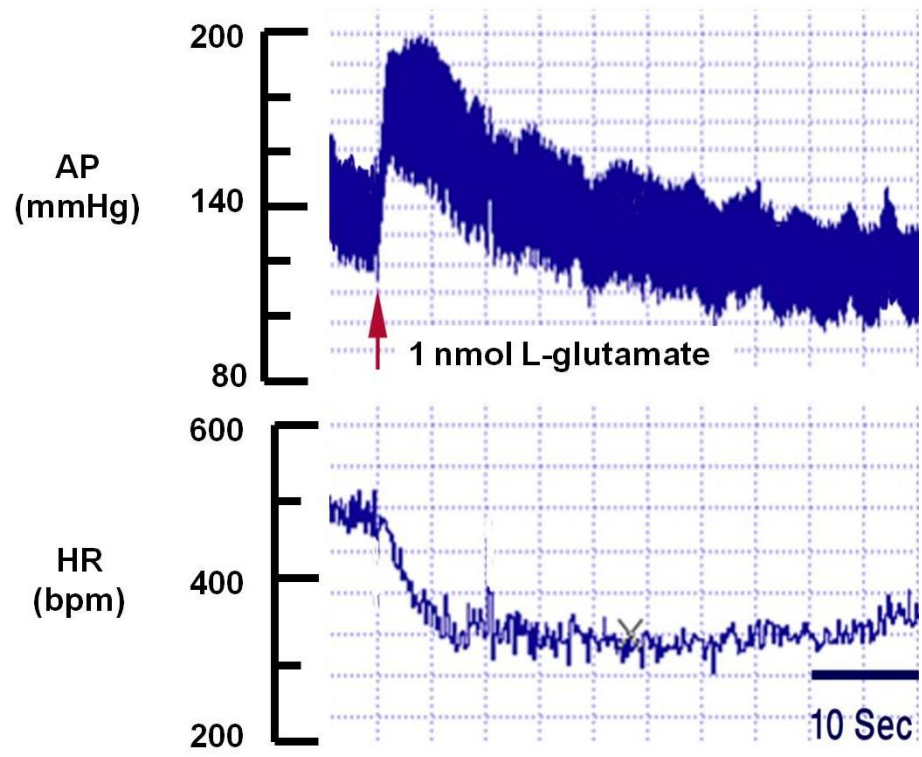
B)
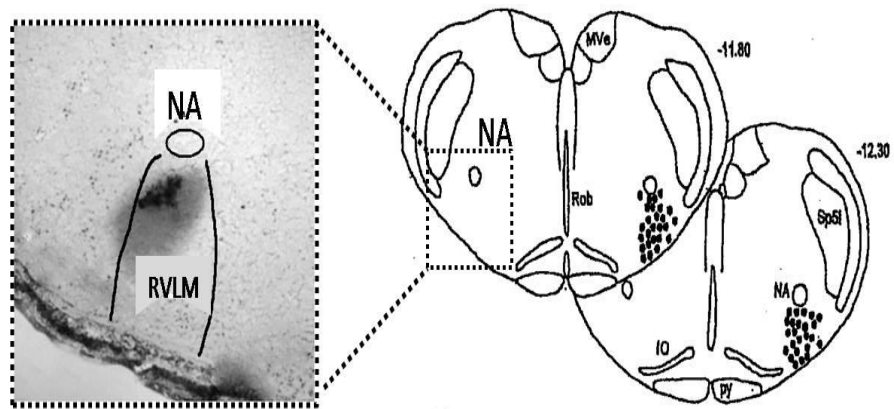
Table. 6.1. Combination of primary and secondary antibodies used in colocalization studies of c-Fos and TH-ir or nNOS-ir in RVLM neurons. All secondary conjugated antibodies were purchased from Jackson Immunoresearch, CA. TH: tyrosine hydroxylase; Ab: antibody.

	c-Fos/TH
1st primary Ab	Mouse anti-TH (1:500; Chemicon., Temecula, CA)
1st secondary Ab	FITC conjugated donkey anti-mouse; 1:200
2nd primary Ab	Rabbit anti-c-Fos (1:2000; Calbiochem, San Diego, CA)
2nd secondary Ab	Cy3 conjugated donkey anti-rabbit, 1:200

	c-Fos/nNOS
1st primary Ab	Mouse anti-nNOS (1:200; BD Biosciences, San Diego, CA)
1st secondary Ab	FITC conjugated donkey anti-mouse; 1:200
2nd primary Ab	Rabbit anti-c-Fos (1:2000; Calbiochem, San Diego, CA)
2nd secondary Ab	Cy3 conjugated donkey anti-rabbit, 1:200

6. 3. 7. Drugs

Muscimol was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in sterile saline.

6. 4. Protocols and experimental groups

6. 4. 1. Experiment 1: Effects of intra-RVLM WIN55,212-2 on BP, HR, p-nNOS and NO

In this experiment (n= 4) conscious rats prepared as above for simultaneous *in vivo* real-time measurement of NO and BP, received 80 nL of vehicle or three successive doses of WIN55,212-2 (100, 200, 300 pmole), as shown in Fig. 6.2 at 10 min intervals. Injections were made unilaterally into the RVLM of all rats. The use of previously reported dose-ranges for WIN55,212-2 (0.5-50 pmole) (Padley, Li et al. 2003) failed to produce any changes in BP or NO in our hands. Ten min after the last dose, a micropunch from the injected RVLM was collected as detailed under methods and used for determining changes in nNOS phosphorylation (n=3) by Western blotting from each rat. The contra-lateral (non-injected) RVLM was also collected and served as control (n=4).

6.4. 2. Experiment 2: Effect of brainstem GABA$_A$ receptor stimulation (muscimol) on the pressor response elicited by i.c. WIN55,212-2

We elucidated the effect of GABA$_A$ receptor stimulation on the i.c. WIN55,212-2-evoked pressor response previously discussed in Chapter 3; animals received muscimol (0.1 µg/rat, i.c) 10 min prior to i.c. WIN55,212-2 (15 µg/rat, i.c) (n=8) or its vehicle (n=5). Hemodynamic measurements were continued for 30 min after WIN55,212-2 or vehicle. The data were

compared to hemodynamic effects of WIN55,212-2 given alone or its vehicle (Chapter 3; experiment 3.5.2). Blood samples were collected to measure plasma NE levels.

6. 4. 3. Experiment 3: Effects of WIN55,212-2 on RVLM-catecholaminergic and nitroxidergic neurons activity (c-Fos) in presence or absence of blockade of CB_1 (AM251) or activation of $GABA_A$ receptors (muscimol)

We have discussed in Chapter 3 that CB_1R-stimulation enhanced RVLM-catecholaminergic neuronal activity (c-Fos-ir). We investigated the neuronal activity, of catecholaminergic and nitroxidergic neurons, indirectly via measuring c-Fos abundance, following i.c. WIN55,212-2 or its vehicle. To elucidate whether these effects are $GABA_A$R-dependent, brain tissues were collected and prepared for quantification of the number of tyrosine hydroxylase immunoreactive neurons (TH-ir), marker of catecholaminergic neurons, or nNOS-ir neurons expressing c-Fos at the conclusion of Exp. 2. Results were compared to those produced from four additional groups of rats treated as indicated earlier (Chapter 3; experiment 3. 4. 2) where rats received WIN55,212-2 (15 μg/rat, i.c) or an equal volume of its vehicle 30 min after i.c. administration of the selective CB_1R antagonist, AM251 (30 μg). Brainstem sections that contained the RVLM were processed for c-Fos/TH-ir or c-Fos/nNOS-ir quantification as described above.

Fig. 6.2. Time line for surgical procedures and experimental protocols for experiment 1.

Blood Pressure recording

| | 0 min | 10 min | 20 min | 30 min | 40 min |

Day -5   -90 min

1 nmol glut

Catheterization

Intra-RVLM cannulation

Veh

100, 200 & 300 pmol
WIN55,212-2

Brain tissue
collection
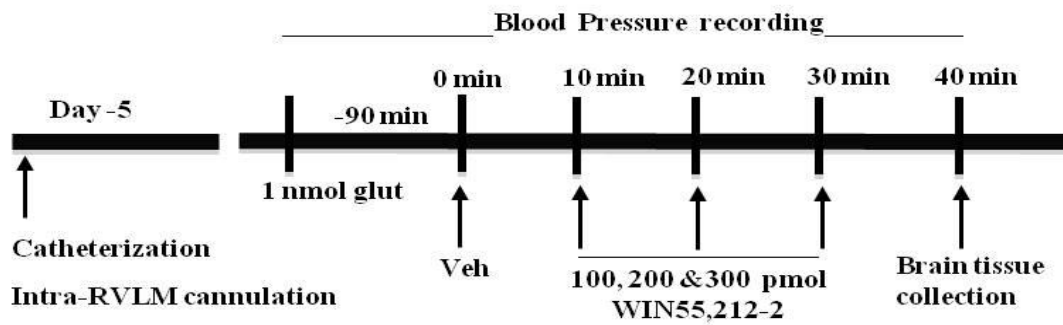
Fig. 6.3. Time line for surgical procedures and experimental protocols for experiments 2 and 3.

Catheterization
i.c. cannulation

Blood pressure
recording

Euthan.
Tissue collect.

day -5

-90 min

0 min

10 min

40 min

Stabliz.
Baseline

muscimol

15 µg WIN55,212-2
or Vehicle
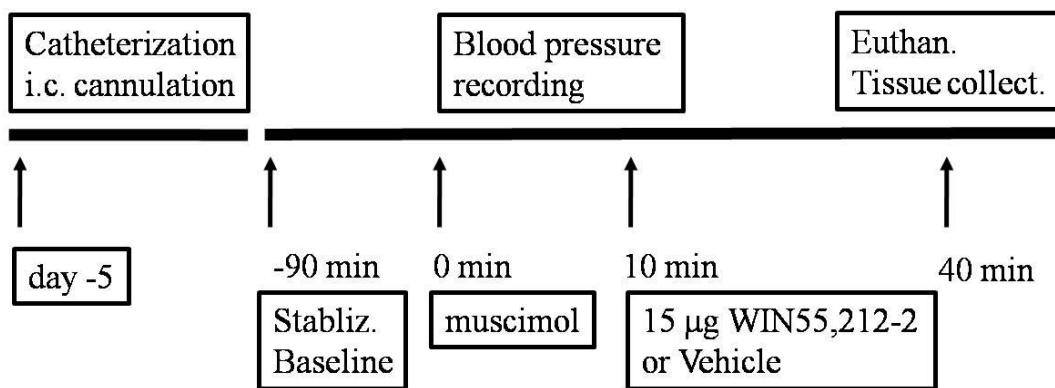
6.5. Statistical Analysis

A one way ANOVA followed by Bonferroni comparison test was used to evaluate the effect of intra-RVLM WIN55,212-2 on p-nNOS or NO (Exp. 1) and the effect of various treatments on co-localization of c-Fos-ir and TH-ir or nNOS-ir neurons of the RVLM (Exp 3). $P < 0.05$ was considered significantly different

6. 6. Results

6. 6. 1. Intra-RVLM WIN55,212-2 elicited dose-dependent increase in blood pressure and RVLM-NO, and enhanced RVLM-p-nNOS

Intra-RVLM WIN55,212-2 elicited dose-dependent increases in blood pressure and RVLM-NO ($P < 0.05$; n=4). The effect on HR was insignificant. The vehicle of WIN55,212-2 had no effect on the measured variables (Fig. 6.4). Additionally, WIN55,212-2 significantly ($P < 0.05$) increased p-nNOS phosphorylation in the RVLM microinjected with WIN55,212-2 compared to the contra-lateral (control) RVLM (Fig. 6.5).

6. 6. 2. Brainstem GABA$_A$R stimulation (muscimol) abolishes CB$_1$R-evoked hemodynamic effects

Muscimol (0.1 μg/rat, i.c) slightly decreased MAP and HR but the values were not significantly different from baseline at the time of WIN55,212-2 injection (Table 6.2). Plasma NE prior to WIN55,212-2 injection was 950 ± 90 pg/ml (n=10) The pressor response and the elevation of plasma NE levels induced by WIN55,212-2 (15 μg/rat, i.c) were abolished by muscimol (0.1 μg/rat, i.c). Muscimol had no effect on WIN55,212-2-evoked bradycardia (Fig.

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6.6). Interestingly, we noticed a brief depressor response caused by WIN55,212-2 in animals pretreated with muscimol.

6. 6. 3. Central CB$_1$R activation (WIN55,212-2) increases RVLM-catecholaminergic (TH-ir) and nitroxidergic neurons (nNOS-ir) activity

WIN55,212-2 (15 μg/rat, i.c) significantly ($P < 0.05$) increased the percentage of TH-ir (Figs. 6.7 and 6.8) and nNOS-ir (Figs. 6.9 and 6.10) neurons expressing c-Fos, compared to control (vehicle). AM251 (30 μg/rat, i.c) or muscimol (0.1 μg/rat, i.c) did not significantly alter the basal TH-ir/c-Fos or nNOS/c-Fos ratio ($P > 0.05$). However, AM251 or muscimol pretreatment significantly ($P < 0.05$) attenuated the WIN55,212-2-evoked increases in number and percentage of TH-ir or nNOS-ir neurons expressing c-Fos. The total number of TH-ir or nNOS-ir (sum of expressing or not expressing c-Fos neurons) was not significantly different in all groups ($P > 0.05$).

Fig. 6.4. Intra-RVLM WIN55,212-2 dose-dependently increases blood pressure and RVLM-NO levels. A) Typical tracings depicting the dose-dependent real time changes in arterial pressure (AP), heart rate (HR) and RVLM-Nitric Oxide (NO) elicited by intra-RVLM microinjection of WIN55,212-2 or its vehicle in conscious freely moving rats. B) Pooled values of intra-RVLM microinjection of 200 pmol CB_1R agonist WIN55,212-2 or its vehicle on real time NO concentration detected electrochemically in RVLM by a nafion coated microelectrode. Values are Mean± SEM. * $P < 0.05$ vs respective control value.
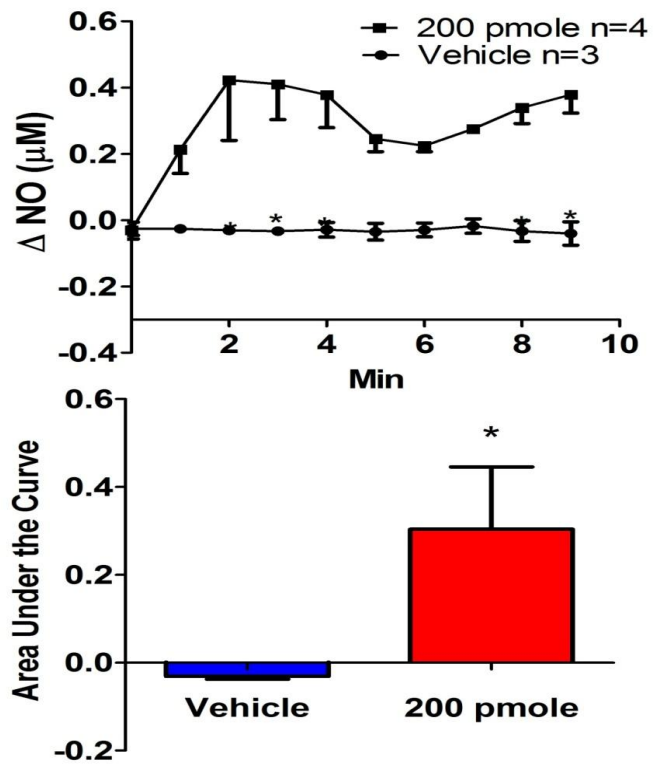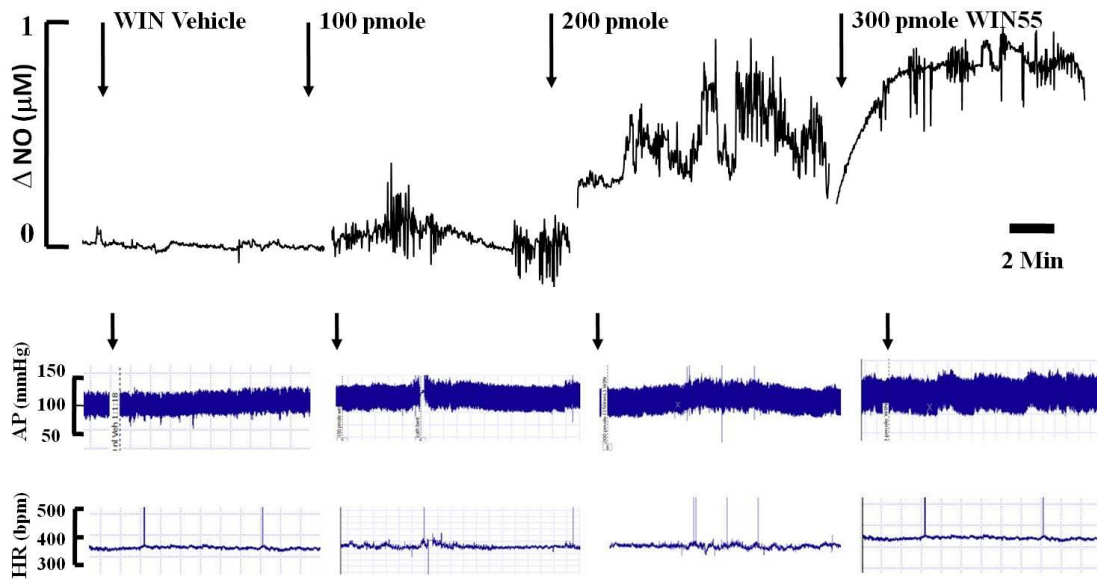
Fig. 6.5. Intra-RVLM WIN55,212-2 enhances p-nNOS level. Quantification of changes in integrated density of RVLM p-nNOS detected by Western blotting following unilateral intra-RVLM WIN55,212-2 as compared to the contra-lateral RVLM (control). Bands of p-nNOS and total nNOS were detected simultaneously from the same membrane using a dual infra-red fluorescence imager. p-nNOS integrated density was normalized to the corresponding total nNOS. Values are Mean± S.E.M.* $P < 0.05$ vs. the contra-lateral (control) RVLM.
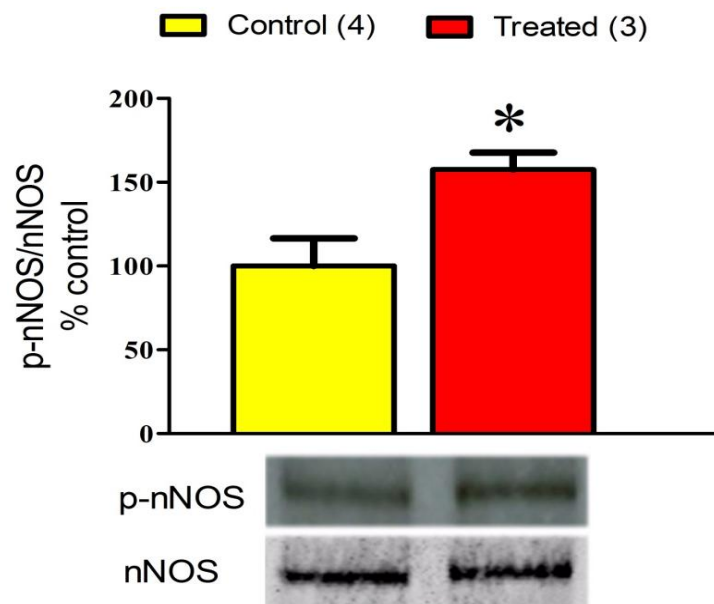
Table. 6.2. MAP (mmHg) and HR (bpm) values before and after pretreatment with the GABA$_A$R agonist muscimol. Values are means ± S.E.M

Treatment	n	Before		After	
		MAP	HR	MAP	HR
Muscimol + Vehicle	5	110.0±4.0	440±12	103.0±4.0	400±12
Muscimol + 15 μg WIN55,212-2	8	106.0±2.0	450±16	99.0±5.0	410±18

Fig. 6.6. Intracisternal muscimol (GABA$_A$R agonist) abolishes CB$_1$R-evoked hemodynamic effects. Time course of changes in mean arterial pressure (ΔMAP, top panel), heart rate (ΔHR, middle panel) and plasma NE (bottom panel) evoked by intracisternal median dose (15 µg/rat) of WIN55,212-2 (Veh + 15 µg WIN55) or an equal volume of its vehicle, indicated by the arrow (top panel), in conscious rats pretreated, 10 minutes earlier, with the selective GABA$_A$R agonist muscimol (0.1 µg/rat, i.c) (Muscimol + 15 µg WIN55) or an equal volume of vehicle (Muscimol + Veh). Values are mean $\pm$ S.E.M. of 5 to 8 observations. * or # $P < 0.05$ vs. respective "Muscimol + Veh" or "Muscimol + 15 µg WIN55" values, respectively.
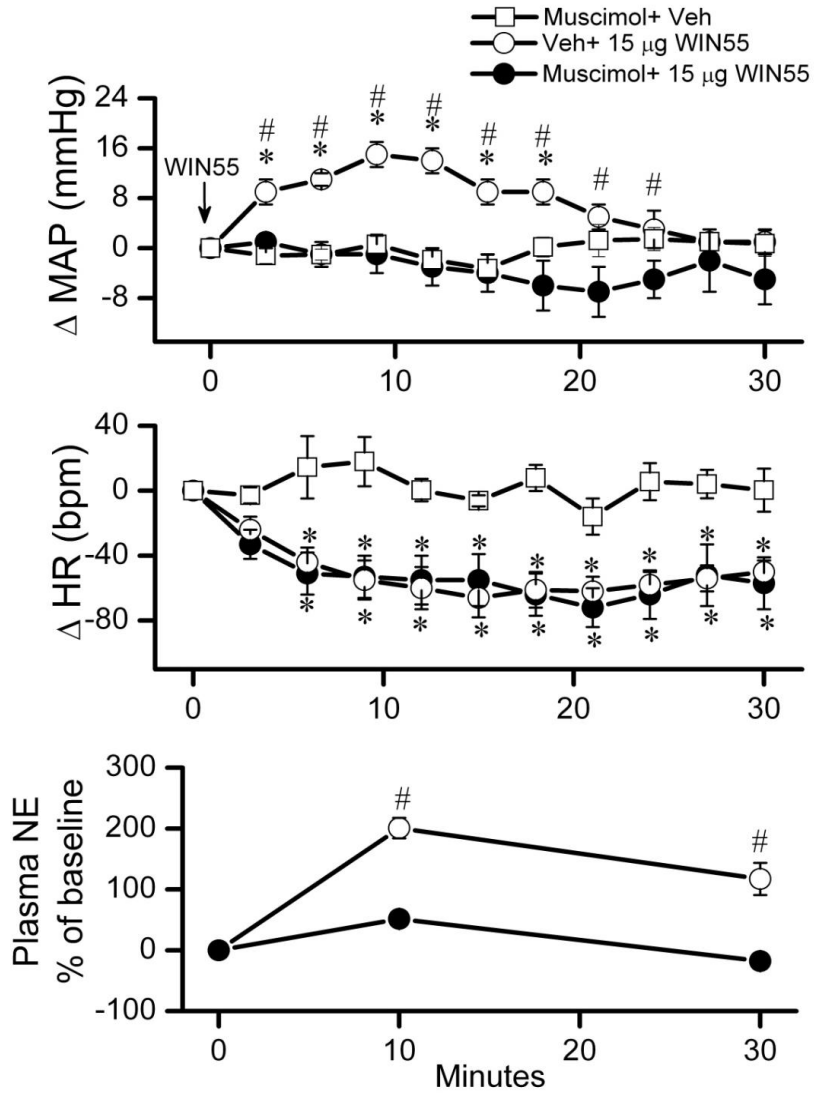
Fig. 6.7. Photomicrographs depicting CB$_1$R-induced c-Fos expression in catecholaminergic neurons in the RVLM. Confocal dual-channel images showing tyrosine hydroxylase immunoreactive (TH-ir) neurons (green) and c-Fos immunoreactive (Fos-ir) cell nuclei (red) in RVLM of rats treated as described under methods section with (A) vehicle, (B) 15 μg WIN55,212-2, (C) AM251 + 15 μg WIN55,212-2 (re-blotted from Fig. 3.6) and (D) Muscimol + 15 μg WIN55,212-2. White or yellow arrowheads indicate single labeled TH-ir neurons or c-Fos-ir cell nuclei, respectively. White arrows denote c-Fos/TH colabeled cells. Scale bar, 20 μm.

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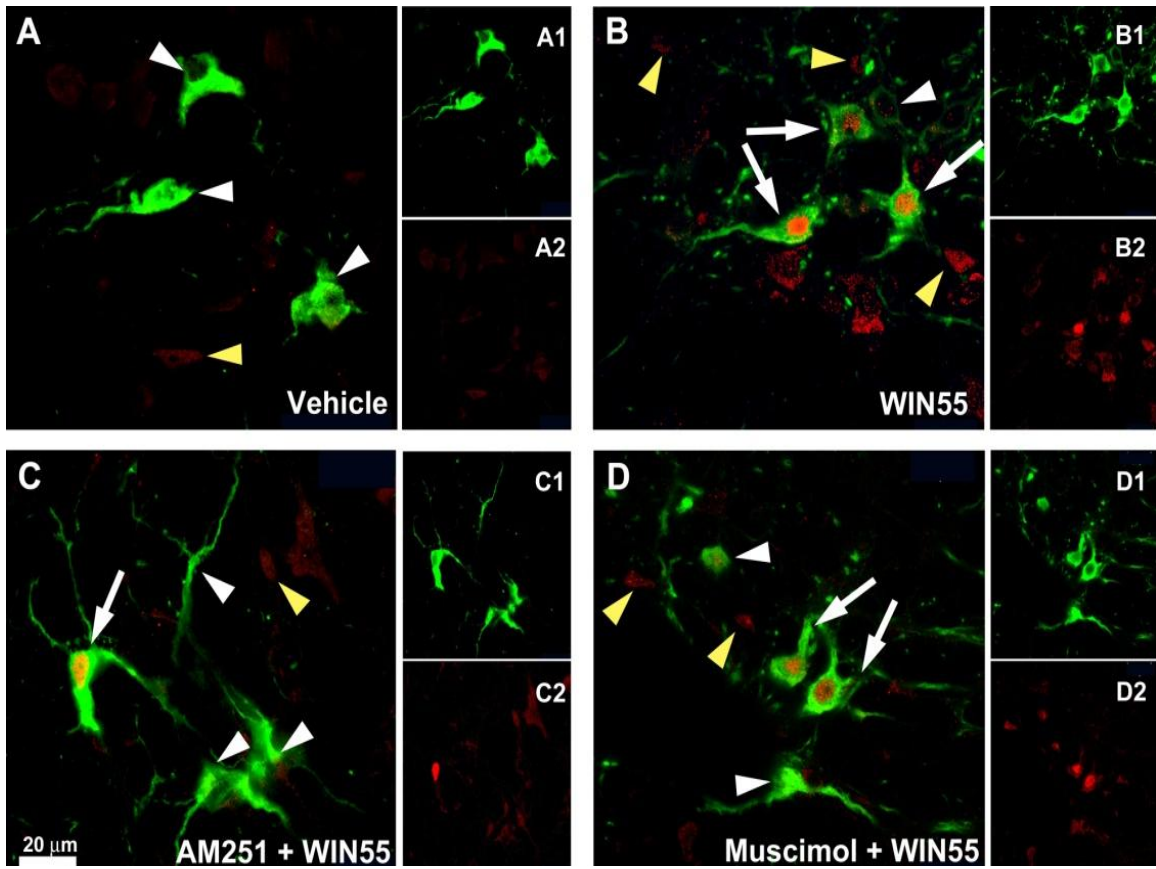
Fig. 6.8. Quantitative analysis of CB$_1$R-induced c-Fos expression in RVLM catecholaminergic neurons. The number of TH-ir neurons, c-Fos/ TH double-labeled neurons and percentage of TH-ir neurons colabeled with c-Fos in the RVLM of rats treated as described under methods with either vehicle, WIN55,212-2 (.15 μg/rat, i.c), AM251 (30 μg/rat, i.c), AM251 prior to WIN55,212-2 (re-blotted from Fig. 3.7), muscimol (0.1 μg/rat, i.c) or muscimol prior to WIN55,212-2 are shown. Bar graphs represent mean ± S.E.M. counts in coronal brainstem sections from 4-6/animal, (n=3-5)/group using one-way ANOVA followed by Bonferroni comparison test. * or # P <0.05 vs respective vehicle or all other treatments values, respectively.
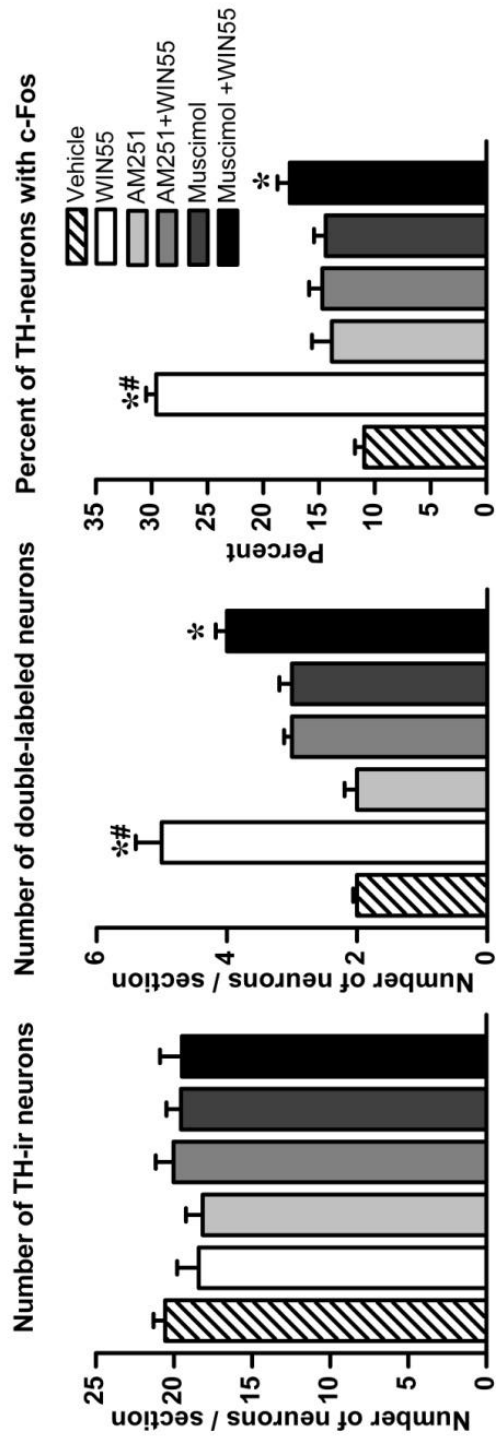
Fig. 6.9. Photomicrographs depicting CB₁R-induced c-Fos expression in nitroxidergic neurons in the RVLM by double labeling immunofluorescence. Confocal dual-channel images showing neuronal nitric oxide synthase (nNOS-ir) neurons (green) and c-Fos immunoreactive (Fos-ir) cell nuclei (red) in RVLM of rats treated as described under methods section with (A) vehicle, (B) 15 μg WIN55,212-2, (C) AM251 + 15 μg WIN55,212-2 and (D) Muscimol + 15 μg WIN55,212-2. White or yellow arrow heads indicate single labeled nNOS-ir neurons or c-Fos-ir cell nuclei, respectively. White arrows denote c-Fos/nNOS colabeled cells. Scale bar, 20 μm.
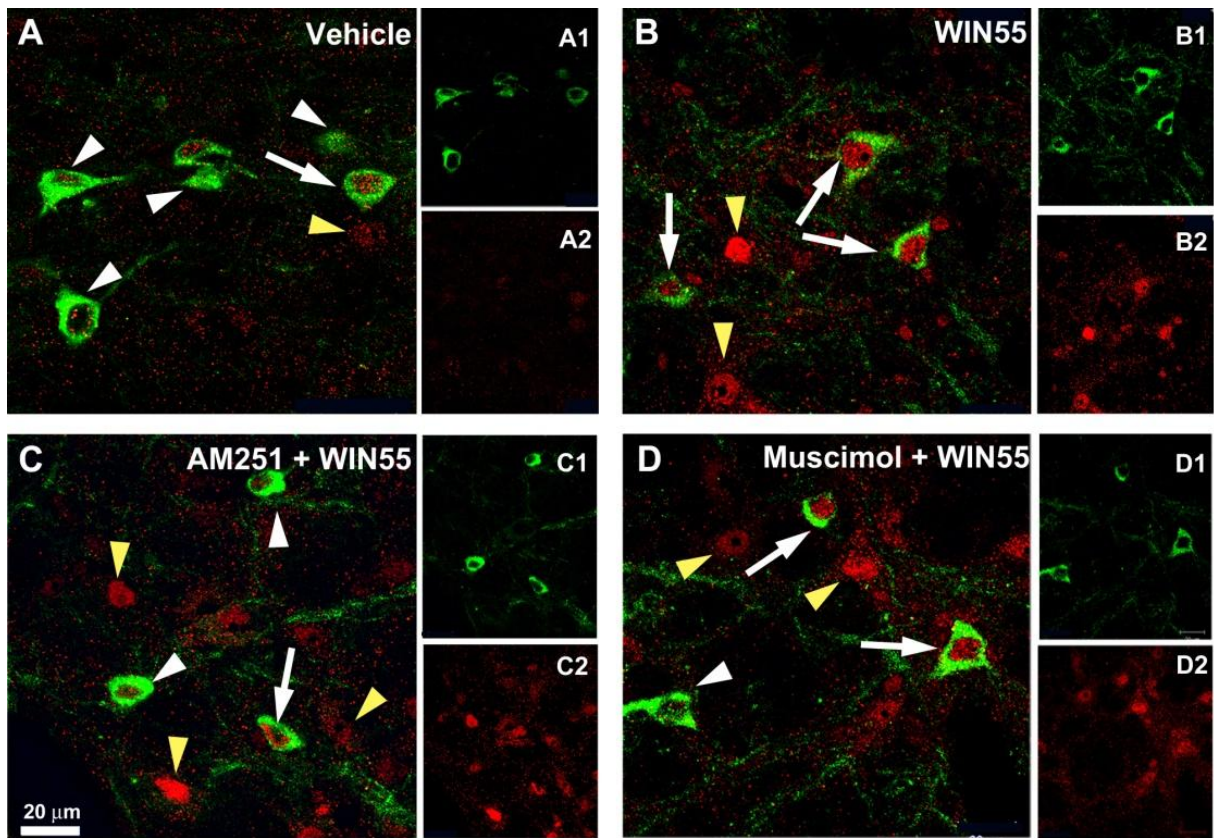
Fig. 6.10. Quantitative analysis of CB$_1$R-induced c-Fos expression in RVLM nitroxidergic neurons. The number of nNOS-ir neurons, c-Fos/ nNOS double-labeled neurons and percentage of nNOS-ir neurons colabeled with c-Fos in the RVLM of rats treated as described under methods with either vehicle, WIN55,212-2 (15 μg/rat, i.c), AM251 (30 μg/rat, i.c), AM251 prior to WIN55,212-2, muscimol (0.1 μg/rat, i.c) or muscimol prior to WIN55,212-2 are shown. Bar graphs represent mean ± S.E.M. counts in coronal brainstem sections from 4-6/animal, (n=3-5)/group using one-way ANOVA followed by Bonferroni comparison test. * or # P <0.05 vs respective vehicle or all other treatments values, respectively.
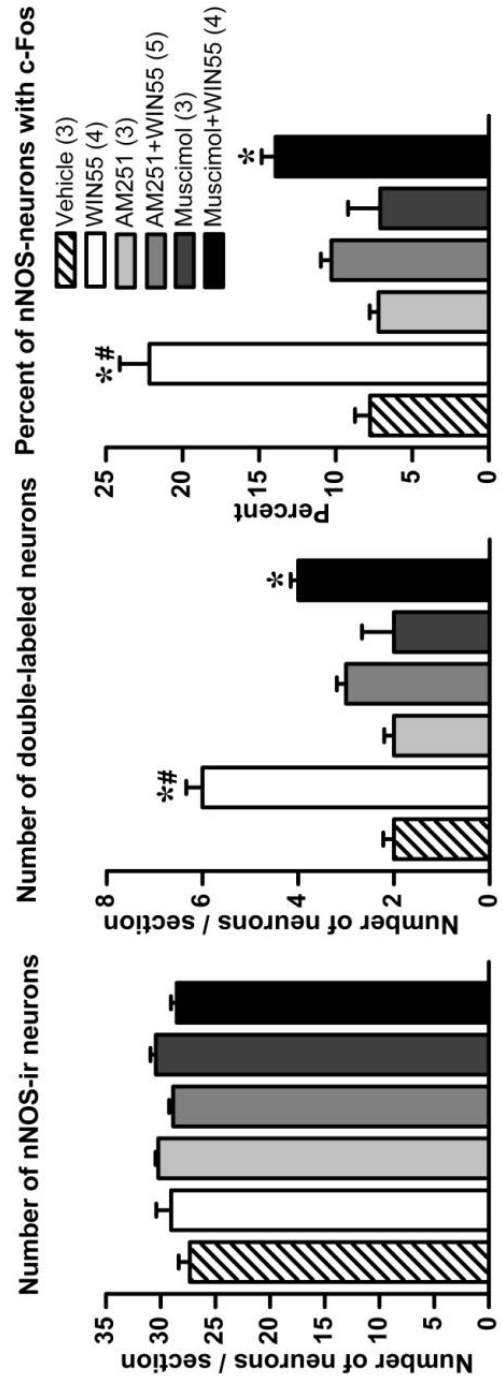
6.7. Discussion

The most important findings of the current study are: (i) intra-RVLM microinjection of the cannabinoid receptor agonist WIN55,212-2 elicited dose-dependent increases in BP and enhancement of RVLM p-nNOS-NO signaling; (ii) stimulation of brainstem CB_1R (WIN55,212-2) activated RVLM-catecholaminergic and nitroxidergic neurons (c-Fos) and elevated plasma NE levels; (iii) these neurochemical, biochemical and cardiovascular responses were virtually abolished by prior central CB_1R blockade (AM251) or activation of central $GABA_AR$ (muscimol). These findings suggest a pivotal role for brainstem p-nNOS-NO-GABAergic signaling in the central CB_1R-evoked sympathoexcitation/pressor response in conscious rats.

In this study, we provided preliminary evidence that implicates RVLM nNOS-NO signaling in the central CB_1R-evoked pressor response. We utilized a technique that permitted simultaneous measurement of blood pressure, heart rate and real time neuronal NO level in conscious rats following unilateral intra-RVLM microinjection of the CB_1R agonist WIN55,212-2 (100, 200 or 300 pmol); this technique has been verified in previous studies in the laboratory (Guichu Li and Abdel A. Abdel-Rahman 2009). In agreement with previous findings (Padley, Li et al. 2003), intra-RVLM WIN55,212-2 elicited dose-dependent increases in blood pressure along with a slight increase in heart rate. These effects were not due to a volume increase, because the WIN55,212-2 vehicle administered in the same volume had no effect on the measured variables. The slight increase in HR is contrary to the bradycardia induced by i.c. WIN55,212-2, which was discussed in Chapter 3. The difference in the heart rate response might be due to the localized effect of WIN55,212-2 within the RVLM compared to the more widespread effect following i.c. injection. Importantly, hemodynamic responses elicited by intra-

RVLM WIN55,212-2 replicated the human response to marijuana/THC smoking (Benowitz, Rosenberg et al. 1979) and also agree with similar findings in anesthetized animals (Padley, Li et al. 2003), despite the difference in the dose range. The justification of the higher dose used in the present study is: (i) the use of microcapillary tubing for the microinjection in the reported study (Padley, Li et al. 2003) vs. a stainless steel microinjector used in the preliminary study for RVLM drug delivery, that provided targeted delivery into specific RVLM neurons; (ii) the conscious state of the animals used in this study vs. anesthetized rats in the reported study, which was shown to alter CB_1R-mediated hemodynamic responses (Lake, Martin et al. 1997).

Evidence suggests that nNOS-generated NO plays a significant role in central CB_1R-mediated responses (Azad, Marsicano et al. 2001; Makara, Katona et al. 2007; Jones, Carney et al. 2008). This study demonstrated, for the first time, that the central CB_1R-evoked pressor response is, at least partly, mediated via enhanced RVLM p-nNOS-NO signaling because intra-RVLM WIN55,212-2 elicited: (i) dose-dependent increases in RVLM-NO that paralleled the pressor response in conscious rats; (ii) significantly increased nNOS phosphorylation; (iii) enhanced the activity (c-Fos) of nNOS-ir and TH-ir neurons was and these responses were abrogated by prior blockade of central CB_1R (AM251). Although possible roles for eNOS or iNOS cannot be excluded and require further investigation, it should be remembered that only RVLM nNOS-derived NO is implicated in centrally-evoked hypertension (Kishi, Hirooka et al. 2001; Ally, Kabadi et al. 2007; Martins-Pinge, Garcia et al. 2007).

We hypothesized that inhibition of GABAergic neurotransmission and localized enhancement of NO generation within the RVLM underlies, at least partly, the sympathoexcitatory/pressor response elicited by WIN55,212-2. In support of this hypothesis is

the finding that prior stimulation of brainstem $GABA_AR$ (muscimol, i.c) abrogated the central CB_1R-evoked pressor response, and the biochemical and neurochemical markers of sympathoexcitation (Fig. 6.6). These findings agree with reported *in vitro* findings that demonstrated inhibition of GABAergic transmission in cultured RVLM neurons following CB_1R activation (Vaughan, McGregor et al. 1999). Further, the present novel neurochemical findings in our model system complement our molecular and pharmacological findings discussed in Chapter 5, which demonstrated that inhibition of PI3K/pAkt and activation of ERK1/2 signaling contribute to the central CB_1R-evoked pressor response because: (i) First, *in vivo* and *in vitro* findings associated reduced PI3K/Akt signaling with reduced postsynaptic $GABA_AR$ phosphorylation and number (Wang, Liu et al. 2003). (ii) Second, activation of ERK1/2 (phosphorylation) caused a reduction in GABAergic transmission (Bell-Horner, Dohi et al. 2006). Finally, activation of NTS CB_1R by the endogenous CB_1R agonist anandamide enhanced baroreflex-mediated sympathoinhibition, at least partly, via presynaptic inhibition of GABA release (Seagard, Dean et al. 2004). It is imperative to note that the differences between the sympathetic neuronal activity due to the CB_1R-evoked inhibition of GABAergic transmission in the RVLM in the present study vs. NTS in the latter study are expected because $GABA_AR$ activation elicits sympathoinhibition in the RVLM vs. sympathoexcitation in the NTS (Dampney, Horiuchi et al. 2003; Dampney, Polson et al. 2003; Samuel H H Chan, Ling-Lin Wang et al. 2003; Mueller and Hasser 2006). Together, the present results and reported studies suggest a pivotal role for the suppression of GABAergic neurotransmission in the brainstem in the neurobiological effects elicited by central CB_1R activation. Equally important, the findings might explain, at least partly, the complex nature of the blood pressure responses elicited by i.c. WIN55,212-2, which gains access to different neuronal pools within the brainstem.

In summary, the present study highlights a pivotal role for p-nNOS-NO/GABAergic transmission within the RVLM in the central CB_1R-evoked sympathoexcitation/pressor response in conscious rats. The findings are the first to establish a causal link between suppression of brainstem GABAergic neurotransmission and the sympathoexcitatory/pressor response elicited by central CB_1R activation. The pharmacological (central CB_1R blockade and $GABA_AR$ activation) along with the biochemical (plasma NE) and neurochemical (Western blot and immunofluorescence) data suggest a role for enhanced nNOS-NO signaling in the RVLM in the suppression of localized GABAergic transmission and the subsequent sympathoexcitation and pressor response. Further studies are needed to establish whether RVLM-nNOS activation plays a causal role in these neuronal events.

CHAPTER SEVEN-GENERAL DISCUSSION AND SUMMARY

The main goal of the current study was to elucidate the mechanisms implicated in the central CB_1R-evoked sympathoexcitation/pressor response. In pursuit of this goal, we characterized the centrally-elicited hemodynamic effects of CB_1R in conscious SD rats. We have confirmed the expression of CB_1R (protein) in the RVLM by: (i) detecting the two bands at 64 and 53 kDa, representing the N-glycosylated and non-glycosylated forms of CB_1R, respectively (Fig. 3.3) (Song C and Howlett 1995); (ii) dual-labeling immunofluorescence, which revealed its expression in the C1 area within the RVLM (Fig. 4.2 C). In the present study, i.c. administration of WIN55,212-2 dose-dependently increased MAP and NE plasma levels, denoting an increase in central sympathetic tone in conscious rats. Notably, the pressor response elicited by central WIN55,212-2 administration fully agrees with reported findings in experimental animals (Niederhoffer and Szabo 1999; Niederhoffer and Szabo 2000; Pfitzer, Niederhoffer et al. 2004), and reflects similar responses observed in humans (Foltin, Fischman et al. 1987; Sidney 2002). Additionally, i.c. WIN55,212-2 elicited a profound and immediate dose-dependent reduction in HR. Our studies were conducted in conscious rats, to circumvent the negative impact of anesthesia that was shown to dramatically compromise cannabinoid-evoked hemodynamic responses (Stein, Fuller et al. 1996; Lake, Martin et al. 1997; Gardiner, March et al. 2001).

Throughout the study we demonstrated that the hemodynamic, biochemical and molecular responses elicited by WIN55,212-2 are CB_1R mediated. This is important because: (i) there are no currently available selective CB_1R agonists; WIN55,212-2, which is routinely used in cannabinoid research, is a mixed CB_1R/CB_2R agonist (Showalter, Compton et al. 1996; Griffin, Atkinson et al. 1998); (ii) both CB receptor subtypes are expressed in the brain (Herkenham,

Lynn et al. 1991; Viscomi, Oddi et al. 2009) including the brainstem, which plays a major role in the sympathoexcitatory/pressor response elicited by CB_1R activation (Padley, Li et al. 2003). The ability of the selective CB_1R antagonist, AM251 (Niederhoffer and Szabo 1999; Niederhoffer and Szabo 2000; Pfitzer, Niederhoffer et al. 2004) to virtually abolish the pressor, biochemical and neurochemical responses elicited by i.c. WIN55,212-2 clearly implicates the CB_1R in the investigated responses. It is important to note, however, that the lack of change in blood pressure, as well as other neurochemical responses, following AM251 administration argues against the involvement of central CB_1R signaling in tonic control of blood pressure in conscious rats.

A main objective of the current study was to provide direct evidence for a functional role for RVLM neurons in the central CB_1R-evoked pressor response. We present the first *in vivo* evidence that central CB_1R-evoked sympathoexcitation/pressor response involves activation of RVLM-catecholaminergic neurons as represented by the significant increase in TH-ir neurons expressing c-Fos, a marker of neuronal activity, following i.c WIN55,212-2. While the CB_1R antagonist AM251 did not alter the number of c-Fos expressing neurons in the RVLM, it abrogated the WIN55,212-2-induced increase in c-Fos expressing neurons. These effects parallel to and further confirm the involvement of the RVLM neurochemical responses in the hemodynamic effects elicited by WIN55,212-2 as discussed above.

In the second part of this study, we provided evidence of a functional crosstalk between central CB_1R and OX_1R signaling and identified the enhanced release of RVLM-orexin-A as a possible neuronal mediator of the CB_1R-evoked pressor response in conscious rats. We investigated the anatomical localization of CB_1R and orexin-A/OX_1R in the RVLM. Multiple

immunofluorescence studies have unraveled that CB_1R labeled neurons and punctuated structures are in close proximity to or colocalized with orexin-A or OX_1R, respectively, in the C1 of the RVLM (immunoreactive to tyrosine hydroxylase) (Chapter 4, Fig. 4.2). In support of our hypothesis, we demonstrated, the novel finding that central CB_1R activation significantly elevated orexin-A levels in the RVLM along with the pressor response (Chapter 4, Fig. 4.3). Interestingly, the finding that activation of cannabinoid receptors via i.c. WIN55,212-2 increased RVLM-orexin-A content, and probably its release, was a CB_1R-mediated response as it was abolished by prior selective blockade of central CB_1R (AM251, i.c.). Because there were no reported studies on the dose of the selective OX_1R antagonist (SB-408124), needed to block central orexin-A-evoked pressor response in rats, we constructed in our model system the dose-pressor response curve elicited by i.c. orexin-A (Fig. 4.4) (Samson, Gosnell et al. 1999; Chen, Hwang et al. 2000). We, then, utilized the dose of the OX_1R selective antagonist SB-408124, which appropriately blocked the pressor response elicited by i.c. orexin-A (Fig. 4.5), to provide the first *in vivo* evidence that prior central blockade of OX_1R (SB-408124) abrogates central CB_1R (WIN55,212-2)-evoked pressor response (Fig. 4.6). This finding is in line with the molecular and behavioral (feeding) studies that suggest a reciprocal interaction between CB_1R and OX_1R (Hilairet, Bouaboula et al. 2003; Ellis, Pediani et al. 2006; Crespo, Heras et al. 2008). Indeed, it is not clear at what level these two receptors interact in central cardiovascular regulating areas. Nevertheless, our study has established a link between CB_1R-evoked release of orexin-A and activation of OX_1R in the RVLM in the pressor response elicited by central CB_1R activation. While the CB_1R-evoked pressor response is dependent on OX_1R signaling, orexin-A-elicited sympathoexcitation seems not to involve CB_1R signaling, because, blockade of brainstem CB_1R (AM251) did not alter the hemodynamic effects elicited by i.c. orexin-A (Fig

4.7). This latter observation strongly suggests that the i.c. orexin-A-mediated pressor response is not dependent on CB_1R signaling. Nevertheless, this finding might be organ/tissue dependent because the CB_1R antagonist SR-141716A attenuated OX_1R-evoked increase in pERK1/2 *in vitro* in cells co-expressing the two receptors (Hilairet, Bouaboula et al. 2003).

Our findings have elucidated the role of PI3K/Akt and ERK1/2 as potential downstream molecular mediators of the central CB_1R-evoked sympathoexcitation/pressor response as suggested by multiple lines of evidence (discussed in Chapters 1 and 5). For example, consistent with a diverse physiological role of PI3K/Akt-ERK1/2 pathway, we showed that a dose-related reduction in pAkt phosphorylation levels in the NTS and RVLM contributes to the i.c. WIN55,212-2-evoked pressor response (Chapter 5, Fig. 5.2). In support of this conclusion are the findings that the inhibition of Akt phosphorylation in the NTS and RVLM preceded the peak WIN55,212-2-evoked pressor response (5 min). Our Western blot findings are consistent with reported findings that CB_1R activation resulted in down-regulation of the PI3K/Akt signaling (Ellert-Miklaszewska, Kaminska et al. 2005; Greenhough, Patsos et al. 2007). However, others have shown that CB_1R activation up-regulated PI3K/Akt signaling in U373 MG human astrocytoma cells (Galve-Roperh, Rueda et al. 2002), hippocampal slices (Derkinderen, Valjent et al. 2003), and *in vivo* (Ozaita, Puighermanal et al. 2007). We are not aware of any reports on the effect of CB_1R activation on PI3K/Akt signaling in the brainstem. Nonetheless, in further support of a causal role for the observed inhibition in Akt phosphorylation in the brainstem in the central CB_1R-mediated pressor response are the findings that pharmacological inhibition of brainstem PI3K-Akt signaling (wortmannin) significantly enhanced the WIN55,212-2 evoked dose-related pressor response (Fig. 5.3). Interestingly, we observed an increase in Akt

phosphorylation elicited by WIN55,212-2 following CB_1R blockade with AM251 in the NTS but not in the RVLM (Fig. 5.5 A and D). This finding clearly highlights differences between neurochemical responses elicited by CB_1R activation in the RVLM vs. NTS. Although such a finding might be expected, given the physiological differences between these two neuronal pools, future studies are warranted to investigate the role of this neuronal response in the central blood pressure effects of CB_1R activation.

Furthermore, the data provide the first evidence that brainstem-ERK1/2 signaling is involved in central CB_1R-mediated sympathoexcitation. Central administration of WIN55,212-2 (i.c.) significantly, elevated pERK1/2 in the NTS and RVLM (Fig. 5.2 B) and (Fig. 5.6 A and D). Abrogation of the WIN55,212-2-mediated hemodynamic responses by PD98059 (Fig. 5.4) and attenuation of the concomitant activation of ERK1/2 pathway by pretreatment with the selective CB_1R antagonist AM251(i.c.) precluded the involvement of CB_2R in these responses (Fig. 5.6 A and D). In view of the crucial role of brainstem pERK1/2 signaling in central control of blood pressure, previous studies from the laboratory (Zhang and Abdel-Rahman 2005; Nassar and Abdel-Rahman 2008) and others (Seyedabadi, Goodchild et al. 2001; Lin, Matsumura et al. 2004; Chan, Wang et al. 2007), in addition to findings from this study, it was suggested that brainstem ERK1/2 has a bi-directional role in central regulation of blood pressure. Based on the molecular findings from Western blotting (Fig. 5.5 B, C, E and F) and (Fig. 5.6 B, C, E and F), we concluded that the effect of WIN55,212-2 on PI3K/Akt may contribute to the enhancement of ERK1/2 phosphoryation because in the presence of the PI3K/Akt inhibitor wortmannin WIN55,212-2-induced ERK1/2 phosphorylation was exacerbated (more evident in the NTS than

RVLM). Additionally, PD98059 alone or in the presence of WIN55,212-2 had no effect on brainstem pAkt phosphorylation levels.

We have investigated whether nNOS-NO plays a significant role in the central CB$_1$R-mediated pressor response. This hypothesis was based on a well-documented role of NOS-NO signaling in the RVLM regulation of autonomic function (Martins-Pinge, Araujo et al. 1999; Martins-Pinge, Garcia et al. 2007; Mayorov 2007). We have demonstrated that intra-RVLM WIN55212,2 microinjection elicited dose-dependent increases in real-time RVLM-NO and blood pressure; the latter was measured using *in vivo* electrochemistry (Chapter 6, Fig. 6.4) and is possibly nNOS-generated because: (i) parallel to the WIN55,212-2 dose-dependent enhancement of NO-release, we detected a significant increase in nNOS phosphorylation in the RVLM, which received WIN55,212-2 compared to the contra-lateral side (control) (Fig. 6.5); (ii) i.c. WIN55,212-2 increased the number of nNOS-ir neurons expressing c-Fos, denoting an increase in nNOS neuronal activity and this effect was abolished following selective CB$_1$R blockade (AM251) (Figs. 6.9 and 6.10); (iii) only RVLM nNOS, but not eNOS or iNOS, derived NO is implicated in centrally evoked hypertension (Martins-Pinge, Garcia et al. 2007).

It is highly likely that central CB$_1$R-elicited sympathoexcitation is mediated via indirect modulation of presympathetic neurons in the brainstem whose activity is regulated by an array of tonic excitatory and inhibitory inputs (Pilowsky and Goodchild 2002; Padley, Li et al. 2003). Notably, CB$_1$R modulates synaptic transmission of both inhibitory (GABA) and excitatory (glutamate) neurotransmitters (Freund, Katona et al. 2003; Piomelli 2003; Drew, Mitchell et al. 2008; Jelsing, Galzin et al. 2009). Interestingly, stimulation of central GABA$_A$ receptors (muscimol) caused the following: (i) abolished the CB$_1$R-evoked pressor response and the

elevation in plasma NE (Fig. 6.6); (ii) attenuated the WIN55,212-2 evoked increase in the activity (c-Fos) of catecholamine (TH-ir) as well as nitroxidergic (nNOS-ir) neurons (Figs. 6.7 and 7.8) and (Figs. 6.9 and 6.10), respectively. These findings are consistent with reported *in vitro* findings that demonstrated CB_1R-evoked inhibition of GABAergic transmission in cultured RVLM neurons (Vaughan, McGregor et al. 1999). Yet, in the NTS, studies have demonstrated a controversial role for CB_1R-mediated presynaptic modulation of excitatory (glutamate) and inhibitory (GABA) neurotransmitters. Anandamide (AEA), an endogenous CB_1R agonist, increased baroreflex-mediated sympathoinhibition in the NTS, presumably, via presynaptic inhibition of GABA release because the response was reversed in presence of the $GABA_AR$ antagonist (Seagard, Dean et al. 2004).

In summary, the present study highlights the predominant sympathoexcitatory effect of brainstem CB_1R activation in conscious rats as shown in Fig. 1.7. Moreover, it demonstrated that CB_1R stimulation enhanced neuronal activity of presympathetic neurons in the RVLM, which are essential for the central regulation of cardiovascular function. We provided the first evidence for a functional crosstalk between cannabinoid and orexins systems within the medullary centers controlling cardiovascular function. CB_1R activation in the brainstem elicited pressor response along with elevation of an orexin-A levels in the RVLM. Importantly, blockade of orexin-A/OX_1R signaling abolished CB_1R-evoked pressor effect. On the other hand, orexin-A/OX_1R pressor response was not altered by prior blockade of central CB_1R. Colocalization studies have unraveled close proximity of orexin-A/OX_1R to CB_1R labeled neurons and punctate-like structures (indicative of presynaptic location), which support the pharmacological findings. Furthermore, the present study delineated a novel role for PI3K/Akt/ERK1/2 signaling in the

brainstem (RVLM and NTS) that seems to contribute, at least in part, to the sympathoexcitatory responses elicited by the central CB_1R activation in conscious rats. The findings of this study demonstrated that CB_1R activation in the RVLM and NTS elicits down-regulation of PI3K/Akt pathway in the RVLM and NTS along with the pressor response. This notion is further supported by exacerbation of WIN55,212-2 evoked hemodynamic responses when PI3K/Akt was inhibited with wortmannin. We speculated that inhibition of PI3K/Akt signaling in the brainstem might underlie the CB_1R-mediated modulation of inhibitory (GABA) neurotransmission. By contrast, the CB_1R-evoked sympathoexcitation was associated with an elevation in the pERK1/2 phosphorylation in the brainstem. Further, suppressing ERK1/2 signaling abolished the central CB_1R-evoked pressor response. Finally, the findings of this study demonstrated that CB_1R activation in the RVLM enhanced neuronal activity in two distinct neuronal populations (catecholaminergic and nitroxidergic) essential for the central regulation of cardiovascular function. These latter neuronal responses may be linked to the modulation of brainstem GABAergic neurotransmission and subsequently to the central CB_1R-evoked sympathoexcitatory and pressor response.

Fig. 7.1 Schematic presentation of the major findings of the study and proposed future studies. See details in the text.
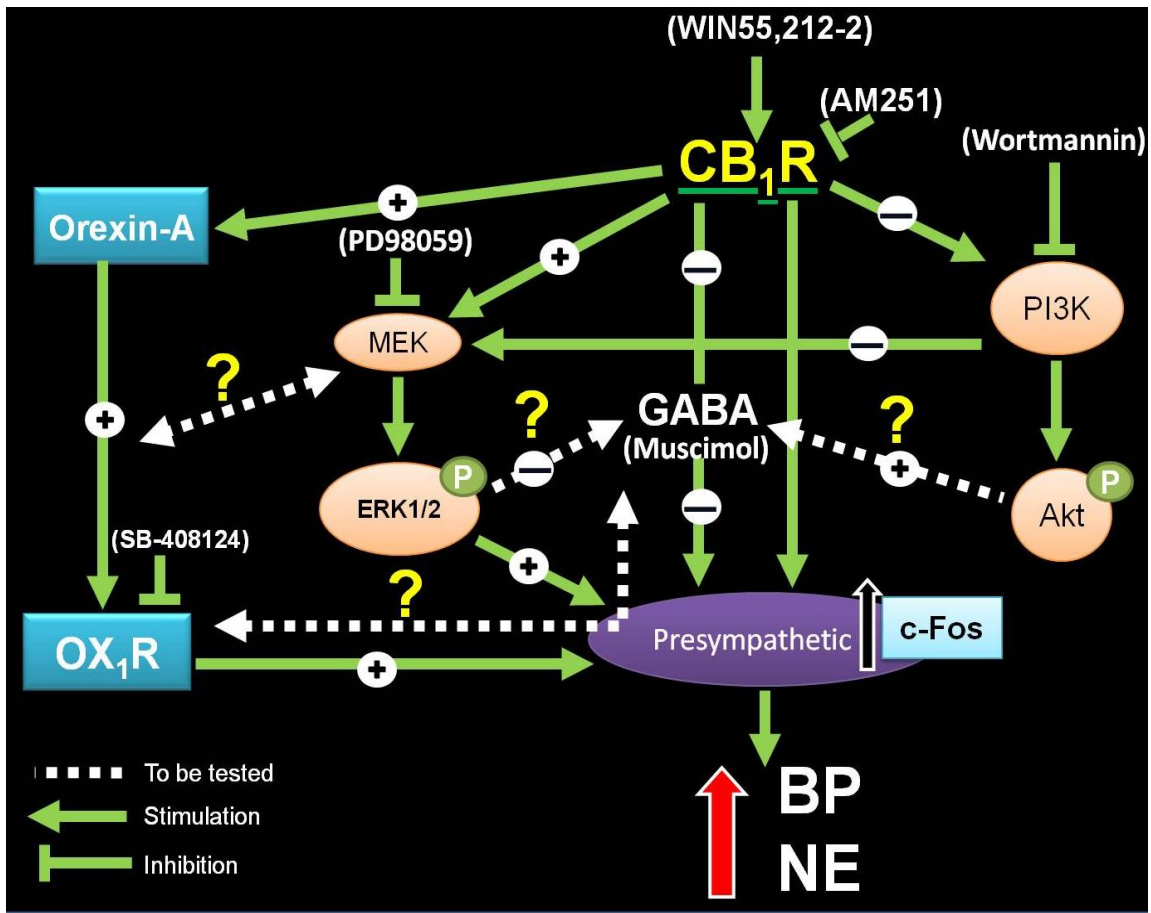
Future directions

The present findings suggest a novel role for brainstem PI3K/Akt/ERK1/2 signaling in the central CB_1R-evoked pressor response (Chapter 5). Importantly, the findings established a causal link between suppression of brainstem GABAergic neurotransmission and the sympathoexcitatory/pressor response elicited by central CB_1R activation (Chapter 6). Notably, *in vivo* and *in vitro* findings associated the reduced PI3K/Akt or enhanced pERK1/2 signaling with modulation of GABAergic transmission (Wang, Liu et al. 2003; Bell-Horner, Dohi et al. 2006). Therefore, it will be interesting to determine whether inhibition of PI3K (wortmannin) or ERK1/2 (PD98059) underlies CB_1R-evoked suppression of GABAergic transmission. We concluded that PI3K/Akt signaling is upstream to ERK1/2 signaling because the significant increase in ERK1/2 phosphorylation by WIN55,212-2 was exacerbated in presence of PI3K/Akt inhibition (wortmannin) (Chapter 5). Further studies are needed to elucidate the signaling pathways between PI3K/Akt and ERK1/2, which seem to influence the balance between sympathoexcitatory and sympathoinhibitory modulators in the brainstem. The pharmacological (central CB_1R blockade and $GABA_AR$ activation) along with the biochemical (plasma NE) and neurochemical (Western blot and immunofluorescence) findings suggest a role for enhanced nNOS-NO signaling in the RVLM in the suppression of localized GABAergic transmission and the subsequent sympathoexcitation and pressor response. The use of the specific nNOS inhibitor (NPLA; $N^{\omega\text{-}}$Propyl-$_L$-arginine) (Kakoki, Zou et al. 2001) will address whether RVLM-nNOS activation plays a causal role in these neuronal events.

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APPENDIX I

ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER
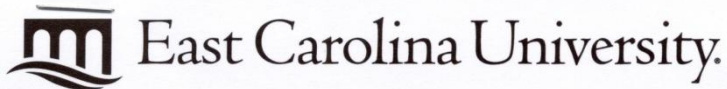
Animal Care and Use Commitee

212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

September 7, 2010

Abdel Abdel-Rahman, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Abdel-Rahman:

The Amendment to your Animal Use Protocol entitled, "Mechanisms of the Cardiovascular Effects of Alcohol", (AUP #W222) was reviewed by this institution's Animal Care and Use Committee on 9/7/10. The following action was taken by the Committee:

"Approved as amended"

**Please contact Dale Aycock prior to any biohazard use

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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1998

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APPENDIX III

APPENDIX III

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Inge Marie de Linde [IMDL@Lundbeck.com] on behalf of CNS Forum [commres445@Lundbeck.com]

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APPENDIX IV

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