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Estrogen Dampens Central Cannabinoid Receptor 1-mediated Neuroexcitation and Pressor Response in Conscious Female Rats

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Abstract

Activation of the rostral ventrolateral medulla (RVLM) cannabinoid receptor-1 (CB_1R) causes nNOS-dependent increases in sympathetic activity, blood pressure (BP) and heart rate (HR) in male rats. However, it remains unknown if the CB_1R-mediated neurochemical and cardiovascular responses are influenced by the ovarian sex hormones, particularly estrogen (E_2). Therefore, we studied the effects of intra-RVLM CB_1R activation (WIN 55,212-2) on BP and HR in conscious female rats under the following hormonal states: (1) highest E_2 level (proestrus sham-operated, SO); (2) E_2-deprivation (ovariectomized, OVX); (3) OVX with E_2 replacement (OVXE2). Intra-RVLM WIN55,212-2 elicited dose (100-400 pmol) dependent pressor and tachycardic responses, in OVX rats, which replicated the reported responses in male rats. However, in SO and OVXE2 rats, the CB_1R-mediated pressor response was attenuated and the tachycardic response reverted to bradycardic response. The neurochemical findings suggested a key role for the upregulated RVLM sympathoexcitatory molecules phosphorated protein kinase B, phosphorated neuronal nitric oxide synthase and reactive oxygen species in the exaggerated CB_1R-mediated BP and HR responses in OVX rats, and an E_2-dependent dampening of these responses. The intra-RVLM WIN55212-2-evoked cardiovascular and neurochemical responses were CB_1R-mediated because they were attenuated by prior CB_1R blockade (AM251). Our findings suggest that attenuation of RVLM neuroexcitation and oxidative stress underlies the protection conferred by E_2, in female rats, against the CB_1R-mediated adverse cardiovascular effects.

Keywords

Cannabinoid receptor-1; Blood pressure; Estrogen; Oxidative stress; Rostral ventrolateral medulla

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CrediT

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1. Introduction

Estrogen modulation of the central cannabinoid receptors 1 (CB_1R) is extensively studied in brain nuclei related to behavioral and reproduction, but not in cardiovascular regulating nuclei [1]. Central CB_1R direct activation [2-4] or following systemic administration of its agonist WIN55, 212-2 [5] causes pressor response in conscious animals. Notably, sympathetic neuronal activity, which is controlled by the rostral ventrolateral medulla (RVLM), plays important role in the central CB_1R-mediated increases in plasma norepinephrine and blood pressure (BP) in conscious rabbits [6] and in anesthetized rats [3]. Further, direct evidence, provided by studies including ours, showed that microinjections of CB_1R agonists into the RVLM increase BP and sympathetic nerve activity [7-9]. Mechanistically, our previous study suggested a pivotal role for phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) in the central CB_1R-mediated sympathoexcitation/ pressor response [7]. Nonetheless, most, if not all, of these studies were conducted in male animals [10]. Therefore, it remains unknown if these RVLM CB_1R-mediated neurochemical and the subsequent BP elevation are influenced by the ovarian hormones, particularly estrogen (E_2).

E_2 is a critical modulator of cardiovascular function in both humans and animals [11-13], estrogen receptor (ER)-β in the hypothalamic paraventricular nucleus (PVN) and RVLM plays an essential protective role against aldosterone/salt-induced hypertension in female rats [14]. And the ERs are distributed in RVLM neurons [11, 15, 16]. Microinjection of E_2 into the RVLM reduces sympathetic tone and BP [12, 13], and this hypotensive response is ERβ mediated, at least pertly, via enhanced inducible nitric-oxide synthase (iNOS)-signaling pathway [12] or PI3K/Akt [17]. Notably, E_2/ERβ dependent changes in other NOS isoforms expression, which included increased endothelial NOS (eNOS) and decreased expression of neuronal NOS (nNOS), are reported in the PVN [18]. These findings seem to suggest tissue specificity of E_2 on NOS signaling in different cardiovascular nuclei.

By marked contrast to central E_2 effects, the enhancements of PI3K/Akt signaling [19-21] and its downstream effector, nNOS [22] underlie the central CB_1R-mediated sympathoexcitation and pressor response, discussed above. It is noteworthy that RVLM nNOS-derived NO causes sympathoexcitation [23-25] and is involved in central BP regulation [24, 26, 27]. There is also evidence that the PI3K/Akt-nNOS-mediated oxidative stress (higher reactive oxygen species, ROS) in the RVLM contributes to sympathoexcitation [28-30]. Based on this knowledge, intra-RVLM E_2 (in either sex) or CB_1R activation (in male animals) similarly activates RVLM PI3K/Akt-nNOS signaling but lead to discordant cardiovascular responses. However, it remains unknown if E_2 will exacerbate or attenuate the RVLM CB_1R-mediated neurochemical and the subsequent cardiovascular responses in female rats.

The aim of the present study was to elucidate the effects of endogenous and exogenous E_2 on the RVLM CB_1R-mediated neuronal responses and the subsequent pressor response in female rats. To achieve this goal, we investigated the effects of RVLM CB_1R activation (WIN55212-2) on BP and RVLM neurochemical responses in conscious sham operated (SO) proestrus (highest endogenous E_2 level), E_2-devoid (ovariectomized; OVX) and OVX

with E_2 replacement (OVXE2) rats. Additional studies were conducted with the selective CB_1R blocker AM251 to ensure the mediation of the neurochemical and cardiovascular responses, elicited by intra-RVLM WIN55212-2, via the RVLM CB_1R in our model system.

2. Materials and methods

2.1 Animals

Female Sprague-Dawley rats (180-200 g; Charles River, Raleigh, NC) were housed at a constant temperature of 23±1°C, humidity of 50±10%, and a 12-h light/dark cycle, with free access to food (Prolab Rodent Chow; Granville Milling, Creedmoor, NC) and water provided ad libitum. All surgical procedure, experimental, and post-operative care procedures were performed in accordance with and approved by the East Carolina University Institutional Animal Care and Use Committee and Guide for the Care and Use of Laboratory Animals (Institute of Laboratory and Animal Resources, 2015).

2.2 Surgery

2.2.1 Surgical procedures were conducted under ketamine (Vedco, Inc. St. Joseph, MO, 90 mg/kg) and xylazine (Akom, Inc. Lake Forest, IL, 10 mg/kg i.p.) anesthesia and sterile conditions. Meloxidyl (Patterson Veterinary, Richmond, VA. 1mg/kg, oral) was administered 30 min before surgery.

2.2.2 Ovariectomy.—As detailed in our previous publication [31], a single longitude incision (approx. 1 cm) beside the middle line was made on the back of the animal, both side of ovaries were isolated and removed. The muscles and the skin were sutured separately. SO was performed by exposing the ovaries without removal.

2.2.3 E_2 replacement.—As described [32], a 1.5 cm veterinary grade silastic tubing (Konigsberg Instruments, Pasadena, CA, 0.062 in I.D, 0.125 in O.D), filled with sesame oil (Sigma-Aldrich, St. Louis, MO, control, sham and OVX rats) or 12.5 μg (25μL) of 17β-estradiol (Sigma-Aldrich, St. Louis, MO), dissolved in sesame oil (OVXE2 rats, sealed with Loctite silicone sealant (Henkel Corporation; Westlake, Ohio), gas sterilized. Two weeks after sham or ovariectomy surgery, under isoflurane (Pivetal, Liberty MO) anesthesia the tubing was implanted subcutaneously on the back of neck for one week.

2.2.4 Arterial catheterization.—As detailed in our study [7], gas sterilized catheters were placed into the abdominal aorta via the femoral artery for BP measurement. The catheter was tunneled subcutaneously and exited at the back of the neck between scapula, anchored with 3M Vetbond™ tissue adhesive (3M Animal Care Products, St. Paul, MN), and plugged with sterile stainless-steel pins. Incisions were closed by surgical clips and swabbed with povidone-iodine solution.

2.2.5 RVLM guide cannulation.—As reported in our previous study [7], rat head was fixed in a stereotaxic frame (David KOPF Instruments, Tujunga, CA). A stainless-steel guide cannula (Small Parts Inc. Logansport, IN; 21.5 in gauge, 14 mm in length) was implanted 2 mm above the RVLM level at coordinates of −3.0 mm posterior, ±2.2 mm lateral, and

−0.5 mm dorsoventral with the interaural line as the reference according to The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 2005). The cannula was fixed with Durelon™ cardoxylate cement (3M ESPE, AG,). Verification of the tip of the microinjection cannula tracks was conducted at the conclusion of the study using the atlas of Paxinos and Watson. Occasionally, we conducted chemical identification with a test dose of L-glutamate (1 nmol in 40 nL), at the beginning of the study, which produced the expected short-lived pressor response as reported [33]. Ample time (30 min) was allowed following this step and the responses to WIN 55,212-2 were similar in rats subjected to this test verification or not.

2.3 BP and HR measurements

The method detailed in our previous studies [7, 34] were followed. Briefly, the rats were allowed to adapt to the environment for at least 30 min. The arterial catheter was flushed with sterile heparinized (Sigma-Aldrich, St. Louis, MO) saline (100 IU/ml) 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 (AD Instruments, Colorado Spring, CO). HR was extracted from BP recording. BP and HR allowed to stabilize for at least 30 min before each intra-RVLM injections.

2.4 Measurement of plasma 17β-estradiol

Blood samples were collected from the femoral catheter before hemodynamic measurements, centrifuged at 5000 g, 4°C for 10 min. The plasma was separated and stored at −20°C. The estradiol level in the samples was measured using ELISA immunoassay (Estradiol EIA kit, Oxford Biomedical Research, Oxford, MI, U.S.A) according to manufacturer's instructions and reported studies [35].

2.5 Western blot

As descripted in our previous publication [7], RVLM tissues were collected at coordinates 12.8 to 11.8 mm relative to bregma (Paxinos and Watson, 2005) with a 0.75-mm punch instrument (Stoelting Co., Wood Dale, IL). The RVLM tissues were homogenized on ice in a lysis buffer with protease inhibitor cocktail (Roche DiagnosticsGmbH, Mannheim, Germany). Protein (40 μg) was separated by 4-12% gel electrophoresis (Novex Tis-Glycine gel, Life Technologies, CA), followed by transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), which were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1h and then incubated overnight at 4°C with a mixture of mouse anti-β-actin (Abcam, Cambridge, United Kingdom, 1:15,000) and rabbit anti-CB_1R (1:200, Santa Cruz, CA, USA), or rabbit anti-phospho-nNOS (Ser1417) antibody (1:200; Thermo-Fisher Scientific, Waltham, MA) and mouse polyclonal anti-nNOS antibody (1:100; BD Biosciences, San Jose, CA), or mouse anti-p-Akt (1:200, Cell Signaling, Danvers, MA) and rabbit anti-Akt (1:200, Cell Signaling, Danvers, MA). Membranes then were incubated for 60 min with secondary antibody mixture containing IRDye680-conjugated goat anti-mouse and IRDye800-conjugated goat anti-rabbit (1:15000; LI-COR Biosciences, San Jose, CA). Bands were detected by Odyssey Infrared Imager and analyzed with Odyssey application software version 3 (LI-COR Biosciences, San Jose, CA).

2.6 Measurement of NO and ROS level

Following the method of NO and ROS measurements descripted in our previous study [36], 30 μm of frozen brain stem sections were incubated with 10 μM of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (NO fluorogenic probes DAF-FM, Invitrogen, Waltham, MA) or 2′,7′-dichlorodihydrofluorescein (ROS fluorogenic probes DCF, Invitrogen, Waltham, MA) at 37°C in the presence of 5% CO_2 for 30 min. Fluorescence intensity was quantified with a Zeiss LSM 510 microscope (Carl Zeiss Inc., Thornwood, NY).

2.7 Drugs

(R)-(+)-WIN 55,212-2 mesylate salt and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) was purchased from Cayman Chemical (Ann Arbor, MI). The emulsifier Alkamuls EL620 (polyethoxylated castor oil) was purchased from Rhodia (Cranbury, NJ). WIN55212-2 and AM251 were dissolved in a mixture of dimethyl sulfoxide/Alkamuls/sterile artificial cerebrospinal fluid (1:1:18) [7].

2.8 Experimental protocol

Experimental design is shown in Fig. 1. Rats were subjected to OVX or SO surgery approximately 2 weeks before subjecting to receive E_2 supplementation or vehicle by silastic tubing implanting, as described above, for 11 days (Fig. 1). This regimen permitted adequate time for ovarian hormones washout in OVX rats and the restoration of physiological E_2 levels in the OVXE2 groups[37]. The SO, OVX or OVXE2 rats were divided into two groups (8 in each group): vehicle and sequential doses (100, 200 and 400 pmol) of the CB_1R agonist WIN55212-2. Each WIN55212-2- dose was injected in 80 nl into the right side of RVLM. A 30 min interval between doses was sufficient for BP and HR to return to baseline levels and in some instances the order of the WIN55212-2 doses was randomized. The same volume of the vehicle (sulfoxide/Alkamuls/sterile ACSF, 1:1:18) was injected into the right RVLM for control. Three other SO groups received intra-RVLM injections of the CB_1R antagonist AM251 (800 pmol), WIN55212-2 (400 pmol) or AM251 20 min before WIN55212-2. These doses and treatment regimen were based on our previous studies [7]. Baseline of BP and HR were recorded for at least 30 min after the rats adapted to the environment. The SO rats used in this experiment were used during the proestrus phase (the highest E_2 level) as determined by vaginal swab microscopy [38], The brains of rats were removed after rat euthanized by Euthasol (euthanasia solution, Virbac AH, Inc. Fort Worth, TX) at the conclusion of the cardiovascular measurements, flash-frozen, and stored at −80°C for ex vivo biochemical and molecular experiments.

2.9 Data analysis and statistics

Values are presented as mean ± SEM and analyzed by two-tailed t-test or one-way repeated measures ANOVA and one-way ANOVA followed by Tukey's Multiple Comparison Test using Prism version 5 (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered significant.

3. Results

3.1 Inverse relationship between plasma E_2 level and body weight

Approximately 3 weeks after OVX or SO surgery and 11 days after E_2 supplementation or its vehicle in OVX rats (Fig. 1), plasma 17β-estradiol levels were substantially higher (75.1 ± 18.2 pg/ml; $p < 0.05$) in proestrus SO rats than in OVX rats (18.7 ± 5.2 pg/ml); the lower E_2 levels in OVX rats were restored to SO levels by E_2 replacement in OVXE2 rats (79.6 ± 20.3 pg/ml) (Fig. 2A). Conversely, the body weight gain in SO (52.9 ± 1.3 g) and in OVXE2 rats (51.4 ± 0.8 g) was significantly ($p < 0.05$) lower than in OVX rats (59.8 ± 1.0 g) (Fig. 2B).

3.2 E_2 dampened the dose-dependent pressor response elicited by intra-RVLM CB_1R activation

There were no differences in baseline mean arteria pressure (MAP) between SO (96.6 ± 7.7 mmHg), OVX (101.1 ± 9.5 mmHg), and OVXE2 (94.3 ± 8.9 mmHg) rats. Intra-RVLM microinjection of WIN55212-2 (100, 200, 400 pmol) dose-dependently increased MAP in SO, OVX, and OVXE2 rats (Figs. 3A-C). The pressor responses elicited by all doses of WIN55212-2 were significantly ($p < 0.05$) higher in OVX than in SO rats, in the presence of ovarian hormones during proestrus (highest endogenous E_2 levels) and following E_2 replacement in OVXE2 rats (Fig. 3D). Further, the dose-dependent bradycardic responses, elicited by WIN55212-2, in SO rats disappeared ($p < 0.05$), and were transformed into dose-related tachycardic responses in OVX rats; E_2 replacement restored the WIN55212-2 dose-related bradycardic response ($p < 0.05$) in OVXE2 rats (Fig. 4).

3.3 CB_1R-mediated phosphorylation of RVLM Akt and nNOS is attenuated by E_2

The basal RVLM protein levels of CB_1R (Fig. 5A), total and corresponding phosphorylated levels of Akt (Fig. 5B) and nNOS (Fig. 5C) were similar ($p > 0.05$) in vehicle treated SO, OVX and OVXE2 rats. However, compared with vehicle, intra-RVLM WIN55212-2 (400 pmol) significantly ($p < 0.05$) enhanced Akt (Fig. 5B) and nNOS (Fig. 5C) phosphorylation in all SO, OVX and OVXE2 rats. Nonetheless, the level of Akt and nNOS phosphorylation was significantly ($p < 0.05$) lower in the presence of E_2 in SO and OVXE2, compared with OVX, rats (Figs. 5B & 5C).

3.4 E_2 mitigated CB_1R-mediated elevations in RVLM NO and ROS

There were no differences ($p > 0.05$) between basal NO and ROS levels in the RVLM of SO, OVX and OVXE2 rats (Fig. 6). While intra-RVLM WIN55212-2 increased the production of NO and ROS in SO, OVX and OVXE2 rats, the increases in both molecules were lower ($p < 0.05$) in E_2-replete (SO and OVXE2), than in OVX, rats (Fig. 6).

3.5 CB_1R blockade abrogated intra-RVLM WIN55212-2-evoked neurochemical and pressor responses

In SO rats, 400 pmol of WIN55,212-2 significantly ($p < 0.05$) elevated MAP and lowered HR (Figs. 7A & 7B). These CB_1R-mediated pressor and bradycardic responses were substantially ($p < 0.05$) attenuated by prior intra-RVLM microinjection of the selective

CB_1R antagonist AM251 (800 pmol). Further, AM251 abrogated ($p < 0.05$) the CB_1R (WINN55212-)-mediated increases in the phosphorylation of Akt (Fig. 7C) and nNOS (Fig. 7D) as well as the elevations in NO (Figs. 7E & 7F) and ROS (Figs. 7G & 7H) in the RVLM. AM251 alone had no effect on MAP or HR (Figs. 7A & 7B).

4. Discussion

The present findings suggest that E_2 dampens the RVLM CB_1R-mediated increase in BP and the predisposing neurochemical/sympathoexcitatory responses. In support of this premise, we showed that intra-RVLM CB_1R (WIN55212-2) activation produced: (1) exaggerated pressor response in E_2-deficient (OVX), compared with E_2-replete (proestrus SO and OVXE2) rats; (2) tachycardia in OVX, vs. bradycardia in SO and OVXE2, rats; (3) greater elevations in RVLM sympathoexcitatory mediators (nNOS and Akt phosphorylation and NO and ROS levels), in OVX, compared with SO and OVXE2, rats. The E_2-dependent dampening of the RVLM CB_1R-mediated neurochemical and pressor responses were reminiscent of pharmacological RVLM CB_1R blockade (AM251). These findings present the first evidence that downregulation of the RVLM neuronal oxidative stress and sympathoexcitation underlie the E_2-dependent dampening of the central CB_1R-mediated cardiovascular excitation in females.

The critical protective role for E_2 in sex differences in cardiovascular diseases has been recently reviewed [39]. Experimental findings including ours support this view because E_2-deficient (OVX) rats exhibit higher ROS levels in cardiovascular regulating nuclei along with higher sympathetic tone, and E_2 supplementation mitigates these anomalies [40]. On the other hand, E_2 plays a counterintuitive proinflammatory role and exacerbated organ injury when the cellular microenvironment undergoes oxidative stress under pathological conditions such as type-2 diabetes [41] or in the presence of ethanol [7, 32, 42]. Despite this knowledge, it is not known if E_2 will confer protection against or will exacerbate the CB_1R-mediated neuronal oxidative stress, sympathoexcitation and pressor responses in females. The present unchartered investigation is important given the increased use of cannabis by both sexes following its legalization by many states in the US.

In conscious male rats, intra-RVLM microinjection of the CB_1R agonist WIN55212-2 increases RVLM sympathetic neuronal activity and subsequently elevates BP via activation of the RVLM PI3K/Akt/nNOS pathway [7, 34]. Intriguingly, activation of the same signaling cascade in the RVLM contributes to the favorable central sympathoinhibitory and hypotensive effects of E_2 in female rats and young women [12, 43-45]. Therefore, we investigated the RVLM neurochemical and subsequent cardiovascular effects elicited by intra RVLM CB_1R activation in ovarian hormones deficient (OVX) rats and in proestrous SO female rats (highest endogenous E_2 levels). Here, we showed the first evidence that the RVLM CB_1R-medited dose-related elevations in BP and HR in OVX rats were dampened, and transformed into bradycardic responses, respectively (Figs. 3 & 4), in proestrous SO rats. We then studied the mechanisms of these novel favorable effects to confirm their dependence on E_2 and to discern the effect of endogenous or exogenous E_2 on the CB_1R-mediated activation of the RVLM PI3K/Akt/nNOS cascade in female rats.

Our experimental approach provided credence for the premise that E_2 plays a critical role in the ovarian hormones-dependent dampening of the adverse neurochemical and cardiovascular elicited by RVLM CB_1R activation for the following reasons. First, RVLM CB_1R activation (WIN55212-2) in ovarian hormones deficient (OVX) rats produced dose-related adverse cardiovascular (Figs. 3 & 4) and RVLM neuroinflammatory responses (Figs. 5 & 6). These responses in OVX rats resembled the male rat phenotype when a similar intra-RVLM WIN55212-2 dose regimen was adopted in our previous study [7]. It is notable that the CB_1R-mediated pressor response results from increased sympathetic nerve activity and plasma norepinephrine in male animals [3, 6-8]. Second, we confirmed the critical role of E_2 in the attenuated CB_1R-mediated cardiovascular responses because this phenotype occurred in proestrous SO rats (highest levels of endogenous E_2) and in OVX rats following E_2 supplementation (OVXE2) (Figs. 3 & 4). Notably, consistent with established clinical and experimental evidence [46-48], we showed a decline in the gain in body weight in E_2 replete (proestrus SO and OVXE2), compared with E_2 deficient (OVX) rats (Fig. 2). These findings insured the successful completion of ovariectomy and validate the physiological relevance of the E_2 replacement regimen in our study.

The novel dampening effect of E_2 on the RVLM CB_1R-mediated pressor response is consistent with E_2 ability to inhibit central sympathetic tone in female rats and young women [49]. However, the E_2-dependent transformation of the tachycardic, to bradycardic, response following RVLM CB_1R activation (Fig. 4) may not be fully explained by the central sympathoinhibitory effect of E_2. It is likely that E_2 sensitization of the baroreceptor reflex response, at least partly, via the activation of central projections that regulate cardiac parasympathetic neurons [50-52], also contributes to this effect. More studies are needed to understand the central effects of E_2 on heart rate regulation, particularly following CB_1R activation.

The molecular mechanisms that underlie the CB_1R-mediated neuronal oxidative stress, sympathoexcitation and subsequently the pressor response and tachycardia are complex. To better understand the unstudied E_2-dependent protective effects against RVLM CB_1R-mediated adverse cardiovascular responses, we investigated the effect of E_2 on the RVLM molecular events that ultimately lead to sympathoexcitation, the precursor for the pressor and tachycardic responses. Specifically, we focused on the role of RVLM nNOS because it contributes to the RVLM neuronal oxidative stress and sympathoexcitation caused by RVLM CB_1R [7]. Consistent with these reported findings in male rats, we showed that similar doses of the CB_1R agonist WIN55212-2 substantially increased the phosphorylation of nNOS and its upstream regulator Akt (Fig. 5) as well as oxidative stress (Fig. 6) in the RVLM of E_2-deficient (OVX) rats. Equally important, we demonstrate new data that these CB_1R-mediated sympathoexcitatory neurochemical responses [7, 24, 26, 27, 53] were blunted in the presence of endogenous or exogenous E_2 in proestrus and OVXE2 rats, respectively (Figs. 5 & 6).

Our findings suggest a pivotal role for the attenuation of RVLM nNOS phosphorylation in the E_2-dependent dampening of the CB_1R-mediated RVLM neurochemical, and the subsequent cardiovascular, responses in female rats. This premise is supported by the findings that in E_2-deficient (OVX) rats, the CB_1R-mediated activation of nNOS, which

elicits sympathoexcitation [24, 25, 54], was attenuated in E_2-replete (proestrus SO and OVXE2) rats (Fig. 5C). This latter effect occurred in the absence of any change in RVLM CB_1R expression (Fig. 5A) suggesting that E_2 attenuated nNOS phosphorylation, at least partly, by dampening the CB_1R-mediated phosphorylation of its upstream activator, Akt (Fig. 5B). The current finding is consistent with E_2 ability to suppress nNOS expression in another sympathoexcitatory brain area, the PVN, which projects to the RVLM [18]. It is also likely that enhanced RVLM iNOS signaling, which plays a role in E_2/ERβ-mediated hypotension [12, 17], contributes to the observed dampening of the CB_1R-mediated pressor response in the presence of E_2 in the present study.

Finally, it is noteworthy to discuss the important role of E_2-dependent dampening of the RVLM nNOS-induced oxidative stress in the favorable cardiovascular effects observed here for the following reasons. First, RVLM nNOS-induced oxidative stress contributes to the CB_1R-mediated sympathoexcitation in male rats [7]. Second, in the RVLM, oxidative stress enhances sympathetic activity and suppresses cardiac baroreflex activity [28, 55, 56] while ROS inhibition or iNOS activation reduces sympathetic activity, BP, and HR [57-59]. Further, enhancement (via direct RVLM CB_1R activation) [7, 34], or inhibition (via intracisternal brainstem CB_1R activation) [60], of RVLM PI3/Akt signaling mediates sympathoexcitation and pressor response. Consistent with these findings, we showed that intra-RVLM CB_1R-mediated ROS production (Fig. 6) and the associated pressor and tachycardic responses (Figs. 3 & 4) were attenuated in E_2-replete rats. Second, the mechanistic link between the RVLM neurochemical and cardiovascular responses in our model system was confirmed by the ability of the selective CB_1R antagonist AM250 to abrogate the intra-RVLM CB_1R-mediated responses (Fig. 7). It is noteworthy that the dampening effects of E_2 on the RVLM CB_1R-mediated molecular and cardiovascular responses were reminiscent of the effects of intra-RVLM CB_1R blockade. While these findings infer E_2-dependent interruption of CB_1R signaling in the RVLM, more studies are warranted to further understand this novel finding.

The present findings suggest that suppression of RVLM neuroexcitation underlies the protection against the CB_1R-mediated deleterious cardiovascular responses in E_2-repelete rats (Fig. 8). Analogous to CB_1R blockade, E_2 dampening of the Akt/nNOS phosphorylation, and ROS generation, mitigated the CB_1R-mediated pressor and tachycardic responses. Collectively, our finding suggest E_2-deficient postmenopausal and surgical menopause women are at greater risk of exhibiting the adverse cardiovascular effects of CB_1R agonist containing drugs.

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Abbreviations:

Akt protein kinase B

AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
BP	blood pressure
CB1R	cannabinoid receptor-1
DAF-FM	4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate
DCF	2',7'-Dichlorodihydrofluorescein
ERs	estrogen receptors
E_2	estrogen
HR	heart rate
MAP	mean arterial pressure
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
OVX	ovariectomized
OVXE2	OVX with E_2 supplementation
PI3K	phosphatidylinositol 3-kinase
ROS	reactive oxygen species
RVLM	rostral ventrolateral medulla
SO	sham operated

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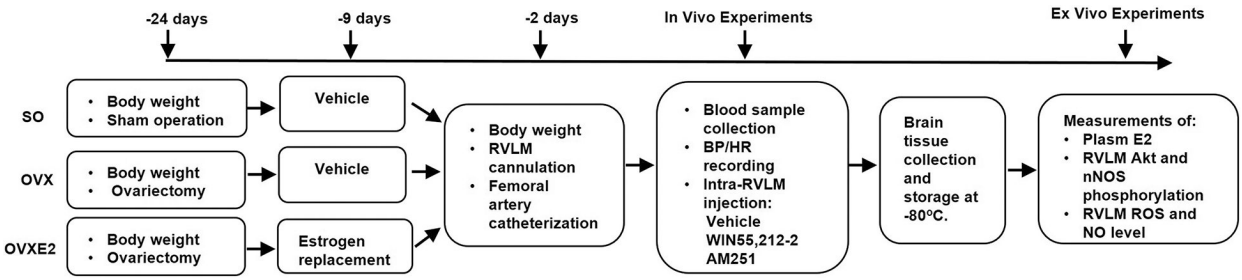

Fig. 1.

A schematic presentation of cardiovascular and biochemical/molecular studies to investigate the effects of intra-RVLM cannabinoid receptor 1 (CB_1R) activation in estrogen (E_2) replete and devoid female rats.

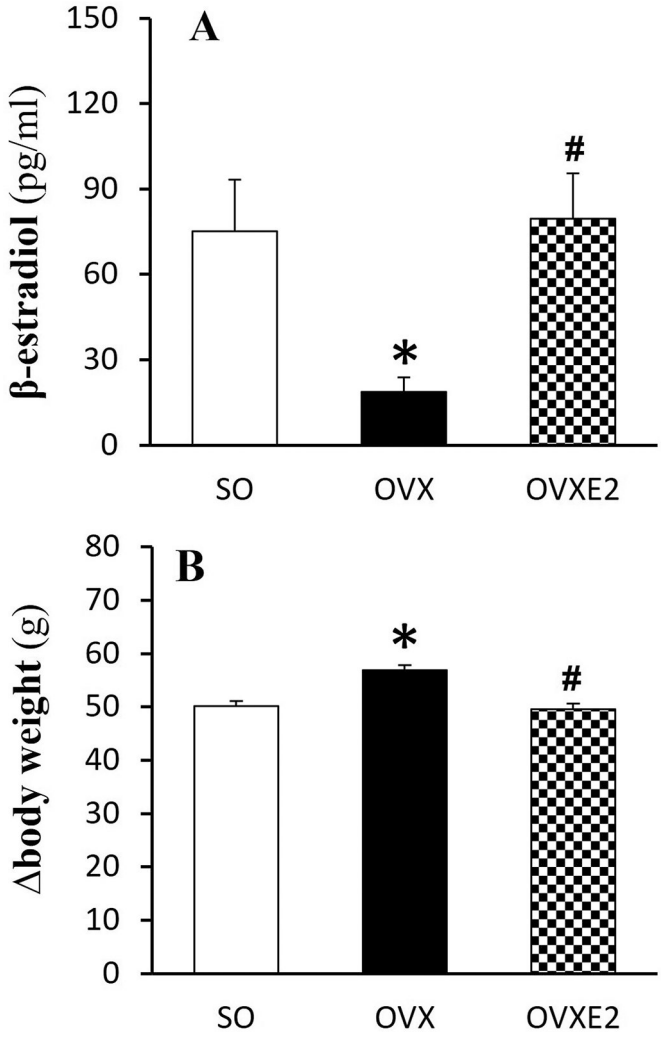

Fig. 2.

The plasma E_2 level (**A**) and the gain in body weight (**B**) in sham operated (SO), ovariectomized (OVX) and OVX with E_2 supplementation (OVXE2) rats before conducting the hemodynamic experiments. The plasma β-estradiol was measured in the proestrus phase of SO rat. Values are mean ± SEM. *$p < 0.05$ versus SO, #$p < 0.05$ versus OVX.

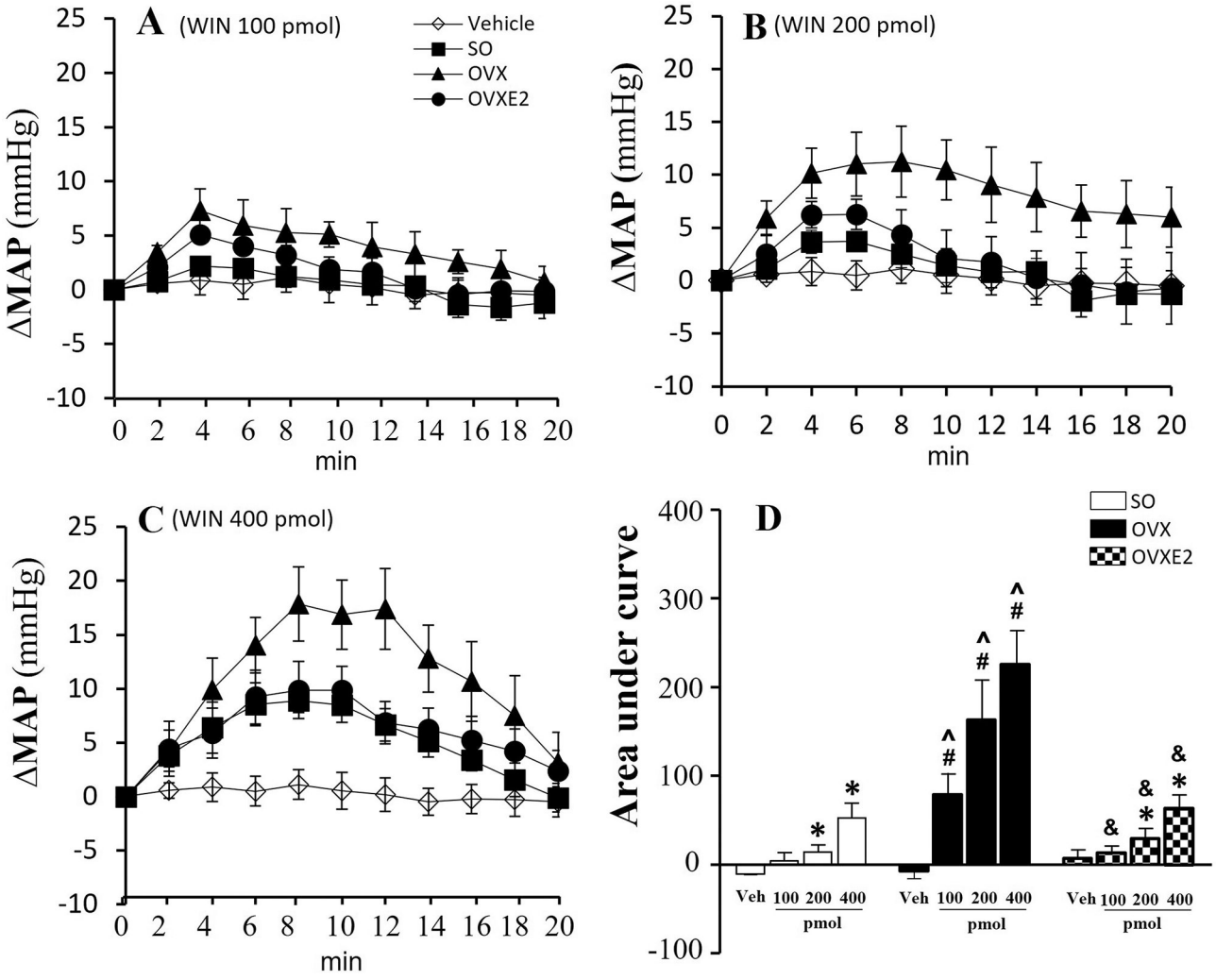

Fig. 3.

Time-course and dose dependent changes in mean arterial pressure (ΔMAP) following intra-RVLM microinjection of the following doses of the CB_1R agonist WIN 55212-2: 100 pmol (**A**), 200 pmol (**B**) and 400 pmol (**C**), or vehicle in SO, OVX and OVXE2 rats. The bar graph (**D**) summarizes the area under the curve of ΔMAP. Values are mean ± SEM. *$p < 0.05$ versus SO vehicle, #$p < 0.05$ versus OVX vehicle, $$p < 0.05$ versus OVXE2 vehicle, ^$p < 0.05$ versus SO treated with same dose of WIN 55212-2, &$p < 0.05$ versus OVX treated with same dose of WIN 55212-2.

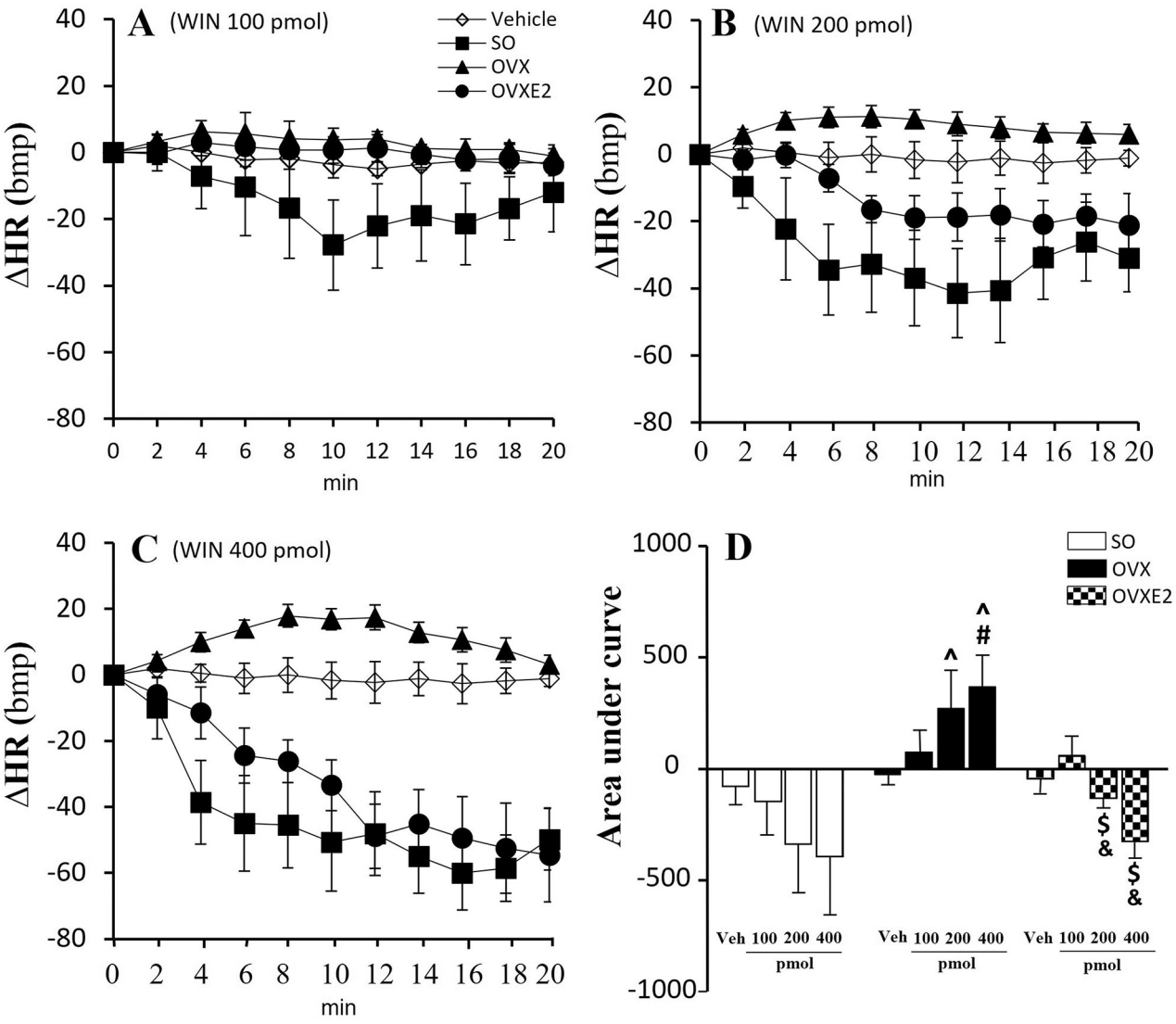

Fig. 4.

Time-course and dose dependent changes in heart rate (ΔHR) following intra-RVLM microinjection of the following doses of the CB_1R agonist, WIN 55212-2: 100 pmol (**A**), 200 pmol (**B**) and 400 pmol (**C**), or vehicle in SO, OVX and OVXE2 rats. The bar graph (**D**) summarized the area under the curve of ΔHR. Values are mean ± SEM. #$p < 0.05$ versus OVX vehicle, \$$p < 0.05$ versus OVXE2 vehicle, ^$p < 0.05$ versus SO treated with same dose of WIN 55212-2, &$p < 0.05$ versus OVX treated with same dose of WIN 55212-2.

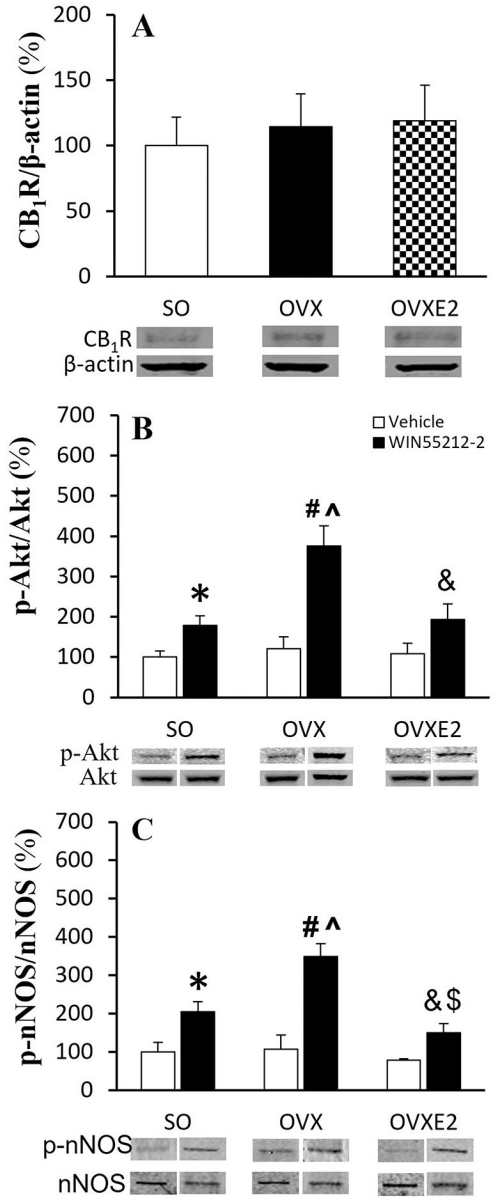

Fig. 5.

CB_1R expression in OVX and SO, and OVXE2 (**A**) and the effect of the CB_1R agonist, (WIN 55212-2) on the phosphorylation of Akt (**B**) and nNOS (**C**) in RVLM of OVX, SO and OVXE2 rats. Representative bands are shown under the bar graphs. Data are expressed as mean ± SEM following normalization to β-actin, total AKT or total nNOS, and comparison with SO vehicle values (100%). *$p < 0.05$ versus SO vehicle, #$p < 0.05$ versus OVX vehicle, $$p < 0.05$ versus OVXE2 vehicle, ^$p < 0.05$ versus SO WIN 55212-2, &$p < 0.05$ versus OVX WIN 55212-2.

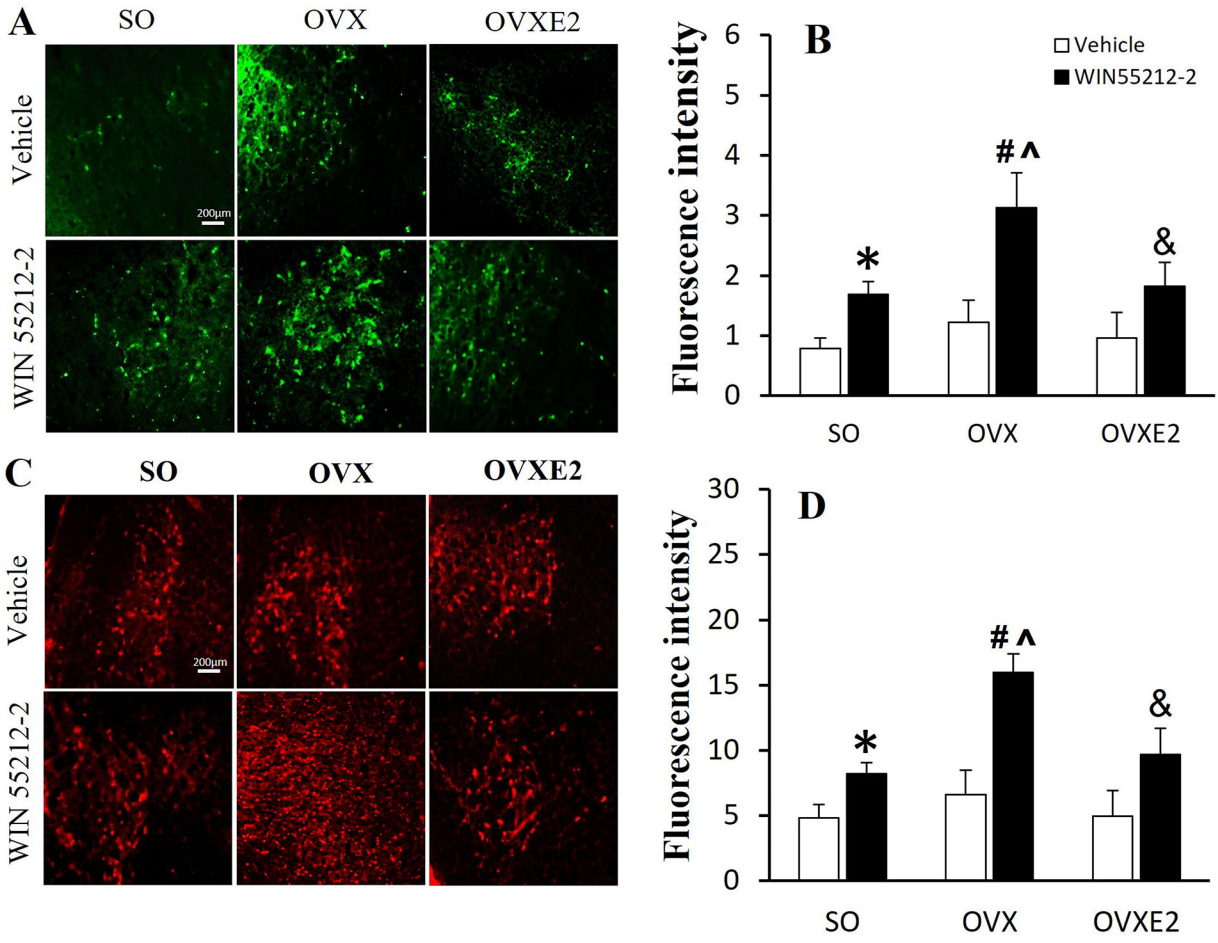

Fig. 6.

The effect of the CB_1R agonist WIN 55212-2 on NO and ROS production in the RVLM of in OVX and SO, and OVXE2 rats. Representative images are shown for NO (fluorogenic probes DAF-AM, **A**) and ROS (fluorogenic probes DCF, **C**). The bar graphs summarize the fluorescence intensity of NO (**B**) and ROS (**D**). *$p < 0.05$ versus SO vehicle, #$p < 0.05$ versus OVX vehicle, ^$p < 0.05$ versus SO WIN 55212-2, &$p < 0.05$ versus OVX WIN 55212-2.

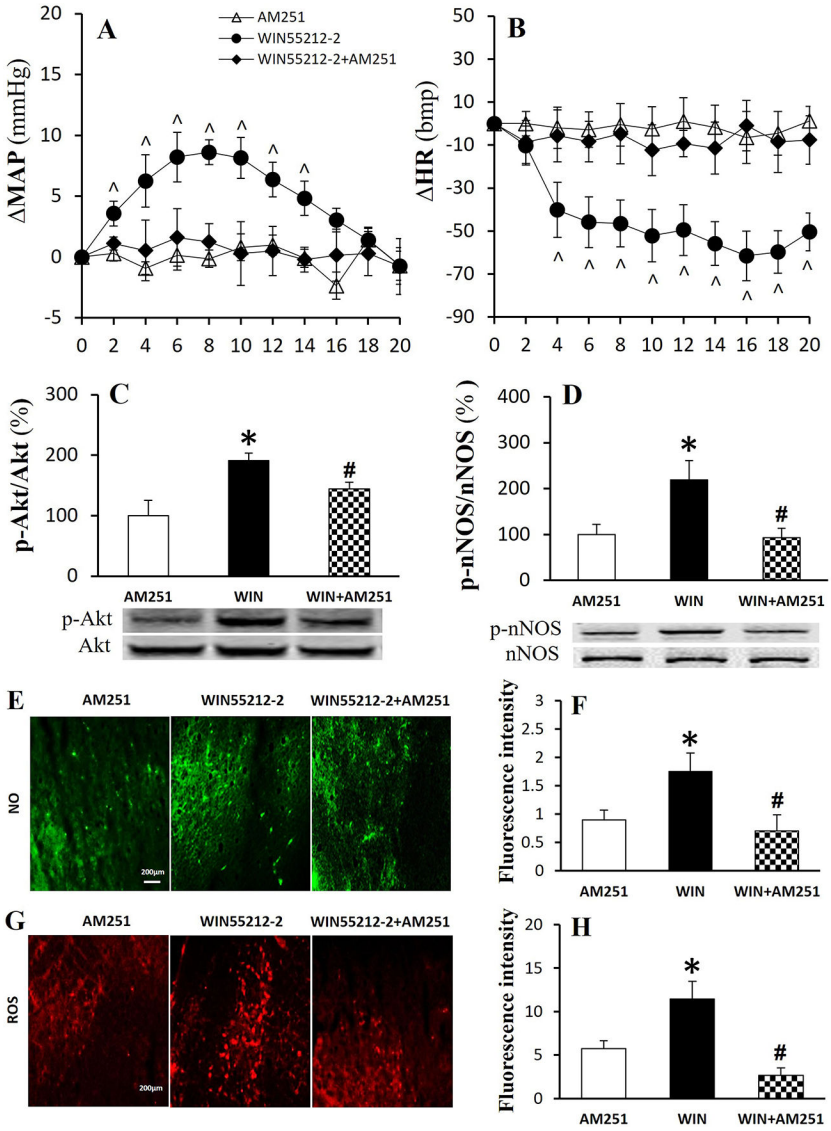

Fig. 7.

The effect of prior CB_1R blockade with AM251 (800 pmol) on intra-RVLM CB_1R (WIN 55212-2; 400 pmol)-evoked mean arterial pressure (ΔMAP, **A**) and heart rate (ΔHR, **B**) along with the following associated molecular responses: (1) phosphorylation of Akt (**C**) and nNOS (**D**) and (2) levels of NO (**E**) and ROS (**F**) in the RVLM of SO rats. Representative bands of p-Akt/AKT and p-nNOS/nNOS are shown under bar graph (**C** and **D**). Data were normalized to corresponding total AKT or total nNOS and compared with AM251 values (100%). Representative fluorescence staining images are shown for NO (DAF-AM, **E**) and ROS (DCF, **G**). The bar graphs summarize the fluorescence intensity of NO (**F**) and ROS (**H**). Data are mean ± SEM. ^$p < 0.05$, versus baseline. *$p < 0.05$ versus AM251, #$p < 0.05$ versus WIN 55212-2.

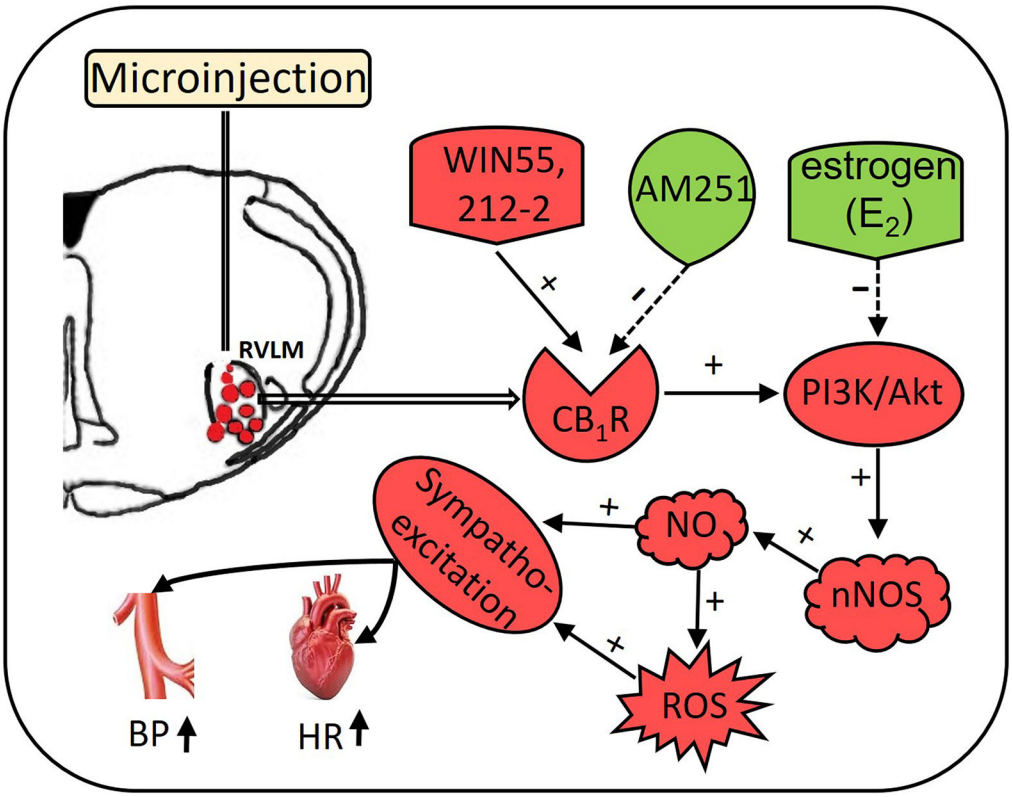

Fig. 8.

Schematic summary of current data. Activation of RVLM CB_1R (WIN55212-2) induces neuronal sympathoexcitatory responses, elevations in sympathoexcitatory mediators (nNOS and Akt phosphorylation, NO and ROS levels), leads to pressor response (BP and HR), which was dampened by E_2 and reminiscent of pharmacological CB1R blockade (AM251).