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Title Page

The Novel Endocannabinoid Receptor GPR18 is Expressed in the Rostral Ventrolateral Medulla and Exerts Tonic Restraining Influence on Blood Pressure

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Running Title Page

a) Running Title: Central GPR18 mediates Depressor Response in Conscious Rats

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Text pages: 37

Tables: 1

Figures: 7

References: 51

Number of Words:

Abstract: 241

Introduction: 640

Discussion: 1500

c) List of Nonstandard Abbreviations:

Abn CBD: Abnormal cannabidiol; (Trans-4-[3-methyl-6-(1-methylethenyl) -2-cyclohexen-1-yl]-5- Pentyl-1, 3-benzenediol)

ADN: Adiponectin

AEA: *N*-arachidonoylethanolamine

BP: Blood pressure

CBD: Cannabidiol

CB_1R: Cannabinoid receptor 1

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CB_2R: Cannabinoid receptor 2

CNS: Central nervous system

DCFH-DA: 2′, 7′-dichlorofluorescein diacetate

DHE: Dihydroethidium

DMSO: Dimethyl sulfoxide

GPCR: G-protein coupled receptor

GPR18: G-protein coupled receptor 18

HR: Heart rate

i.p.: Intraperitoneal

ir: Immunoreactive

i.v.: Intravenous

MAP: Mean arterial pressure

NAGly: N-Arachidonoyl glycine

NO: Nitric Oxide

O-1918: 1, 3-Dimethoxy-5-methyl-2-[(1*R*, 6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene

RVLM: Rostral ventrolateral medulla

SR141716: Rimonabant

TH: Tyrosine hydroxylase

d) Recommended Section Assignment: Cardiovascular Pharmacology

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Abstract

Systemic administration of the GPR18 agonist abnormal cannabidiol (Abn CBD) lowers blood pressure (BP). Whether GPR18 is expressed in the CNS and plays a role in BP control is not known despite the abundance of the GPR18 ligand N-arachidonoyl glycine (NAGly) in the CNS. Therefore, we first determined if GPR18 is expressed in the presympathetic tyrosine hydroxylase (TH) immunoreactive (ir) neurons of the brainstem cardiovascular regulatory nuclei. Second, we investigated the impact of GPR18 activation and/or blockade on BP and heart rate (HR) and neurochemical modulators of sympathetic activity/BP. Immunofluorescence findings revealed GPR18 expression in TH-ir neurons in the rostral ventrolateral medulla (RVLM). Intra-RVLM GPR18 activation (Abn CBD) and blockade (O-1918) elicited dose-dependent reductions and elevations in BP, respectively, along with respective increases and decreases in HR in conscious male Sprague-Dawley rats. RVLM GPR18 activation increased neuronal adiponectin (ADN) and NO and reduced reactive oxygen species (ROS) levels while GPR18 blockade reduced neuronal ADN and increased oxidative stress (ROS) in the RVLM. Finally, we hypothesized that the negligible hypotensive effect caused by the endogenous GPR18 ligand NAGly could be due to concurrent activation of CB_1R in the RVLM. Our findings supported this hypothesis because NAGly-evoked hypotension was doubled following RVLM CB_1R blockade (SR141716). These findings are the first to demonstrate GPR18 expression in the RVLM, and to suggest sympathoinhibitory role for this receptor. The findings yield new insight into the role of a novel cannabinoid receptor (GPR18) in central BP control.

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Introduction

Endogenous and exogenous cannabinoids exert complex cardiovascular effects due, at least partly, to their activation of diverse cannabinoid receptors (CBR) and their location within the cardiovascular system and different brain nuclei (Randall et al., 2004). The two classical CBRs (CB_1R and CB_2R) are implicated in behavior and cardiovascular regulation (Randall et al., 2002). However, the cardiovascular responses might be confounded by the use of anesthetics because CBs produce hypotension in anesthetized animals (Varga et al., 1995), which is consistent with vasorelaxation in vitro (Jarai et al., 1999), but produce pressor response in conscious rats (Gardiner et al., 2002; Ibrahim and Abdel-Rahman, 2011). Further, many studies implicated several novel receptors in the diverse cardiovascular effects of synthetic and natural cannabinoids (Offertáler et al., 2003). For example, Abn CBD caused mesenteric vasodilation and hypotension in mice lacking CB_1/CB_2 receptors via the activation of a novel CB receptor (Jarai et al., 1999). This new Gi/Go coupled receptor, which mediates endothelium-dependent vasodilation (Begg et al., 2005; Mackie and Stella, 2006), has been named the endothelial 'anandamide receptor' or the 'Abn CBD' receptor. Recent findings indicate that N-arachidonoyl glycine (NAGly), Abn CBD and Δ^9tetrahydrocannabinol (THC) (McHugh et al., 2012) act as selective agonists while O-1918, an analog of cannabidiol (CBD), acts as an antagonist at this receptor (Offertáler et al., 2003; McHugh et al., 2010).

Recent studies have identified the orphan G protein-coupled receptor GPR18 as the 'Abn CBD' receptor and NAGly as its endogenous ligand (Kohno et al., 2006; McHugh et al., 2010; McHugh, 2012). It is possible that Abn CBD and O-1918 might act via

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another putative cannabinoid receptor, GPR55 (Johns et al., 2007; Godlewski et al., 2009). However, more recent studies showed that although GPR18 and GPR55 share some ligands, they do not respond to them in the same manner or with the same efficacy (McHugh et al., 2010; Okuno and Yokomizo, 2011).

GPR18 is found in humans, rodents and canine (Gantz et al., 1997), and its mRNA is most abundantly expressed in testis, spleen and brain stem among other tissues (Vassilatis et al., 2003). Although GPR18 mRNA is expressed in human and rodent brainstem (Vassilatis et al., 2003) and NAGly is abundant in the brain (Huang et al., 2001), there are no studies on the expression and function of GPR18 in brainstem cardiovascular/sympathetic activity regulating nuclei. Notably, NAGly or Abn CBD activation of GPR18, which is coupled to the Gi/o family (Kohno et al., 2006), enhances ERK1/2 phosphorylation and PI3K/Akt signaling (Offertáler et al., 2003; Mo et al., 2004) as well as glycinergic transmission in the nervous system (Jeong et al., 2010). Further, NAGly causes vasorelaxation via NO release in rat small mesenteric arteries (Parmar and Ho, 2010) and N-palmitoyl glycine (a palmitic acid conjugate of NAGly) increases the levels of NO (Rimmerman et al., 2008). Whether these signaling effects result in functional changes in sympathetic activity and ultimately in BP has not been investigated. Notably, the findings that the endogenous GPR18 ligand NAGly has the potential to increase anandamide (AEA) (endogenous CB_1R agonist) (Burstein et al., 2002), and that central CB_1R activation increases BP (Ibrahim and Abdel-Rahman, 2012) might influence the final BP response mediated by NAGly activation of RVLM GPR18.

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The main objectives of the present study were to determine if GPR18 is expressed in RVLM presympathetic neurons, and to elucidate its role in central control of BP. To achieve these goals, we conducted integrative dose-response studies that permitted measurements of BP and HR responses caused by direct activation and/or blockade of the RVLM GPR18 in conscious rats. We also investigated the possibility that concurrent activation of RVLM CB_1R, which mediates sympathoexcitation, might explain the dampened hypotensive response produced by the endogenous GPR18 ligand NAGly. Finally, the integrative pharmacological studies were complemented with ex vivo neurochemical studies to elucidate the molecular mechanisms implicated in the central GPR18-mediated hypotensive response.

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Materials and Methods

Preparation of the Rats. Male Sprague-Dawley rats (300-350 g; Charles River Laboratories, Raleigh, NC) were used in the present study. All rats were housed two per cage in a room with controlled environment at a constant temperature of 23 ± 1^{O}C, humidity of 50 ± 10%, and a 12 hr light/dark cycle. Food (Prolab Rodent Chow, Prolab RMH 3000; Granville Milling, Creedmoor, NC) and water were provided ad libitum. All surgical, postoperative care and experimental procedures were performed in accordance with, and approved by, the Institutional Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 2011). Arterial catheterization, intra-RVLM cannulation and blood pressure measurements were performed as reported in our previous studies (Mao and Abdel-Rahman, 1995; Zhang and Abdel-Rahman, 2002) and as detailed in the supplement.

Western Blot and Neurochemical Studies. Animals received a lethal dose of sodium pentobarbital (i.p.), and following decapitation, brains were removed, flash frozen in 2-methylbutane on dry ice, and stored at -80°C until use as detailed in the supplement.

Immunohistochemistry. The procedure reported in Current Protocols in Neuroscience for immunohistochemistry for light microscopy (Ince et al., 1997) was followed and detailed in the supplement.

Immunofluorescence. Co-localization studies were conducted according to the protocol used in previous reports (Wang and Abdel-Rahman, 2005; Matias et al., 2008) and as detailed in the supplement.

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Measurement of Nitrate/Nitrite. RVLM punches were obtained from the rats of different experimental groups and homogenized in 300 µl phosphate buffered saline (PBS). The homogenate was centrifuged (14,000 rpm) for 20 min and the protein in the supernatant was quantified using Bio-Rad protein assay system. The supernatant, 140 µl, was ultra-filtered using Amicon Centrifugal Filter Units (10 kDa) and centrifuged (14,000 rpm) for 1 hour. The NOx content was measured using a Nitrate colorimetric assay kit (Catalog # 780001) according to manufacturer's instructions (Cayman Chemical Company; Ann Arbor, MI) and as detailed in reported studies (Misko et al., 1993; El-Mas et al., 2009).

Dihydroethidium (DHE) Staining for ROS Detection. For measurement of ROS**,** fresh unfixed brainstem sections (20 µm) were incubated with 10 µM dihydroethidium (DHE) (Molecular Probes, Grand Island, NY) at 37°C in the presence of 5% CO_2 in a moist chamber for 30 min. Positive and negative controls were used to validate the assay (see experimental groups and protocols). Images were visualized with a Zeiss LSM 510 microscope. Three-five images were acquired from 4 brainstem sections for each experimental condition. Quantification was conducted using Image J software (NIH) and changes in total fluorescence intensity, normalized to control, were calculated as reported (Collin et al., 2007).

Measurement of Reactive Oxygen Species by DCFH-DA. RVLM specimen from treated and control groups were homogenized in PBS. The homogenate was centrifuged (14,000 rpm) for 20 min. Protein in the supernatant was quantified using Bio-Rad protein assay system. 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was dissolved in DMSO (12.5 mM) and kept at -80°C in the dark. It

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was freshly diluted with 50 mM phosphate buffer (pH 7.4) to 125 µM before experiment. DCFH-DA was added to RVLM homogenate supernatant (10 µl) in a 96-well microtiter plate for a final concentration (25 µM). 2′, 7′-Dichlorofluorescein (DCF) was used for a 6-point standard curve. Quantification was conducted by examining fluorescence intensity using a microplate fluorescence reader at excitation 485 nm/emission 530 nm. Kinetic readings were recorded for 30 min at 37°C. ROS level was calculated by relative DCF fluorescence per µg protein. Positive and negative controls were used to validate the assay as in our previous studies (McGee and Abdel-Rahman, 2012).

Experimental Groups and Protocol

Anatomical expression of GPR18 in the RVLM. Coronal sections were obtained from naïve rats (n=5) for detecting GPR18 protein by immunohistochemistry and by Western blotting as described under methods. Positive (testis and spleen) and negative (liver) controls, based on reported studies (Gantz et al., 1997), were simultaneously run with RVLM GPR18 to confirm the Western blot findings. Further, we used the GPR18 blocking peptide, recommended by the manufacturer, to verify the GPR18 antibody specificity. Spatial distribution of GPR18, in relation to tyrosine hydroxylase (TH)-ir neurons, in the RVLM was investigated by dual labeling immunofluorescence in brainstem sections containing the RVLM as described under methods and in our previous studies to verify the co-localization of the c-Fos immuno-reactive cell nucleus and TH-ir neurons in the RVLM (Ibrahim and Abdel-Rahman, 2011).

Functional role of RVLM GPR18 in BP and HR regulation. There are no reported studies on the effect of the activation or blockade of RVLM GPR18 on blood pressure in

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conscious or even anesthetized rats. Therefore, we conducted preliminary studies in conscious instrumented rats to identify a dose range for the microinjected GPR18 agonist Abn CBD on BP. Thereafter, 4 groups of conscious unrestrained rats (n=5-6) were employed for investigating the dose-BP/HR responses elicited by intra-RVLM microinjections of the GPR18 agonists NAGly (0.5, 1, 2 or 4 μg) or Abn CBD (0.2, 0.4 or 0.8 μg) or the antagonist O-1918 (0.2, 0.4, 0.8 μg); control rats received equal volume (80 nl) of vehicle (methyl acetate). Following stabilization of BP and HR at baseline, the rat in a particular group received intra-RVLM microinjections of only the GPR18 agonist or antagonist, and control rats received equal amount of the vehicle. Three additional groups of rats (n=6 each) were included to determine the involvement of baroreflexes in the tachycardic and bradycardic response observed following Abn CBD and O-1918 microinjection, respectively. Vagal (atropine 1 mg/kg; i.v.) and β-adrenergic receptor blockade (propranolol 1 mg/kg; i.v.), which abolishes baroreflex mediated bradycardia and tachycardia, according to established protocol (Coleman, 1980), was induced in all rats. Thirty min after atropine and propranolol administration, the rats in a particular group received intra-RVLM Abn CBD (0.2, 0.4 or 0.8 μg), O-1918 (0.2, 0.4, 0.8 μg) or equal volume of vehicle.

Effect of O-1918 on the BP and neurochemical responses elicited by intra-RVLM Abn CBD. Based on the dose-response findings of experiment 2, discussed above, 0.4 μg of Abn CBD or O-1918 (80 nl) was utilized in this experiment to test the hypothesis that enhancement of adiponectin and NO generation in the RVLM underlie the GPR18-mediated hypotensive response. The effects of RVLM GPR18 activation (Abn CBD) on BP and neurochemical responses were investigated in the absence or presence of the

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selective GPR18 blockade (O-1918) in four groups of conscious male rats (n=5-6 each). After stabilization of BP and HR at baseline, the rats in a particular group received intra-RVLM vehicle or Abn CBD (0.4 μg) 30 min after methyl acetate (vehicle) or O-1918 (0.4 μg). BP and HR were monitored after Abn CBD administration and the animals were euthanized during the hypotensive response in the Abn CBD group and the corresponding time in the O-1918 + Abn CBD group. The brains were collected and processed for neurochemical studies.

Effect of microinjecting ADN into the RVLM on BP, NO and ROS levels. In this experiment, we investigated the impact of microinjecting ADN into the RVLM, on mean arterial pressure and heart rate. Animals in this experiment received increasing doses of adiponectin (0.25, 0.5, 1, 2, 4 pmol); the doses were based on adding two lower and two higher doses than the reported 1 pmol ADN microinjected into the area postrema (Fry et al., 2006). Neurochemical effects of ADN were investigated in the brains collected after the conclusion of the cardiovascular studies; the contralateral (untreated) RVLM tissues were used as controls.

Effect of RVLM CB_1R blockade on the cardiovascular effects of NAGly. In this experiment, we investigated the impact of CB_1R blockade, with the selective blocker SR141716, (Hohmann et al., 2005), on the BP response elicited by intra-RVLM NAGly because NAGly regulates AEA (CB_1R agonist) levels (Huang et al., 2001; Burstein et al., 2002). Four groups of conscious rats (n = 5-6 each) received one of the following intra-RVLM treatment combination: (i) DMSO (solvent for SR141716) + Vehicle; (ii) SR141716 (0.1 μg) + Vehicle; (iii) DMSO + NAGly (1 μg) or (iv) SR141716 + NAGly. SR141716 or DMSO was administered into the RVLM 30 min before NAGly or vehicle

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and the dose of SR141716 was based on reported studies (Hohmann et al., 2005). DMSO was diluted in ACSF (1:16) and this DMSO/ACSF mixture had no effect on BP, which is consistent with our previous findings (Nassar et al., 2011). The BP and HR measurements continued for 30 min after which the rats were euthanized and the brains were collected and stored at -80°C for subsequent biochemical studies.

Drugs. Abn CBD, NAGly, O-1918 and SR141716 were purchased from Cayman Chemical (Ann Arbor, MI). Methyl acetate, propranolol hydrochloride, atropine sulfate and dimethyl sulfoxide were purchased from Sigma Aldrich (St. Louis, MO). Adiponectin was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Sterile saline was purchased from B. Braun Medical (Irvine, CA). DMSO was used as the vehicle for SR141716. Methyl acetate was used as the vehicle for Abn CBD, O-1918, and NAGly and was tested in at least three animals without any significant changes in MAP and HR from the basal levels.

Data Analysis and Statistics. All values were expressed as mean ± S.E.M change from their respective baselines. The dose response curves were analyzed using repeated measures ANOVA using SPSS 16.0 statistical package for Windows (SPSS Inc., Chicago, IL) for differences in treatment trends. All other statistical analyses were done using a one-way or repeated-measures ANOVA with Bonferroni post hoc test and Student's t test, Prism 5.0 software (GraphPad Software Inc., San Diego, CA) was used to perform statistical analysis and $P < 0.05$ was considered significant.

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Results

Expression of GPR18 in tyrosine hydroxylase immunoreactive neurons in the RVLM. Western blot (Fig. 1A) and immunohistochemical (Fig. 1B) findings revealed the expression of GPR18 in RVLM neuronal tissues. Positive and negative controls using tissues rich in (testis and spleen), or devoid of (liver), GPR18 verified the GPR18 findings (Fig. 1A). Further, dual labeled immunofluorescence findings revealed the localization of GPR18 in tyrosine hydroxylase-ir neurons of the RVLM (Fig. 1C).

Activation of RVLM GPR18 causes hypotensive response. These studies were conducted to elucidate the functional role of RVLM GPR18 in BP control. Compared with the vehicle (methyl acetate), intra-RVLM microinjection of the GPR18 agonist Abn-CBD caused dose-related reductions in BP along with tachycardic responses (Figs. 2A, B). On the other hand, microinjection of the GPR18 antagonist O-1918 caused dose-dependent increases in BP along with bradycardic responses (Figs. 2A, B). Prior autonomic blockade with atropine and propranolol (1 mg/kg each) had no significant effect on the dose-related reductions and elevations in BP caused by Abn CBD and O-1918, respectively, but fully abrogated the associated HR responses (Figs. 2 C, D). Notably, however, intra-RVLM microinjection of the endogenous GPR18 agonist NAGly caused small ($P>0.05$) hypotensive (-5 ± 1 mmHg; $n=6$) and inconsistent HR responses (Figs. 2E, F). BP and HR values prior to Abn CBD or NAGly were not significantly different (Table 1). Compared to the vehicle control, RVLM GPR18 activation (Abn CBD) increased ADN and NO levels while blockade (O-1918) reduced ADN level (Figs. 3A, B) in the RVLM.

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Intra-RVLM ADN reduces BP and lowers ROS and elevates NO levels in RVLM. ADN caused dose dependent reductions (-2 ± 2 to -12 ± 1 mmHg; *n*=4) in BP (Fig. 4A), but not in HR; however, slight increases in HR of the control group resulted in significant differences in HR at the 0.25 and 1 pmol doses (Fig. 4B). Compared to the control, ADN increased NOx (Fig. 4C) and reduced ROS (Fig. 4D) levels in the RVLM.

RVLM CB_1R blockade unmasks NAGly-evoked hypotension. This experiment was conducted to determine if NAGly concomitant (indirect) activation of RVLM CB_1R masks the GPR18-mediated hypotensive response. Fig. 5A shows co-localization of GPR18 and CB_1R in the presympathetic neurons of the RVLM of naïve rats inferring potential interaction between the two receptors. Pharmacologic studies showed that selective RVLM CB_1R blockade with SR141716 (0.1 μg) caused modest but significant ($P<0.05$) BP reduction (-8 ± 2 mmHg; *n*=6) while HR was not significantly changed (Figs. 5B and C). Further, despite gradual return of BP to baseline level within 30 min of SR141716 administration, prior CB_1R blockade significantly ($P<0.05$) enhanced the hypotensive response (-11 ± 1 mmHg; *n*=6) caused by intra-RVLM NAGly (Fig. 5B). The effect of SR141716 at CB_1R lasts at least 2hrs as reported in a different model system (Jarbe et al., 2010). Therefore, NAGly-evoked hypotension was not a result of the additive hypotensive responses caused by NAGly and SR141716 (Fig. 5B). HR was not influenced by any of the treatments (Fig. 5C). Neurochemical findings showed that SR141716/NAGly treatment significantly increased ADN and NO levels (Figs. 3A, B) and reduced ROS levels (Fig. 7A) in the RVLM compared to NAGly alone.

O-1918 abrogated Abn CBD evoked hypotension and neurochemical responses. To confirm the involvement of GPR18 in Abn CBD evoked BP and RVLM

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neurochemical responses, Abn CBD was microinjected after the GPR18 antagonist O-1918. Intra-RVLM O-1918 (0.4 μg) abrogated ($P<0.05$) the reduction in BP (Figs. 6A, B) and the increases in RVLM ADN (Fig. 3A) and NO level (Fig. 3B) caused by intra-RVLM Abn CBD (0.4 μg). The selected Abn CBD and O-1918 doses were based on the dose response curves for these drugs (Figs. 2A, B). Notably, at the time of Abn CBD or its vehicle administration, the BP of O-1918 pretreated rats had declined towards pretreatment level (Fig. 6A), which rules out functional antagonism as a potential reason for O-1918 abrogation of the hypotensive effect of Abn CBD. Importantly, O-1918 blockade of GPR18 (Abn CBD)-mediated responses was evident for at least 1 hr (Caldwell et al., 2013). The HR responses were not significantly different among the different treatment groups during the observation period (Fig. 6B).

GPR18 activation reduces ROS generation in the RVLM. Given the established link between ROS generation in the RVLM and sympathoexcitation/pressor response (Kishi et al., 2004), this experiment was conducted to determine the impact of GPR18 activation (Abn CBD) or blockade (O-1918) on RVLM ROS level in neuronal tissues collected during the BP responses caused by these interventions. Compared with vehicle, Abn CBD significantly ($P<0.05$) reduced (Fig. 7A) while O-1918 significantly ($P<0.05$) increased ROS levels in the RVLM (Figs. 7A, B). Further, O-1918 abrogated the reduction in RVLM ROS caused by Abn CBD (Figs. 7A); these neurochemical responses paralleled the BP responses described above (Figs. 6A, B).

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Discussion

The following are the most important findings of the present study, which is the first to elucidate the function of RVLM GPR18 in conscious rats: (i) GPR18 is expressed in the TH-ir (presympathetic) neurons of the RVLM; (ii) activation and blockade of RVLM GPR18 causes dose dependent reduction and elevation in BP, respectively; (iii) GPR18 blockade (O-1918) abrogated Abn CBD evoked hypotension; (iv) concomitant CB_1R activation dampens the hypotensive response caused by the endogenous GPR18 ligand NAGly; (v) RVLM GPR18 activation increases ADN and NOx, and reduces ROS levels in the RVLM while its blockade produced the opposite neurochemical responses; (vi) Intra-RVLM ADN reduced BP and RVLM ROS, and increased RVLM NOx. Collectively, the findings identify novel sympathoinhibitory role for RVLM GPR18 mediated, at least partly, by reducing oxidative stress in the RVLM.

While GPR18 mRNA is expressed in humans and mice (Vassilatis et al., 2003), we are the first to demonstrate GPR18 protein expression in the RVLM of rats (Figs. 1A, B). Further, GPR18 is spatially located within the RVLM TH-ir neurons (Fig. 1C), which modulate the sympathetic activity (Sved et al., 2003; Guyenet, 2006) inferring GPR18 involvement in central control of sympathetic tone and BP. To elucidate the RVLM GPR18 functional role, we microinjected selective GPR18 agonist or antagonist into the RVLM of conscious rats. Abn CBD causes CB_1R/CB_2R independent peripheral vasodilation (Randall et al., 2004; Johns et al., 2007); in the latter study, Abn CBD was administered i.v. in anesthetized mice in much higher doses (30 mg/kg). Differences in the route of administration, dose, and anesthesia might explain the differences in the onsets and durations of the hypotensive responses and HR responses in the two

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studies. Here, we report the first evidence that intra-RVLM Abn CBD caused dose-dependent hypotensive response (Fig. 2A). This response was GPR18-mediated because it was abrogated (Fig. 6A) by O-1918, a selective GPR18 antagonist (McHugh et al., 2010). Further, intra-RVLM O-1918 elicited dose-related pressor response (Fig. 2A). Prior cardiac vagal and adrenergic blockade virtually abolished the HR, but not the BP, responses caused by GPR18 activation or blockade (Figs. 2C, D), which suggest that the HR responses are mediated, at least partly, via cardiac baroreflex responses.

It is imperative to comment on the complexity of the observed HR responses. While the reciprocal relationships between HR and BP responses elicited by Abn CBD or O-1918 (Fig. 2 and supplement Fig. 1S) support the involvement of the baroreceptors in the HR responses, it is notable that atropine and propranolol also block baroreceptor-independent cardiac responses. It is highly unlikely that Abn CBD produced direct chronotropic effects because it was microinjected into the RVLM in substantially lower doses than those used systemically (Johns et al., 2007). Nonetheless, it is possible that the central sympathoinhibitory effect of intra-RVLM Abn CBD might have dampened the tachycardic response. This possibility might explain the plateaued tachycardic response despite the dose-dependent hypotensive response caused by Abn CBD (Fig. 2), and the decline of the tachycardic response from its peak before the hypotensive response reached its nadir (Fig. 1S). Collectively, these findings suggest that RVLM GPR18 exerts tonic restraining sympathoinhibitory influence on BP, and that such central effect might contribute to the complexity of the observed HR responses.

The finding that intra-RVLM NAGly, the endogenous GPR18 ligand (Kohno et al., 2006), only modestly reduced BP (Fig. 2E) might: (i) cast doubt about the biological

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significance of RVLM GPR18, and (ii) infer that Abn CBD mediated hypotension was a consequence of local redox changes in the RVLM rather than direct agonism at RVLM GPR18. Notably, NAGly can inhibit the enzyme fatty acid amide hydrolase (FAAH) leading to increased AEA levels (Huang et al., 2001; Burstein et al., 2002). AEA may then activate central CB_1R, which mediates sympathoexcitation/pressor response (Ibrahim and Abdel-Rahman, 2011), and ultimately counterbalance the GPR18 dependent reduction in BP caused by NAGly. In support of this notion are the following findings: (i) GPR18 and CB_1R are co-localized in RVLM neurons (Fig. 5A), which partly agrees with our findings that demonstrated CB_1R expression in RVLM TH-ir neurons (Ibrahim and Abdel-Rahman, 2011); (ii) the ability of intra-RVLM SR141716 (CB_1R blockade) to lower BP (Fig. 5B) is consistent with a sympathoexcitatory/pressor function for central CB_1R (Ibrahim and Abdel-Rahman, 2011); (iii) while NAGly alone had no effect on ADN, NOx or ROS levels, prior CB_1R blockade (SR141716) uncovered the NAGly ability to increase ADN and NO (Figs. 3 A, B), and to reduce ROS level (Fig. 7A) in the RVLM along with lowering BP (Fig. 5B). These novel findings replicated the redox and BP effects of Abn CBD (Figs. 3, 7A). Together, these data support the conclusion that the reductions in RVLM oxidative stress and BP are caused by Abn CBD direct agonism at GPR18 and suggest a functional role for GPR18-CB_1R interaction in the RVLM in modulating the local redox state and BP. It is imperative to comment on the pharmacological perspectives of our study because the endogenous GPR18 ligand NAGly lowered BP only following blockade of CB_1R, which might infer that CB_1R blockade is required for uncovering the GPR18-mediated responses. Our present findings argue against generalizing this notion because the GPR18 agonist Abn CBD,

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which does not interact with CB_1R directly or indirectly (Jarai et al., 1999; Offertáler et al., 2003), lowered BP without prior CB_1R blockade.

It is important to comment on the complexity of RVLM NOS-derived NO in BP regulation because NO is implicated in GPR18-evoked hypotension (this study) and CB_1R-mediated hypertension (Ibrahim and Abdel-Rahman, 2012). Findings of the latter agree with a sympathoexcitatory role for RVLM NO (Chan et al., 2003). It is likely that the RVLM NO effect on BP depends on the source of NO, and its effect on local sympathoinhibitory (GABA) and sympathoexcitatory (L-glutamate) neuromodulators. For example, while GABA inhibition is implicated in the NO-dependent CB_1R-mediated pressor response (Ibrahim and Abdel-Rahman, 2012), eNOS-derived NO mediates increases in RVLM GABA level, and hypotension (Kishi et al., 2001; Kishi et al., 2002). More studies are warranted to delineate the mechanisms of the differential role of RVLM NO in modulating sympathetic activity/BP, and to investigate the possibility that GPR18-dependent NO generation enhances RVLM GABA release/signaling in future studies.

A common anti-inflammatory role for ADN (Nanayakkara et al., 2012) and GPR18 (Vuong et al., 2008) infers a role for ADN in GPR18 signaling. We present the first evidence that GPR18 activation in the RVLM increases neuronal ADN (Figs. 3A) along with findings that support a functional role for ADN in GPR18 signaling because RVLM GPR18 blockade (O-1918): (i) reduced RVLM ADN (Fig. 3A) and elevated BP; (ii) abrogated the GPR18 (Abn CBD)-mediated BP and neurochemical responses (Figs. 3, 6). Next, we show, for the first time, that ADN produced dose-dependent reductions in BP (Fig. 4A), increased RVLM NOx (Fig. 4C), and reduced RVLM ROS levels (Fig. 4D). The ADN doses were based on a reported microinjected dose of ADN in area postrema

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(Fry et al., 2006). In the latter study, ADN caused modest pressor response and inconsistent changes in HR. Differences in the neuroanatomical targets, and use of anesthesia in the reported study might account for the differences in BP responses. Further, a very recent study (Song et al., 2013) showed that the active (globular) ADN fraction replicates ADN-evoked neuroprotection via a reduction in oxidative stress. Together, these findings implicate RVLM ADN in the GPR18-mediated reductions in neuronal oxidative stress (ROS) in the RVLM and BP.

We reasoned that the GPR18-mediated neurochemical responses, discussed above, would ultimately reduce BP via ROS reduction in RVLM because enhanced and suppressed ROS production in the RVLM leads to elevation and reduction in BP, respectively (Kishi et al., 2004; Yoshitaka, 2008). Using two different methods, we showed that activation of RVLM GPR18 reduced neuronal ROS while its blockade increased neuronal ROS and abrogated the GPR18-mediated ROS reduction (Fig. 7). These redox findings, which paralleled the ADN (Fig. 3A) and BP (Figs. 6A, B) responses, reinforce a well-established role for oxidative stress in RVLM neurons in sympathoexcitation and BP elevation. Further, the findings lend credence to our conclusion that ADN-dependent reduction in RVLM ROS plays a crucial role in GPR18-mediated hypotension.

In summary, the present study yields new insight into the role of the novel cannabinoid receptor GPR18 in central (RVLM) control of BP. We present the first evidence that RVLM GPR18 mediates reductions in oxidative stress and BP in conscious rats. The neurochemical findings suggest that increases in ADN and NO and reduced ROS production in the RVLM play significant role in GPR18-mediated

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hypotension. In the RVLM, CB_1R serves a counterbalancing role against GPR18, which explains the negligible hypotensive response caused by the endogenous GPR18 ligand NAGly in our model system. Future studies are warranted to delineate the GPR18 signaling implicated in the neurochemical effects described in this study, and to investigate the role of GPR18 signaling in hypertension. Such studies will advance our knowledge of the role of endocannabinoids in the neural control of BP and might lead to the development of novel antihypertensive drugs.

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Authorship Contributions

Participated in Research Design: A. Penumarti and A. A. Abdel-Rahman

Conducted Experiments: A. Penumarti

Performed data analysis: A. Penumarti

Contributed to the writing of the manuscript: A. Penumarti and A. A. Abdel-Rahman

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Figure Legends

Figure 1. (A) Expression of GPR18 (38kDa) in the rat RVLM compared with testes and spleen (positive controls) and liver (negative control). **(B)** Immunohistochemical staining showing the expression of GPR18 in the RVLM of perfused naïve rat brains. **(C)** Dual labeled immunofluorescence of perfused naïve rat brains showing co-expression of GPR18 and Tyrosine hydroxylase (TH) expressing neurons in the RVLM.

Figure 2. Effect of intra-RVLM Abn CBD or O-1918 (0.2, 0.4 or 0.8 μg) **(A, B)** on mean arterial pressure (MAP) and heart rate (HR) in conscious male rats. **(C, D)** Effect of vagal and beta adrenergic blockade with atropine (1mg/kg) and propranolol (1mg/kg), respectively, on the MAP and HR elicited by intra-RVLM Abn CBD or O-1918 (0.2, 0.4 or 0.8 μg). Effect of the endogenous ligand of GPR18, NAGly (0.5, 1, 2 or 4 μg) **(E, F)** on the MAP and HR. Values are mean ± S.E.M. of 5-6 observations. *P < 0.05 vs. control (Vehicle).

Figure 3. Western blots and total nitrate/nitrite levels in the RVLM (NOx content; index of NO) showing the effects of NAGly (1 μg), Abn CBD (0.4 μg), O-1918 (0.4 μg), or O-1918 (0.4 μg)/Abn CBD (0.4 μg) or SR141716 (0.1μg)/NAGly (1 μg) treatment on adiponectin (ADN) expression **(A)** and nitrate/nitrite (NOx) level **(B)** in the RVLM. Values are mean ± S.E.M. of 5-6 observations. *P < 0.05 vs. vehicle; #P < 0.05 vs. Abn CBD; ^P < 0.05 vs. NAGly values.

Figure 4. Effect of intra-RVLM adiponectin, ADN (0.25, 0.5, 1, 2 and 4 pmol) **(A, B)** on mean arterial pressure and heart rate in conscious male rats (n=4), compared with values obtained from vehicle (ACSF)-treated rats (n=3). Total nitrate/nitrite levels (NOx;

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index of NO) **(C)** and DCFH-DA measured ROS **(D)** in the RVLM following ADN microinjection, compared with the corresponding values in the contralateral (control) RVLM. *$P < 0.05$ vs. contralateral RVLM levels.

Figure 5. (A) Dual labeled immunofluorescence of perfused naïve rat brains showing co-expression of GPR18 and CB_1R in the RVLM neurons. Time course changes in ΔMAP **(B)** and ΔHR **(C)** caused by intra-RVLM microinjection of DMSO/Vehicle, DMSO/NAGly (1 μg), SR141716 (0.1μg)/Vehicle and SR141716 (0.1μg)/NAGly (1 μg). The animals in each group received intra-RVLM microinjections of either DMSO (diluted 1:16 in ACSF) or SR141716 (0.1μg) at -30 min followed by vehicle (methyl acetate) or NAGly (1 μg) at time "0". Pretreatment with SR141716 (CB1R blockade) uncovered NAGly (GPR18)-mediated hypotension. Values are means ± S.E.M. of 5 observations. *$P < 0.05$ compared with the corresponding control value.

Figure 6. (**A, B**) Time course of changes in MAP and HR following intra-RVLM microinjection of vehicle/Abn CBD (0.4 μg), O-1918 (0.4 μg)/vehicle or O-1918 (0.4 μg)/Abn CBD (0.4 μg), compared with corresponding vehicle/vehicle values. The animals in each group received intra-RVLM microinjections of either vehicle (methyl acetate) or O-1918 (0.4 μg) at -30 min followed by vehicle (methyl acetate) or Abn CBD (0.4 μg) at time "0". Pretreatment with O-1918 abrogated the hypotensive effect produced by Abn CBD and the associated tachycardic response. The bar graphs (C-F) depict the area under the curve (AUC) data generated from the time-course values over the pretreatment (-30 to 0 min) and treatment (0-30 min) period. Compared with vehicle, the two groups that were pretreated with O-1918 exhibited significant elevations in BP

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and reductions in HR (Figs. 6C & E). Treatment with Abn CBD caused significant reduction in BP and increase in HR, and these responses were abrogated in O-1918 pretreated rats (Figs. 6D & F). Values are mean ± S.E.M. of 5-6 observations. *P < 0.05 vs. vehicle.

Figure 7. (A) Effect of vehicle, NAGly (1μg), Abn CBD (0.4 μg), O-1918 (0.4 μg), O-1918 (0.4 μg)/Abn CBD (0.4 μg) and SR141716 (0.1μg)/NAGly (1μg) on RVLM ROS levels shown by Dihydroethidium (DHE) staining visualized with confocal microscopy. Values are mean ± S.E.M (n=5-6 rats). *P < 0.05 vs. vehicle values; #P < 0.05 vs. Abn CBD; ^P < 0.05 vs. NAGly values. **(B)** DCFH-DA measured ROS levels in terms of relative fluorescence units (RFU) in the RVLM following treatment with vehicle, Abn CBD (0.4 μg), O-1918 (0.4 μg) and O-1918 (0.4 μg) /Abn CBD (0.4 μg). The values of NAGly (1 μg) were not significantly different from the control and are not shown for clarity. Values are mean ± S.E.M (n=5-6 rats). *P < 0.05 vs. vehicle values; #P < 0.05 vs. Abn CBD.

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Table 1. Mean Arterial Pressure (MAP, mmHg) and Heart Rate (HR, beats/min) values immediately before intra-RVLM administration of the GPR18 agonists or their vehicles.

Group	n	MAP	HR
Vehicle	5	110 ± 5	364 ± 12
Abn CBD	6	100 ± 8	376 ± 9
NAGly	6	113 ± 2	327 ± 20
O-1918	6	108 ± 3	343 ± 7
Atropine/Propranolol + Vehicle	6	120 ± 5	353 ± 8
Atropine/Propranolol + Abn CBD	6	120 ± 11	342 ± 24
Atropine/Propranolol + O-1918	6	113 ± 5	340 ± 16
ADN	4	111 ± 5	336 ± 17
DMSO/Vehicle	5	109 ± 7	325 ± 24
DMSO/NAGly	6	115 ± 5	356 ± 17
SR141716/Vehicle	6	107 ± 6	352 ± 12
SR141716/NAGly	6	110 ± 8	360 ± 22
Vehicle/Vehicle	5	108 ± 5	351 ± 9
Vehicle/Abn CBD	6	113 ± 4	362 ± 13
O-1918/Vehicle	6	117 ± 6	345 ± 18
O-1918/Abn CBD	6	112 ± 7	366 ± 12

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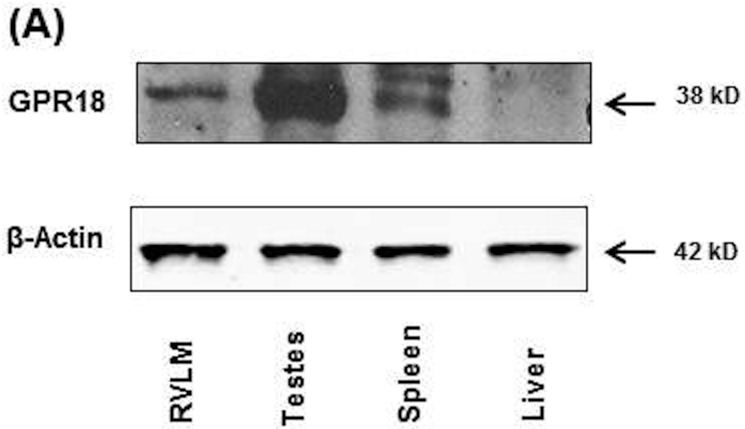
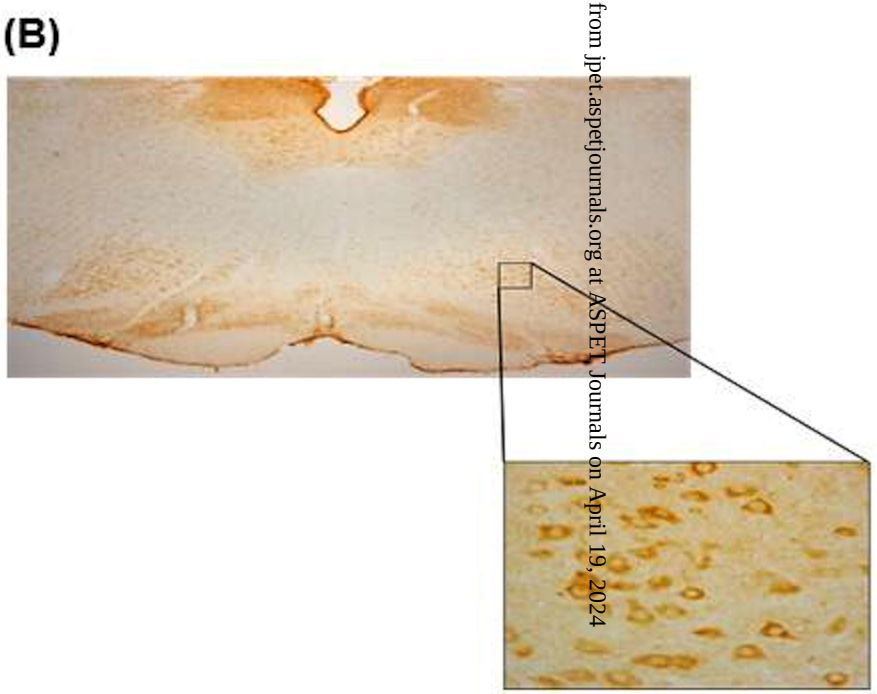
Figure 1





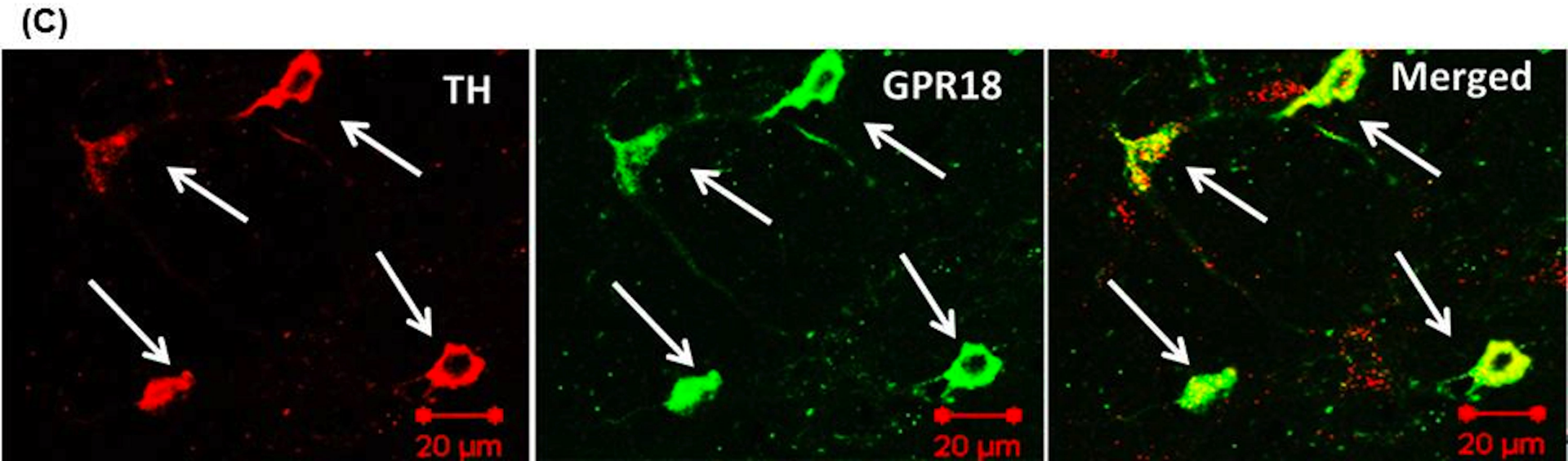


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Figure 2

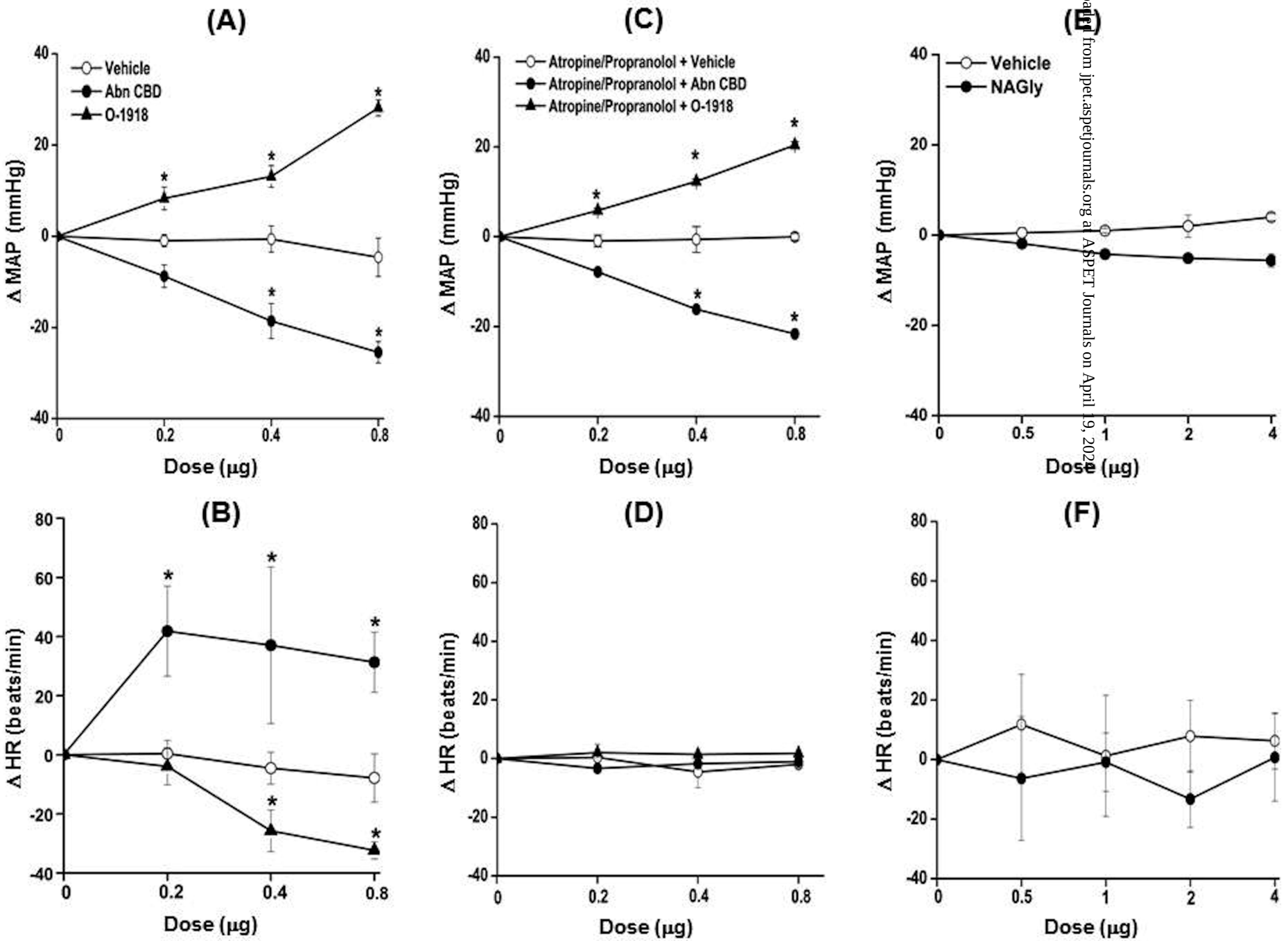

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Figure 3

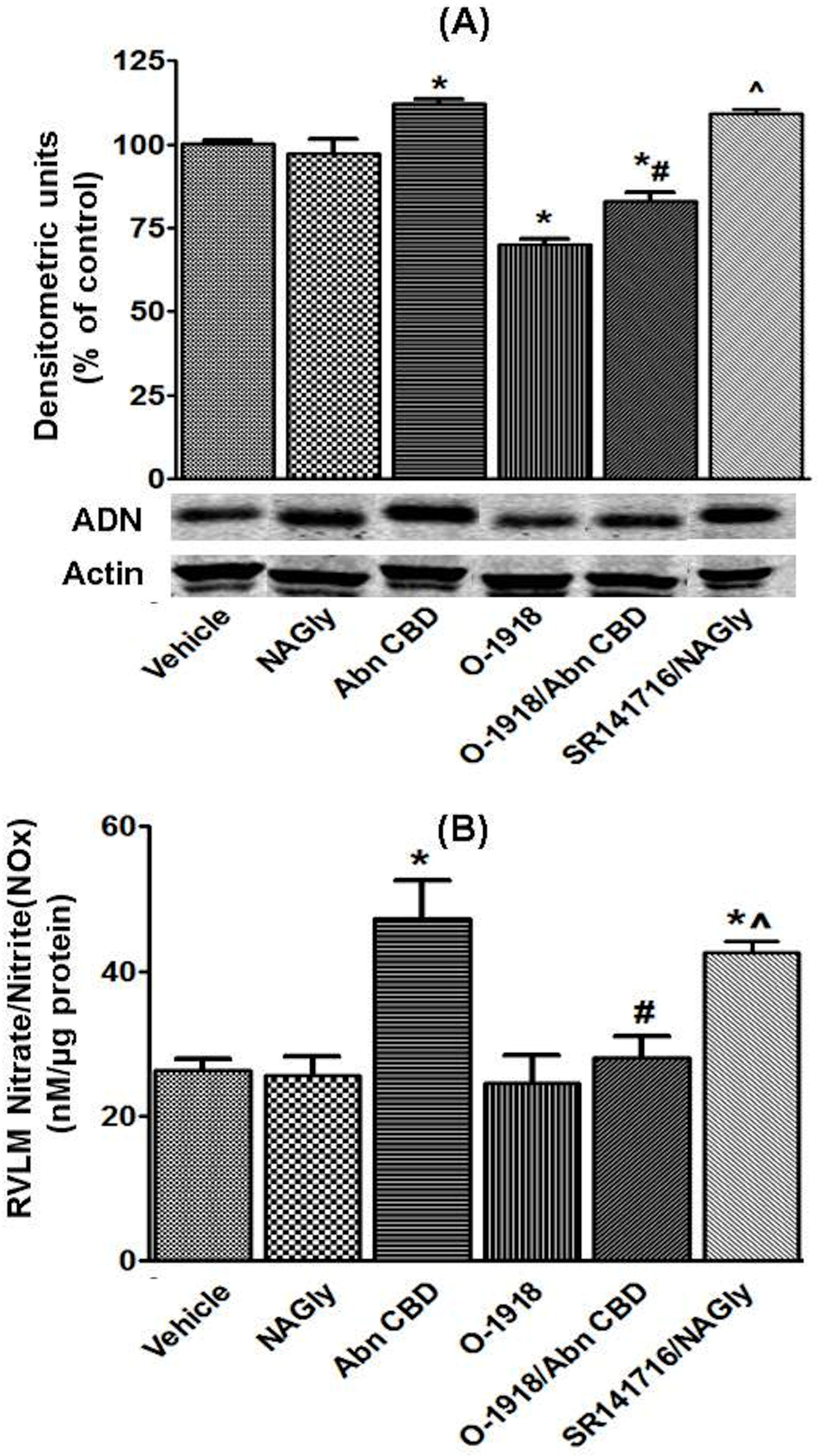

(A)
Densitometric units
(% of control)
125
100
75
50
25
0
*
*
*#
^
ADN
Actin
Vehicle
NAGly
Abn CBD
O-1918
O-1918/Abn CBD
SR141716/NAGly
(B)
RVLM Nitrate/Nitrite(NOx)
(nM/µg protein)
60
40
20
0
*
#
*^
Vehicle
NAGly
Abn CBD
O-1918
O-1918/Abn CBD
SR141716/NAGly

Figure 4

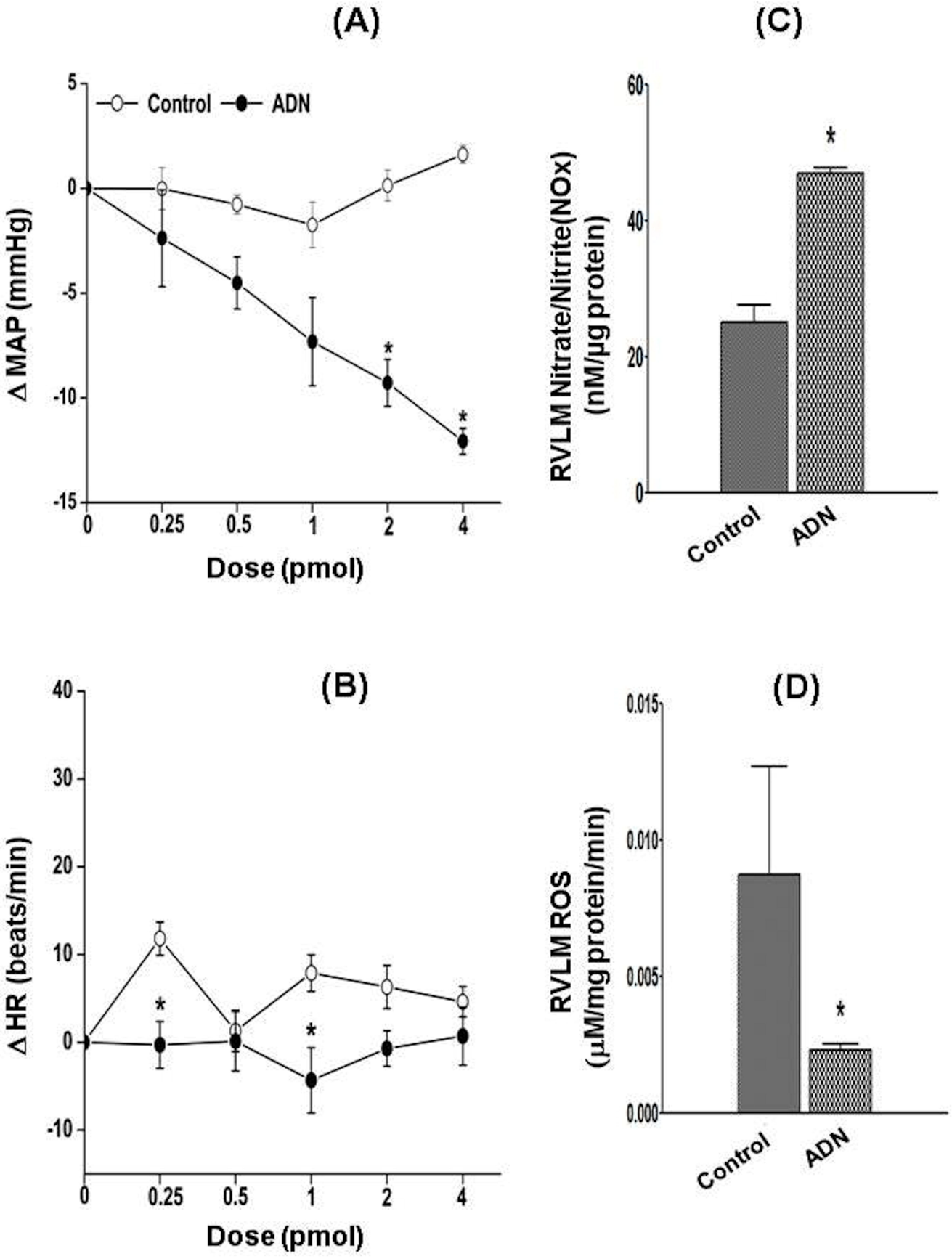

(A)
Control
ADN
Δ MAP (mmHg)
Dose (pmol)
(C)
RVLM Nitrate/Nitrite(NOx) (nM/μg protein)
Control
ADN
(B)
Δ HR (beats/min)
Dose (pmol)
(D)
RVLM ROS (μM/mg protein/min)
Control
ADN

Figure 5

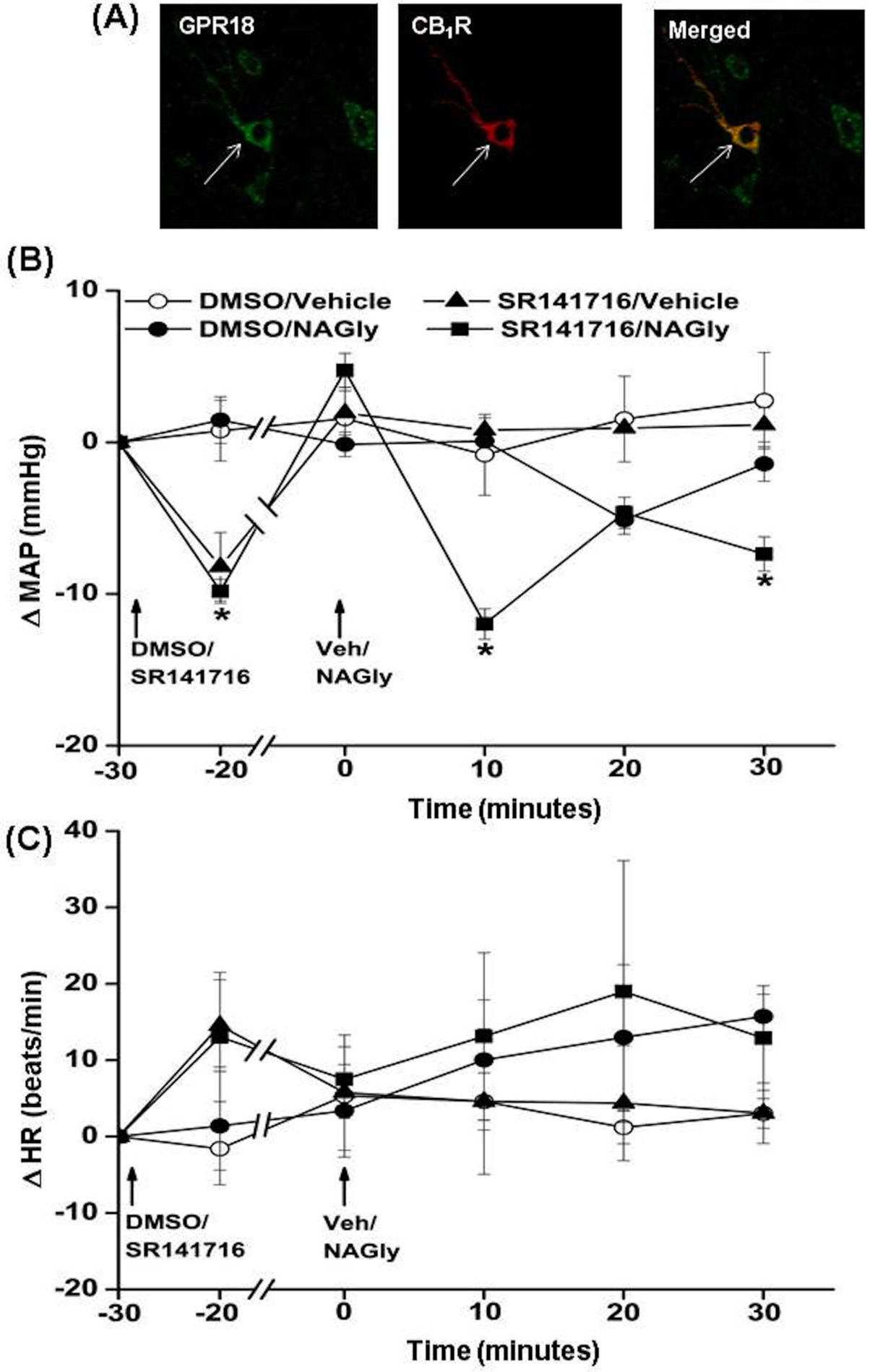

(A)
GPR18
CB1R
Merged
(B)
DMSO/Vehicle
SR141716/Vehicle
DMSO/NAGly
SR141716/NAGly
Δ MAP (mmHg)
10
0
-10
-20
DMSO/
SR141716
Veh/
NAGly
*
*
*
-30
-20
0
10
20
30
Time (minutes)
(C)
Δ HR (beats/min)
40
30
20
10
0
-10
-20
DMSO/
SR141716
Veh/
NAGly
-30
-20
0
10
20
30
Time (minutes)

Figure 6

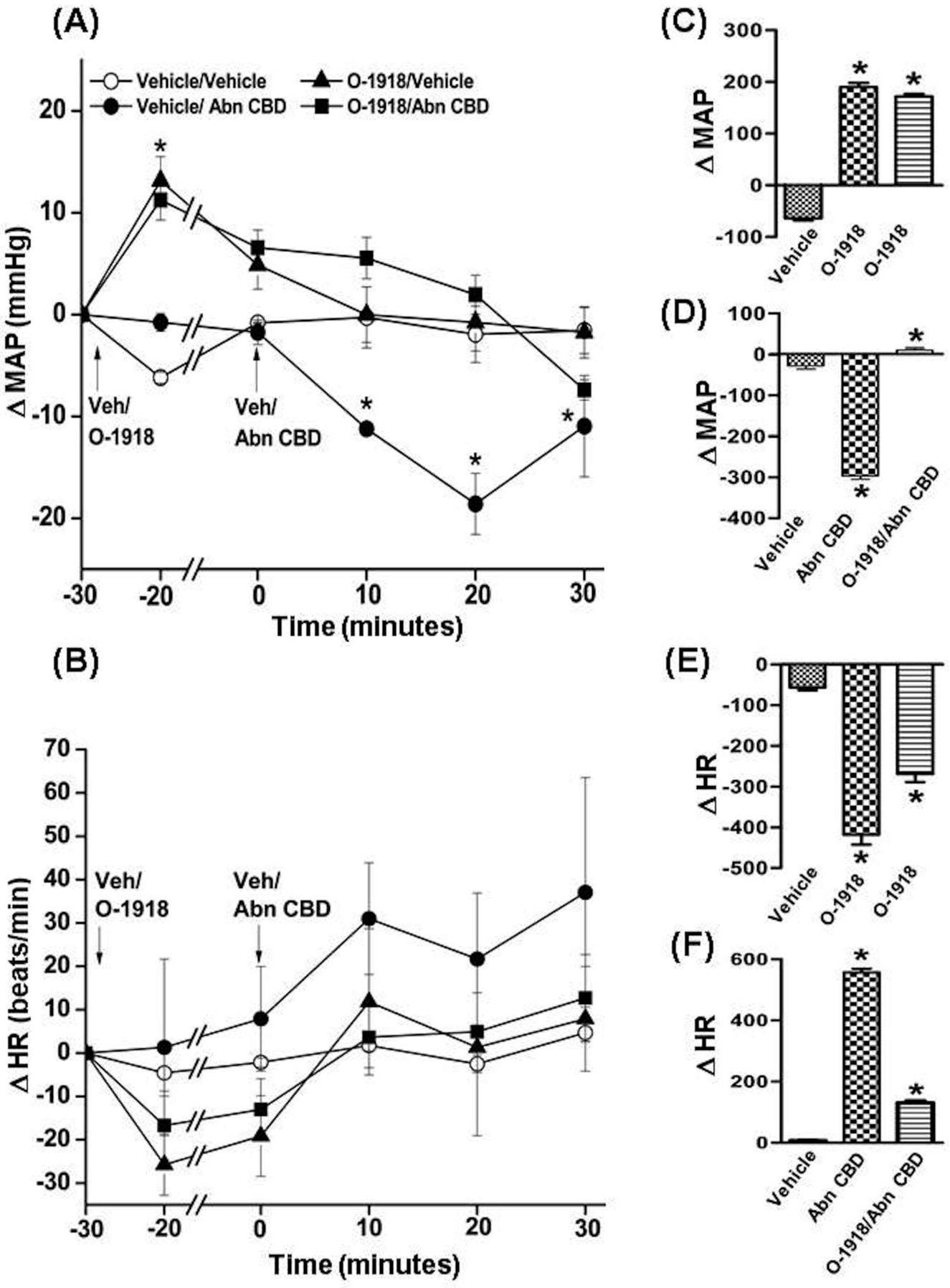
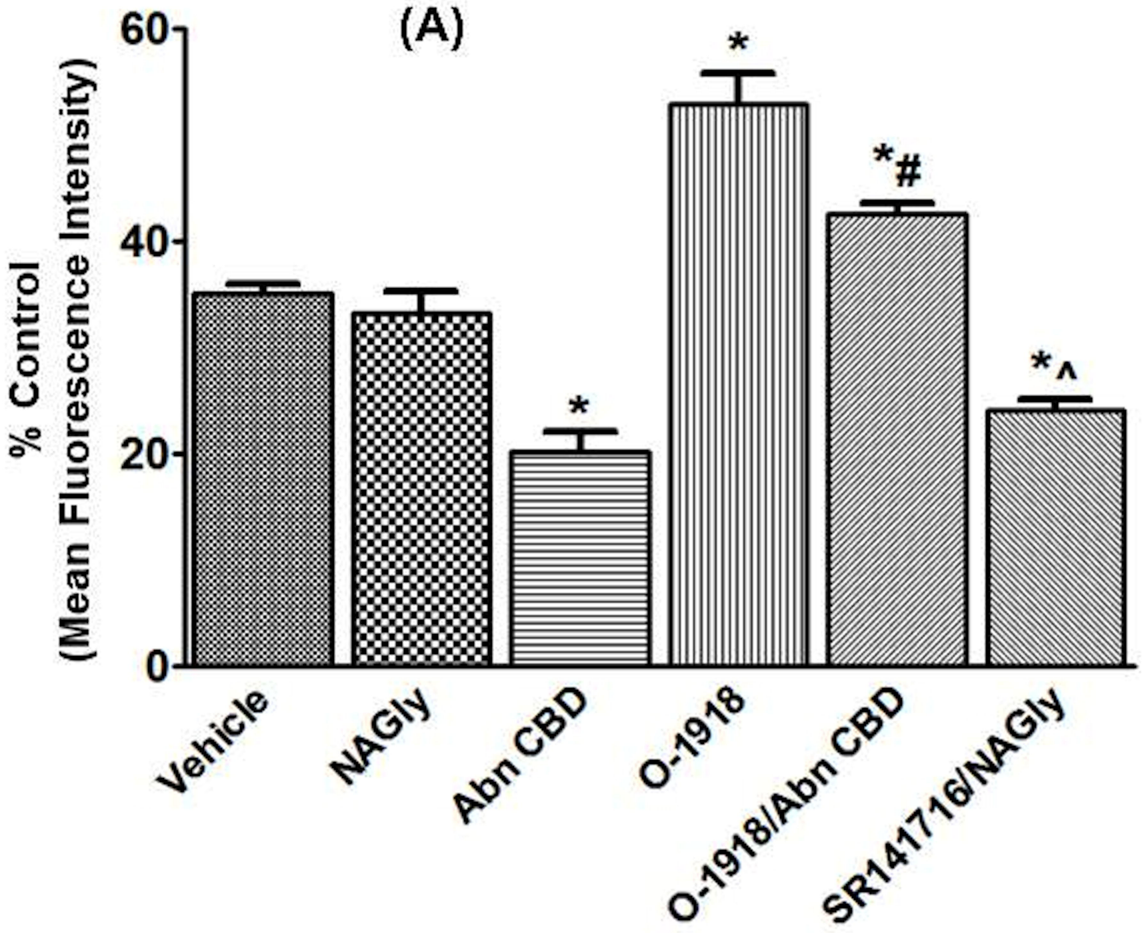
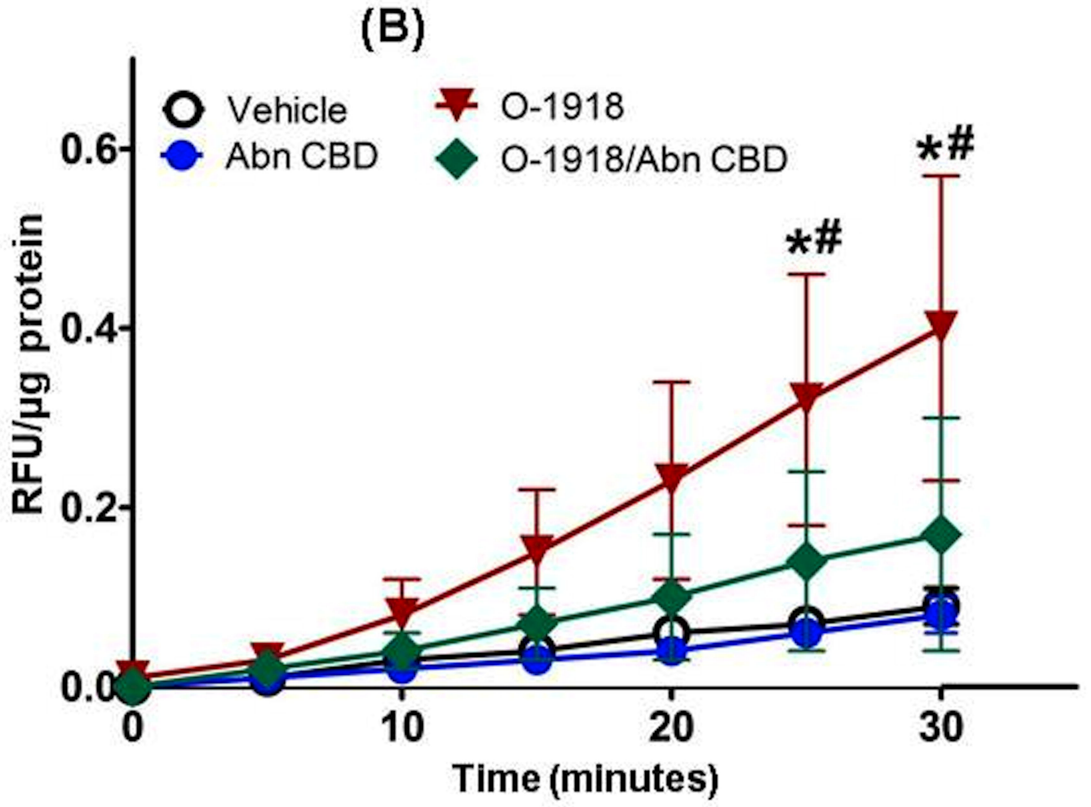

(A)
Vehicle/Vehicle
O-1918/Vehicle
Vehicle/ Abn CBD
O-1918/Abn CBD
Δ MAP (mmHg)
Veh/
O-1918
Veh/
Abn CBD
Time (minutes)
(B)
Δ HR (beats/min)
Veh/
O-1918
Veh/
Abn CBD
Time (minutes)
(C)
Δ MAP
Vehicle
O-1918
O-1918
(D)
Δ MAP
Vehicle
Abn CBD
O-1918/Abn CBD
(E)
Δ HR
Vehicle
O-1918
O-1918
(F)
Δ HR
Vehicle
Abn CBD
O-1918/Abn CBD

Figure 7


(A)
% Control
(Mean Fluorescence Intensity)
60
40
20
0
*
*
*#
*^
Vehicle
NAGly
Abn CBD
O-1918
O-1918/Abn CBD
SR141716/NAGly


(B)
Vehicle
Abn CBD
O-1918
O-1918/Abn CBD
RFU/µg protein
0.6
0.4
0.2
0.0
*#
*#
0
10
20
30
Time (minutes)