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Paraventricular Nucleus V_{1a} Receptor Knockdown Blunts Neurocardiovascular Responses to Acute Stress in Male Rats after Chronic Mild Unpredictable Stress

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Abstract

Chronic stress and depression impart increased risk for adverse cardiovascular events. Autonomic dysregulation, particularly sympathoexcitation, has long been associated with poor cardiovascular outcomes. Vasopressin (AVP) receptors with the paraventricular nucleus (PVN), known as an integrating locus for hemodynamic and autonomic function, have been implicated in behavior and stress. The present studies were designed to test the hypothesis that knockdown of vasopressin V_{1a}R within the PVN in male Sprague Dawley rats subjected to chronic mild unpredictable stress (CMS) would result in lower resting hemodynamics and renal sympathetic nerve activity (RSNA) and mitigate the responses to acute stressors. Male rats underwent CMS for 4 weeks; controls were housed in standard caging. Twenty days into the paradigm, the PVN was injected with either small interfering RNA (siRNA) directed against V_{1a}R or scrambled RNA (scrRNA). Arterial pressure, heart rate and RSNA were ascertained by telemetry with the animals in their home cages. Pretreatment with siRNA to V_{1a}R prevented the increase in arterial pressure to PVN microinjection with exogenous AVP. Basal mean arterial pressure (MAP) was significantly higher in scrRNA-treated but not in siRNA-treated CMS rats vs control rats. Paradoxically, basal RSNA was approximately two-fold higher in siRNA-treated CMS rats. Acute emotional stress delivered as 15-sec air-jet resulted in greater peak and duration of the MAP and RSNA responses in scrRNA-treated CMS rats vs control; siRNA treatment inhibited the responses. The 15-sec exposure to ammonia to test the nasopharyngeal reflex, whose circuitry does not include the PVN, produced similar increases in arterial pressure, heart rate, and RSNA in controls and both groups

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of CMS rats. Thus, CMS increases arterial pressure and predisposes to greater hemodynamic and RSNA responses to acute emotional stress. The higher basal RSNA in siRNA-treated rats may be due to functional and/or anatomical neuroplasticity occurring during more protracted inhibition of V_{1a}R PVN signaling. Vasopressinergic signaling via V_{1a}R in PVN modulates the cardiovascular and sympathetic responses to both the chronic and acute stress.

Keywords

Vasopressin; Blood pressure; Renal sympathetic nerve activity; Air-jet stress

1. Introduction

Depression, particularly major depressive disorder, is associated with increased risk of cardiovascular disease, stroke, and all cause mortality even when adjusted for traditional risk factors such as hypertension, smoking, and dyslipidemias [1–4]. The relationship between cardiovascular disease and depression is evident worldwide but is more prominent in urban areas regardless of income level. Although more women suffer depression, cardiovascular risk is greater in men with depressive symptoms [2, 5]. In a large, case-controlled, international study involving 52 countries across diverse ethnic groups (INTERHEART), the psychosocial index which includes depression and acute life stressors displays a population attributable risk of 28.8% for myocardial infarction. After adjustment for age, sex, obesity, diabetes, hypertension, activity, alcohol intake, and ApoB/Apol1, the population attributable risk remains elevated at 32.5% with an odds ratio of 2.67 (99% CI: 2.21–3.22) [6]. Moreover, a major cardiovascular event is more likely to result in death in depressed patients compared with non-depressed individuals [1, 3, 4, 7, 8].

Dysregulation of the autonomic nervous system has long been associated with adverse cardiovascular outcomes [9, 10]. The paraventricular nucleus (PVN) is a critical central regulator of autonomic and humoral responses to a variety of acute [11–13] and chronic [14–17] physical and psychosocial stressors. Vasopressin (AVP) is a neurohormone synthesized by PVN magnocellular neurons. Brain AVP and its receptors, V_{1a}R and V_{1b}R, have been implicated in both autonomic control and behavior, whereas V_2R are primarily located in peripheral organs [18, 19]. Importantly, studies in both humans and rodents support a role for central AVP in depression and anhedonic models, respectively [20–24]. Rats lacking AVP due to a point mutation in the gene encoding for the hormone display attenuated depressive and stress-associated behaviors [20, 25, 26]. Furthermore, mRNA expression of both V_{1a}R and V_{1b}R is increased within PVN of rats after four weeks of chronic mild unpredictable stress [27]. In humans, autopsy tissue from PVN of depressed individuals exhibits augmented AVP and abundance of PVN V_{1a}R compared with PVN tissue from non-depressed [23, 28].

Ludwig and Leng [29] have shown that vasopressinergic neurons release AVP within the PVN from soma and dendrites. In turn, this somatodendritic release of AVP can permit communication between the magnocellular and parvocellular regions of the PVN [30], thereby potentially linking the regulation of psychosocial responses with that of the

autonomic nervous system. A considerable body of evidence supports the role of centrally-acting AVP in modulating the release of adrenocorticotropic hormone (ACTH) which, in turn, would modulate the hypothalamo-pituitary-axis (HPA) response to stress [31, 32]. In contrast, direct evidence for involvement of PVN AVP in the autonomic response during chronic or acute stress is more limited, despite the finding that circulating catecholamines are elevated in depressed individuals [7]. Brainstem and spinal cord loci crucial for regulating cardiovascular function via the sympathetic and parasympathetic nervous systems receive direct inputs from the PVN [33]. Notably, greater than one-third of the parvocellular PVN neurons projecting to the spinal cord sympathetic preganglionic motoneurons express AVP mRNA [34]. Microinjection of AVP into PVN stimulates sympathetic efferent activity leading to increased blood pressure and heart rate [27, 35]. Recent studies utilizing combined antagonism of $V_{1a}R$ and $V_{1b}R$ support their role in sympathoexcitation in chronic stress, but lack sufficient selectivity [27]. What is even less clear is whether AVP within the PVN is involved in the acute stress response and, further, if that response is augmented when superimposed on chronic stress.

1.1. Hypothesis for Current Study

In light of the above, we hypothesize that silencing vasopressinergic signaling by $V_{1a}R$ in PVN in male Sprague Dawley rats subjected to chronic mild unpredictable mild stress (CMS) will result in lower resting blood pressure, heart rate, and renal sympathetic nerve activity (RSNA). In addition, the response to psychological stress via acute air-jet will be blunted by $V_{1a}R$ knockdown, but not the response mediated by chemoreceptor activation. The present studies are designed to assess arterial pressure, heart rate, and RSNA by telemetry in conscious, unrestrained male CMS and control rats whose PVN has been pre-treated with small interfering RNA (siRNA) directed toward $V_{1a}R$ or scrambled RNA (scrRNA). The acute responses to air-jet or ammonia will also be ascertained.

2. Methods

Experiments were done in male Sprague Dawley rats (225–250 g) procured from Envigo (Indianapolis, IN). Upon arrival, the rats were permitted to acclimate with free access to water and standard rat chow (Envigo) for 4–5 days while housed under controlled light:dark cycle (lights on 06:00–18:00) and temperature (21–23°C). All husbandry, procedures, and protocols were approved by Wayne State University's Institutional Animal Care and Use Committee (#19–08-1225) in compliance with ARRIVE guidelines and the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

2.1. Chronic Mild Unpredictable Stress (CMS)

After acclimation, the rats were assigned on a random basis to remain in standard housing as above (control) or to undergo chronic mild unpredictable stress (CMS) for four weeks. The CMS protocol involved exposure to differing stressors applied in random order over a 7-day period then repeated over the 4-week period (Fig. 1A) as described by Grippo et al [36] and applied with minor modification by our laboratory [27]. The stressors included cage tilt at 45° for 6 hr, overnight water deprivation followed by replacement of an empty water bottle for 1 hr followed by paired housing for 8 hr with water, resumption of single housing with

continuous lighting for 24 hr, then separate housing with damp bedding for 24 hr, followed by paired housing with a 3-hr period of white noise and a 6-hr period with a strobe light (overlapping), and finally a 24-hr period of single housing. The 7-day routine was repeated over a 4-week period. Control rats were housed singly with free access to rodent chow and water.

As indicated in Fig. 1B, on day 20 of the Protocol, the PVN of rats were injected bilaterally with either scrambled RNA or siRNA directed toward the $V_{1a}R$ (siRNA) and returned to the protocol. At the completion of the four weeks (day 28), rats were equipped with telemetry transmitters for hemodynamic as well as nerve recording. The rats then were singly housed under standard conditions. Hemodynamics and renal sympathetic nerve activity (RSNA) were recorded starting at 24 hr after full recovery from anesthesia in their home cages. Acute stress testing was performed on days 32 to 34.

2.2. Surgical Procedures

2.2.1. PVN injections—On day 20 of the protocol, rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) ip and positioned in a stereotaxic apparatus (Kopf, Tujunga, CA). An injection cannula was directed to the magnocellular region of the PVN at the following coordinates: −1.8 caudal to bregma, 0.6 lateral to the midline, and −8.0 ventral to the surface of the skull. A mixture of four pooled ON-Target plus siRNAs directed against either $V_{1a}R$ (siRNA) or scrambled RNA (scrRNA) totaling 1.25 pmole in 150 nl DEPC-treated water was injected over 2 min. The cannula was then withdrawn. A fresh injection cannula inserted on the contralateral side and the same mixture injected. After recovery (~6 hr), the rats were returned to their cages and resumed control or CMS conditions. Separate animals (n = 3) were injected with 150 nl 1,1-di-ictadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate to assess the location and extent of spread.

2.2.2. Hemodynamic radiotelemetry transmitter placement—The rats were anesthetized with sodium pentobarbital 50 mg/kg ip and given a dose of buprenorphine SR (0.3 mg/kg ip) for preemptive analgesia. The hemodynamic transmitter (TA11PA-C40; Data Sci. Intl., St. Paul, MN) was placed using sterile technique as previously described [37]. An incision was made in the right groin and the femoral artery was exposed. The proximal section was occluded and the gel-filled catheter attached to the transmitter was introduced and then advanced into the distal aorta. The catheter was stabilized with medical adhesive, and the body of the transmitter was then inserted subcutaneously and sutured to the muscle. The skin was apposed and closed with surgical staples.

2.2.3. Nerve radiotelemetry transmitter placement—After placement of the hemodynamic transmitter, the rat was repositioned for insertion of the nerve transmitter unit (F50-W-F2; Data Sci. Intl., St. Paul, MN). Briefly, the approach follows that previously reported by our laboratory [37]. Using a retroperitoneal approach, a flank incision was made and the left renal nerve was identified and isolated under a stereomicroscope. The intact nerve was placed on the wire electrode attached to the transmitter. The ground wire was sutured to the iliopsoas muscle. The proximal ends of the electrodes were stabilized by anchoring with 6–0 sutures to the adventitia of the aortic wall. The quality of the

nerve signal was established by evaluating the raw nerve tracing on the oscilloscope (Hameg, Elgin, IL) and assessing the nerve sounds by Grass AM 8 audio monitor (Grass Technologies, Warwick, RI). The nerve and electrodes were then embedded in silicone elastomer (Kwik-Cast, World Precision Instruments, Sarasota, FL). The body of the transmitter was then inserted subcutaneously and sutured to the underlying muscle on the left flank. The skin was sutured with vicryl 2–0 suture (Ethicon, Johnson & Johnson, New Brunswick, NJ). All rats were permitted to recover for a minimum of 3 days, singly housed in standard caging prior to testing.

2.3. Design and Verification of $V_{1a}R$ siRNA Knockdown

We developed four siRNAs targeted to sequences in the $V_{1a}R$ gene. A mixture of four ON-Target plus siRNAs were obtained and designed to target sequences at positions 767–785, 979–997, 1214–1232 and 1379–1397 of the rat $V_{1a}R$ (accession #NM_053019.2; Dharmacon Inc., Lafayette, CO). The non-targeting (scrambled) siRNA pool consisted of the four sequences (UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA). The siRNAs were dissolved in double-distilled, DEPC-treated water to provide the concentration of 1.25 pmole/150 nl.

Separate male Sprague Dawley rats not on main protocol underwent PVN injections bilaterally 14, 21, and 28 days after injection (n = 3 for each period). Rats were lightly anesthetized with 2% isoflurane and decapitated by guillotine. Brains were rapidly harvested, flash frozen, and stored at −70°C for no more than 4 weeks. To assess the level of knockdown we excised the PVNs bilaterally while keeping the tissue frozen over dry ice. Total RNA was isolated, purified, reverse transcribed, and analyzed by real time RT-PCR amplification for efficiency of knockdown. Total RNA was isolated, purified, reverse transcribed, and analyzed by real time RT-PCR amplification for efficiency of knockdown (see below). We have published our strategy of successful direct injection of constructs without a viral vector [38, 39]. Although higher concentrations of the injected constructs are required with naked siRNA, this approach avoids spread to adjacent areas and immune mediated effects [40]. In accordance with these results, we elected to inject the rats with siRNA or scrRNA on day 20 of the protocol to permit evaluation of baseline hemodynamics and RSNA in CMS and controls as well as further testing on days 32–34.

To further verify that the siRNA directed at $V_{1a}R$ effectively blocked the action of AVP at the PVN, rats were anesthetized and surgically prepared as above with hemodynamic transmitters only. Two rats (one scrRNA and one siRNA) were also equipped with a nerve transmitter. In addition, they had guide cannulas placed directed to the PVN using the stereotaxic coordinates above except the depth was −7.0 dorsoventral to permit the injection cannula, when introduced, to protrude 1.0 mm. A dummy cannula was inserted after surgery. Two days after surgery, the rats were injected with either siRNA or scrRNA injections (n = 3 and 3). Two weeks later, 100 ng AVP was injected unilaterally into the PVN and the change in MAP recorded using LabChart Pro 8 (ADInstruments, Colorado Springs, CO). The sites of injection, boundaries of the block of PVN tissue taken for analysis, and extent of spread for these studies and the protocols below are depicted in Supplemental Figure 1S.

2.4. Testing Protocols

2.4.1. Basal testing after CMS—On days 1 and 2 after placement of the transmitter surgeries, each rat was brought into the laboratory for 60–90 min while remaining in its home cage. Three days after placement of the telemetry transmitters, hemodynamic parameters were recorded for 24 hr (06:00 to 06:00) and RSNA for 4 hr (08:00 to 12:00) with the rats in their home cages. The average measurements were taken as baseline values for the CMS and control conditions.

2.4.2. Acute stress testing—At the time of acute stressor testing, each rat remained housed in its home cage and was permitted to acclimate for 30 min. All acute testing was performed between 08:00 and 12:00 on either day 32, 33, or 34. Acute stressors were applied in random order. Baseline values for MAP and RSNA were then recorded continuously over the next 30 min prior to exposure to the acute stressor. Monitoring and recording continued during the acute stressor and for a minimum of 30 min thereafter. *Air-jet stress*. At the end of the 30-min baseline period, each rat was subjected to a 15-sec stream of compressed air (1.5 atm) directed to the top of the rat's head so that the rat did not see the tubing. *Nasopharyngeal reflex.* Baseline parameters were recorded as above, a cotton-tipped applicator was soaked in 100% ammonia and held close to the external nares for 15 sec and MAP and RSNA recorded for a minimum of 30 min. On the day after the testing protocols were completed, the rats were euthanized with sodium pentobarbital, 210 mg/kg i.p., background RSNA recordings were obtained after death was assured, and the brains were harvested to assess the level of V_{1a}R knockdown.

2.5. RNA purification and RT-PCR amplification of V_{1a}R

Total RNA was isolated and purified from homogenized tissue punch biopsies of PVN by using RNeasy Plus mini kit according to the manufacturer's instructions (QIAGEN, Ann Arbor, MI). Each of the purified RNAs was reverse transcribed to obtain first strand cDNA following previously published methods. Briefly, a 30 μl reverse transcription (RT) reaction consisted of 2μl of the RNA template, 3μl of 10xRT buffer (500mM Tris-HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, 100 mM DTT), 1.5 μl of 0.1 M DTT, 1.5 μg of Random Hexamers, 3 μl of dNTPs mix (containing 1.25 mM of each of dCTP, dGTP, dCTP, and TTP), 2 μl of RNAs in RNAse inhibitor, 40 units/μl; (Promega, Madison, WI), and 3 μl of Molony Murine Leukemia Virus Reverse Transcriptase (M-MuLV-RT; 200 units/μl; New England Biolabs, Ipswich, MA). Each reaction mixture minus dNTPs, RNAsin, and MMLV-RT was first incubated at 70°C for 5 min. To each reaction was then added the required amounts of dNTPs, RNasin, and MMLV-RT, and incubated at 37°C for 60 min. At completion, the reactions were stored at −80°C until needed.

PCR amplification for rat V_{1a}R and GAPDH cDNAs was carried out as follows. Briefly, a 25 μl PCR reaction consisted of 3μl and 1 μl of the above RT reaction as template for V_{1a}R and GAPDH amplification, respectively. Additional components of the PCR reaction included 2.5 ul of 10x PCR buffer [100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01%(w/v) gelatin], 2 μl of dNTPs mix (containing 1.25 mM of each of dCTP, dGTP, dCTP, and TTP), 200 ng each of respective forward and reverse primers, and 0.125 μl of Amplitaq polymerase (5 units/μl; Applied Biosystems, Bedford,

MA). Forty cycles of amplification were carried out with denaturation step of 30 sec at 95°C, annealing step of 30 sec at 62°C, and extension step of 30 sec at 72°C. In the case of $V_{1a}R$ amplification, at the conclusion of 40 cycles, each of the PCR reactions was diluted 100-fold, and 1 μl of this diluted reaction was then utilized as template for a second, "nested" PCR amplification essentially as above except that different forward and reverse primers (termed nested primers) were used and PCR amplification was carried out for 20 cycles. The PCR amplified DNAs for GAPDH and the "nested" reactions for $V_{1a}R$ were then analyzed on 2.5% agarose gel. The sequences of the PCR primers for GAPDH were 5' GGTCATCATCTCCGCCCCTTC 3' (upstream primer) and 5' CCACCACCCTGTTGCTGTAG 3' (downstream primer) and have been described [41]. For $V_{1a}R$ amplification the primers consisted of 5' ACTGCTGGGCTACCTTCATCC 3' (upstream primer) and 5' CCTTGGCGAATTTCTGCGCC 3' (downstream primer). For the "nested" PCR, the primers consisted of 5′ TCATCTGCTACCA-CATCTGGCG 3′ (forward primer F2) and 5' TGTGATG-GAAGGGTTTTCTGAAT 3' (reverse primer R1) as previously reported [41, 42].

2.6. Analyses and Statistics

Expression of $V_{1a}R$ after scrRNA and siRNA was assessed on the same gel and quantitated using ImageJ software. Only rats with knockdown of $V_{1a}R > 90\%$ in the siRNA group were used in the final analyses. Evaluation of hemodynamics and RSNA was performed by an investigator blinded to both the group (control vs CMS) and treatment (scrRNA vs siRNA). Blood pressure and heart rate during the 24-hr baseline period were sampled at 500 Hz for 30 sec every 60 min at a sampling rate of 500 samples per sec. These values were then averaged over the 24-hr period. Baseline RSNA measurements were obtained at 1kHz by averaging 1-sec intervals sequentially over a 4-hr period between 08:00 and 12:00 using Ponemah software (Data Sciences Intl., New Brighton, MN). The RSNA signal was filtered at 50 Hz, amplified, rectified, and integrated. At the time of acute stress testing, hemodynamics and RSNA were recorded continuously during a 30 min baseline period, during the stimulus, and for a minimum of 30 min afterward [37]. The reported integrated RSNA values for each rat were corrected for its unique background noise. Renal nerves with a noise threshold > 0.5 μV.s were excluded *a priori.* For the AVP stimulation studies, baseline parameters were obtained during 30 min and then the peak response at 5 min was assessed for each animal [35].

The study was powered to assess a difference of 6 mmHg in MAP and 1.2 μV.s in integrated RSNA with a standard deviation of 3 mmHg and 0.6 μV.s, respectively, with 95% power at an α level of 0.05. All data are presented as mean ± SE. This required 8 rats per group. Since there were no differences in control rats treated with either scrRNA or siRNA, they were assessed as one group in accordance with the principles of humane animal experimentation to reduce, refine, and replace when possible. It became evident that the duration of the responses to acute stimuli was prolonged in CMS rats, so that the AUC from the time of stimulation (t_0) until the time of return to baseline (t_n) was ascertained for each acute stimulus using the trapezoidal rule given by the following equation where n is the number of equal intervals and y is the difference between the value of MAP or RSNA during response to the baseline at a given point. The intervals, n, were taken every 1.0 sec.

$$AUC = ((t_n - t_0)/2n) \times \left(y_0 + 2y_1 + 2y_2 + \ldots 2y_{(n-1-)} + y_n\right)$$

Comparisons across baseline values, peak values, and AUC for each group was accomplished by one-way ANOVA followed by Tukey-Kramer *post hoc* analysis. Analysis of peak responses vs baseline for the same animal was performed using the paired t-test. A value of $P < 0.05$ was considered significant.

3. Results

Control unstressed and CMS rats had similar weights on entry into the protocol but scrCMS-treated rats weighed 24 ± 4 g and siRNA-treated rats weighed 19 ± 3 g less than control rats by the end of the 4 weeks and prior to acute stress testing.

3.1. Verification of $V_{1a}R$ knockdown

Maximal knockdown of gene $V_{1a}R$ gene expression from PVN tissues occurred at 14 days: 95 ± 1% (Fig. 2), but remained substantively suppressed at 21 and 28 days: 93+1% and 95+4%, respectively (n = 3 each). Tissue remote from PVN (e.g., lateral hypothalamus) did not display knockdown of $V_{1a}R$ (data not shown). Recent studies published from our laboratory showed that injection of vehicle did not change MAP, heart rate, or integrated RSNA (27). These studies were not repeated in accordance with ARRIVE guidelines to reduce the number of animals used.

The response of hemodynamic parameters to exogenous AVP injected into the PVN was used in pilot studies to verify that the siRNA constructs blocked signaling via $V_{1a}R$ comparable to that observed with a $V_{1a}R$ antagonist. The increase in MAP induced by exogenous AVP applied to the PVN in scrRNA- or siRNA-treated control rats at 14 days was significantly suppressed. Pre-treatment with scrRNA did not change the response to AVP (Table 1). Fig. 3 shows that not only was the increase in blood pressure inhibited in the siRNA-treated rat, but the AVP-induced increase in RSNA was also diminished when compared with the rat treated with scrRNA. Given the above findings, the studies in control and CMS rats were performed 12–14 days after PVN treatment with scrRNA or siRNA. $V_{1a}R$ gene expression did not meet the criterion of $>90\%$ knockdown in one siRNA-treated CMS rat which was eliminated from the final analyses.

3.2. Basal hemodynamics and RSNA

MAP averaged over the 24-hr period from day 12 to 13 after PVN injection was significantly higher in the CMS rats treated with scrRNA compared with control rats, whereas MAP in the CMS group treated with siRNA directed against $V_{1a}R$ was no different than that of the control rats. Baseline RSNA did not differ between control and scrRNA-treated CMS rats at the end of the CMS.Baseline RSNA of conscious siRNA-treated CMS rats in their individual home cages averaged over the 24-hr period was significantly higher than integrated RSNA of either control rats or scrRNA-treated CMS rats. Heart rate did not differ among the groups (Table 2). The higher baseline RSNA in siRNA-treated rats was confirmed in the baseline tracings obtained prior to acute stress as well (Table 3). The lower MAP and higher

integrated RSNA of the siRNA-treated CMS rat can also be appreciated in the raw and integrated nerve recordings shown in Fig. 3.

3.3. Hemodynamic and RSNA responses to air-jet stress

MAP, heart rate, and integrated RSNA values in the 30 min prior to acute stressors displayed the same pattern as those observed during the 24-hr averaged values. Peak responses to either air-jet stress or exposure to ammonia were significantly different from the respective pre-test values for all three parameters. Specifically, prior to exposure to air-jet, MAP was significantly higher in the scrRNA-treated CMS group and RSNA was higher in the siRNA-treated CMS group versus the control group. Heart rates did not differ among the groups (Table 3). Fig. 4A shows that the response of MAP to air-jet stress was greater in scrRNA treated CMS rats compared with control rats. The increase in MAP by CMS rats treated with siRNA to $V_{1a}R$ was significantly lower than that of the scrRNA CMS rats and no different than that of control rats. The increase in heart rate with air-jet stress was similar across the groups: control, 54 ± 5 bpm; scrRNA CMS, 43 ± 1 bpm, and siRNA CMS, 43 ± 21 bpm ($P > 0.05$).

The absolute value of the peak RSNA response to air-jet stress was significantly greater in the siRNA-treated CMS rats vs control rats. Although the peak RSNA response to air-jet stress was also higher in scrRNA CMS rats, the absolute value was not significantly different than that of the control rats (Table 3). When the differences in pre-stress (baseline) RSNA expressed in absolute voltage were taken into account and the change in RSNA from baseline to peak values was assessed, both scrRNA-treated and siRNA-treated CMS rats displayed significantly greater increases in RSNA than control rats (Fig. 4C); however, when expressed as percent of baseline RSNA, the scrRNA- treated CMS rats displayed a significantly higher peak response 388 ± 10%baseline ($P < 0.001$) than either control or scrRNA CMS rats: 315 ± 9 and 295 ± 11%baseline, respectively. Notably, the duration of the response by the scrRNA CMS group was more than twice as long as that of either the control or siRNA-treated CMS group. When the combination of the magnitude and duration of the response to air-jet was taken into account by assessment of the AUC, scrRNA CMS rats displayed significantly greater MAP and integrated RSNA responses. In contrast, pre-treatment with siRNA against $V_{1a}R$ within the PVN resulted AUCs for both MAP and RSNA that were remarkably similar to the values from the control group (Fig. 4B and D). Typical recordings of air-jet responses of control, scrRNA- and siRNA-treated CMS rats are shown in Fig. 4E. The interaction of CMS and peak acute air-jet stress was significant for both MAP ($P < 0.005$) and integrated RSNA ($P < 0.03$); the interaction with CMS and AUC for air-jet stress were also significant: MAP ($P < 0.001$) and integrated RSNA ($P < 0.001$). No interaction was observed for heart rate.

3.4. Hemodynamic and RSNA responses to nasopharyngeal stimulation

Likewise, prior to nasopharyngeal reflex testing, MAP was higher in the scrRNA CMS group compared with either control or siRNA CMS groups, and heart rates were similar across all three groups (Table 3). Peak changes in MAP were similar among the groups (Fig. 5A). Pre-test heart rates did not differ among the groups (Table 3). Reflex bradycardia was observed in control rats, −59 ± 5 bpm. The decrease in heart rate after exposure to ammonia

was augmented in scrRNA-treated CMS rats, −126 ± 44 bpm, and siRNA-treated CMS rats, −99 ± 16 bpm, but the differences did not achieve significance.

Prior to nasopharyngeal reflex testing, baseline integrated RSNA was significantly elevated in the siRNA-treated CMS rats as previously noted (Table 3). Absolute peak responses after exposure to ammonia were greater in both scrRNA- and siRNA-treated CMS rats compared with the peak response of control rats. The duration of RSNA response was significantly prolonged in the scrRNA-treated CMS rats. However, when the change in peak response from baseline MAP and RSNA values was assessed, no differences were apparent among control, scrRNA-treated CMS, or siRNA-treated CMS rats (Fig. 5C). When expressed as percent of baseline RSNA, scrRNA-treated CMS rats displayed a significantly greater response 326%baseline, than either control 193 ± 24%baseline ($P < 0.001$) or siRNA-treated CMS rats (222 ± 11%baseline ($P < 0.001$). Notably, the AUCs for both MAP and RSNA were similar among the groups (Fig. 5B and D). Representative recordings of the nasopharyngeal responses of control, scrRNA- and siRNA-treated CMS rats are shown in Fig. 5E.

4. Discussion

The present studies support the hypothesis that selective knockdown of $V_{1a}R$ within the PVN of rats subjected to CMS decreases resting arterial pressure. Unexpectedly, baseline integrated RSNA was enhanced compared with either control or CMS rats pre-treated with scrambled RNA. In CMS rats, acute psychosocial stress with air-jet resulted in blood pressure and RSNA responses that were more robust particularly when the duration of the responses was taken into account. The acute air-jet responses were prevented by $V_{1a}R$ knockdown. In contrast, chemoreceptor activation via the nasopharyngeal reflex was similar among all the groups. Importantly, the siRNA directed against $V_{1a}R$ substantially reduced its PVN expression. Successful knockdown of the $V_{1a}R$ was further verified by the attenuated pressor and sympathetic responses to AVP microinjected into the PVN.

These findings provide strong data linking $V_{1a}R$ signaling within the PVN in chronic stress and specific acute stressors, such as air-jet. Central AVP has been implicated in human depression [23,28] and anhedonia, the latter being a validated surrogate for depression in rodents [20–26, 47]. Moreover, the expression of both $V_{1a}R$ and $V_{1b}R$ mRNA is increased within PVN of rats after four weeks of chronic mild unpredictable stress [27]. The current studies focused on the impact chronic stress exerts on hemodynamic and sympathetic activity using cutting-edge technology for nerve recording in freely moving animals in their home cages. Admittedly, the studies would have been strengthened by formal behavioral testing. Confirmation with behavioral experiments using knockdown approaches will be essential in providing important additional data regarding long-term $V_{1a}R$ plasticity in stressful environments.

An increasing body of evidence indicates that neuropeptides play a role in psychosocial behaviors and disorders [22]. The PVN is strategically positioned to integrate the stress, neuroendocrine, and autonomic responses [43, 44]. AVP, particularly within the PVN, has

been implicated in stress, anxiety, and depression [20, 23, 45, 46]. $V_{1a}R$ expression is substantially increased in PVN of CMS rats [27].

Rats exposed to CMS, a validated model exhibiting several features consistent with depression including anhedonia [47], have been shown to display abnormal cardiovascular and autonomic function including baroreflex dysfunction of both cardiac and sympathetic nerve activity [27, 36, 48]. The current studies used telemetry to assess both hemodynamics and RSNA in rats in their home cages. The present findings confirmed that basal blood pressure was elevated, but heart rate was not increased in scrRNA-treated CMS rats. Notably, activation of spinally projecting neurons from PVN to the upper thoracic level where sympathetic innervation of the heart are primarily oxytocinergic fibers [49, 50], whereas those projecting to T9-T13 that regulate RSNA are vasopressinergic fibers [51, 52]. Although basal integrated RSNA assessed by telemetry was not statistically greater, RSNA was consistently 20 ± 3% higher in the scrRNA-treated CMS rats compared with controls but, nonetheless, lower than the ~35% and ~50% greater basal RSNA and LSNA, respectively, that have been reported [27, 48]. These observations highlight the potential for confounding influences and subtle stressors imposed by studies performed using arterial catheters and externalized nerve electrodes despite careful protocols to acclimate conscious rats to the testing chambers even within our own laboratory and with an identical chronic stress paradigm.

Treatment with siRNA against $V_{1a}R$ in control rats was approximately twice as effective in blunting the increase in MAP than pharmacological antagonism of $V_{1a}R$ [27]. Nonetheless, the rise in MAP was not completely abrogated supporting the participation of other vasopressinergic pathways, such as $V_{1b}R$ mechanisms.

CMS rats treated with siRNA directed to $V_{1a}R$ exhibited a two-fold higher basal RSNA compared with either control or scrRNA-treated rats. At first glance this observation appears paradoxical since somato-dendritically released AVP stimulates parvocellular neurons via a $V_{1a}R$ mechanism resulting in augmented sympathetic efferent activity [30]. Although acute pretreatment with a $V_{1a}R$ antagonist attenuates the increase in RSNA after microinjection of AVP into the PVN of CMS rats, it is noteworthy that $V_{1a}R$ antagonism alone does not decrease resting RSNA. Rather, combined maximally inhibitory doses of both $V_{1a}R$ and $V_{1b}R$ blockers are required to decrease basal RSNA as well as MAP and heart rate in CMS rats [27]. In this context, several possible explanations may account for the observed greater basal sympathetic activity in siRNA-treated CMS rats. Pharmacological blockade is less specific than genetic knockdown. The widely used $V_{1a}R$ inhibitor, [1-β-mercapto-β, β-cyclopentamethylene-proprionyl^{1},O-methyl)Tyr^{2}, Arg^{8}]vasopressin (also known as Manning compound), has a K_i of ~95 nM against $V_{1b}R$ [53]. Despite being 100 times more selective at $V_{1a}R$ than at the oxytocin receptor [54], Manning compound could potentially inhibit signaling via either of the other receptor subtypes. Microinjection of antagonists localizes inhibition of receptors at the site of injection. The silencing RNA blocks synthesis of $V_{1a}R$, thereby also knocking down expression of $V_{1a}R$ more remotely in axon projections of PVN spinally projecting neurons [55]. Moreover, in contrast to acute pharmacological inhibition, the silencing RNA approach brings about a specific and more prolonged deficiency in $V_{1a}R$ signaling. The additional time may permit functional as well as structural neuroplasticity

[56]. Notably, psychological [57] and physical [58] stressors themselves modulate the transcriptional and translational control of both $V_{1a}R$ and $V_{1b}R$. CMS itself, increases relative expression of mRNA of both vasopressin receptors in the PVN [27]. Chronic decrease in $V_{1a}R$ could therefore independently result in increased expression of $V_{1b}R$, whereas treatment with scrRNA would not provoke such a response. Thus, knockdown of $V_{1a}R$ in CMS may further augment $V_{1b}R$ signaling which is also known to increase sympathetic output [35, 59]. In addition, $V_{1a}Rs$ excite GABAergic PVN neurons such that a chronic decline in $V_{1a}R$ signaling may diminish tonic inhibitory inputs to sympathetic outflow [60, 61]. Taken together, these findings underscore two important factors. First, genetic knockdown strategies whether by siRNA or by other means permit neuroplasticity that may not be evident with acute pharmacological inhibition. Second, tonically elevated basal sympathetic nerve activity in different groups undergoing chronic paradigms may be obscured when reported as percent baseline. Nerve telemetry permits less extraneous interference than externalized wires so that variations in baseline among subjects within a group are minimized allowing assessment of non-normalized activity.

Clearly, the peak arterial pressure response to air-jet stress response was amplified in scrRNA-treated CMS rats versus control rats and was reduced in siRNA CMS rats. Although the absolute peak response of RSNA by the siRNA-treated rats was comparable to that of the scrRNA-treated animals, the duration of the response was substantially reduced such that the area under the curve was no different than that of the control group. These observations are consistent with earlier findings demonstrating that overexpression of $V_{1a}R$ in PVN increases blood pressure and heart rate variability in response to acute air-jet stress [62]. Overall, the results support the concept that chronically stressed male rats not only have higher basal arterial pressure and RSNA, but that they are also predisposed to a more robust physiologic response when presented with an acute emotional stress event that is, at least in part, due to $V_{1a}R$ signaling in the PVN.

When confronted with a physiologic stressor, each group exhibited the similar pressor, bradycardic, and sympathoexcitatory responses that are characteristic of the nasopharyngeal reflex [63, 64]. Although the duration of the response was longer in scrRNA-treated CMS rats, the magnitude of the responses did not differ among the three groups, even when the duration was taken into account. Notably, when reported as % baseline, scrRNA-treated CMS rats displayed a greater response than either other group, thus highlighting the importance of the type of analysis used to evaluated nerve activity. Importantly, the circuitry of the nasopharyngeal reflex involves afferent projections from the medullary dorsal horn of the trigeminal nerve to brainstem autonomic sites: rostral ventrolateral medulla, nucleus tractus solitarius, the A5 area and the peribrachial complex, but not the PVN [65, 66]. Although acute hypoxia that occurs with the reflex induces sympathoexcitation, it does not involve PVN vasopressinergic neurons [67]. The similarity of responses among the groups is consistent with the interpretation that PVN $V_{1a}R$ are not involved in the nasopharyngeal reflex. Moreover, injection of siRNA into PVN likely knocked down $V_{1a}R$ expressed not only on vasopressinergic neurons but also on presympathetic neurons projecting to the rostral ventrolateral medulla (RVLM). Son et al [30] demonstrated dendro-dendritic interrelationships between the magnocellular neurons and presympathetic neurons within the PVN that project to RVLM and were antagonized by a selective $V_{1a}R$ inhibitor. Thus,

the increase in RSNA after air-jet stress in CMS rats and the attenuation of this response in siRNA-treated rats suggests that there is robust crosstalk between magnocellular and presympathetic neurons. These findings implicate intranuclear AVP release acting via $V_{1a}R$ on PVN neurons that project to RVLM controlling RSNA and cardiovascular responses to stress. That nasopharyngeal stimulation which does not involve the PVN provokes increases in blood pressure and sympathetic output but is not inhibited by $V_{1a}R$ knockdown in PVN further corroborates using naked siRNA to knock down $V_{1a}R$ rather than a viral vector constrained the knockdown within the PVN and, further, that dendritically released AVP is selective for stressors that involve the PVN. In addition, the augmented response to acute emotional stress provided by prior chronic unpredictable mild stress may not necessarily generalizable to physiological stressors.

4.1. Limitations and Further Considerations

Telemetric hemodynamics and nerve activities to minimize unintended stress to the animals imparted a distinct advantage to the present studies, nonetheless some limitations remain. We only studied male rats. This was done primarily to permit comparison with previous reports using the same CMS paradigm in male rats [14, 15, 27, 36, 47, 48, 56, 59, 61]. Certainly, studies in female rats are warranted and should include careful attention to hormonal status.

Although the CMS paradigm has been validated [47, 68], parallel experiments to evaluate the impact of $V_{1a}R$ knockdown on behavior would have been highly complementary. In order to avoid any confounding influences on the hemodynamic and sympathetic responses, we avoided extensive behavioral testing. The CMS rats, however, did have ~5.6 – 7% lower body weights compared with controls consist with reported body weight changes in the validations studies [47]. Weights of the siRNA-treated rats vs scrRNA-treated CMS rats did not differ from each other and may have been due to the injection of siRNA at T20D rather than at the beginning of the CMS protocol.

The timing of PVN injections with siRNA on day 20 (T20D) of the CMS protocol was chosen to optimize silencing of the $V_{1a}R$ pathway rather than at the beginning of CMS. The chosen time was a compromise to permit the injection to be remote enough from the end of the protocol and acute stress testing yet minimize the impact of the injection as a stressor itself and still maximally knockdown $V_{1a}R$. Longitudinal data over the course of CMS may have been ideal; however, unless depicting moment-by-moment changes in MAP or heart rate, even a 6- or 12-hr average of these parameters would not be meaningful when the intermittent stressors are presented at different times of day or night and for different intervals. We attempted to record the impact on circadian rhythm, particularly of RSNA, at the end of CMS but encountered technical issues with the data acquisition of RSNA over a 24-hr period. If the technical aspects can be overcome, the impact of chronic stress on diurnal variation of sympathetic activity would be invaluable. In contrast to hemodynamic telemetry which is $>$ 99% successful, the success rate of telemetric RSNA recordings in our hands for the 5–7 days required by the experimental design was only ~ 65 – 70%.

$V_{1a}R$ and $V_{1b}R$ expression are increased two-fold and seven-fold in CMS rats requiring pharmacological inhibition with higher doses of antagonists to both receptors [27]. In light

of the observation that knockdown of $V_{1a}R$ enhanced RSNA in CMS rats, it would have been of value to know whether $V_{1b}R$ receptor expression is further enhanced by silencing of the $V_{1a}R$ pathway. It would also have been advantageous to have more precise cellular information regarding the extent of $V_{1a}R$ knockdown other than harvesting whole PVN. Additional studies with siRNA targeted to $V_{1b}R$ alone and in combination with $V_{1a}R$ should also be considered.

5. Conclusion

In summary, the present studies using hemodynamic and nerve telemetry in male rats freely moving in their home cages confirm that CMS increased basal arterial pressure. The increase in arterial pressure was prevented by selective knockdown of PVN $V_{1a}R$. Contrary to studies in rats conditioned to study chambers [27, 48], a significant increase in basal RSNA was not evident in scrRNA-treated CMS rats. Paradoxically, basal RSNA was enhanced by silencing PVN signaling via $V_{1a}R$ which may have been due to either functional or anatomical neuroplasticity not evident with acute pharmacological blockade of the receptor. Furthermore, when duration of the responses was considered, prior CMS enhanced the arterial pressure and RSNA responses to an emotional stressor but not a physiological stressor, such as the nasopharyngeal reflex. Overall, the findings support an integral role for vasopressinergic signaling within the PVN via $V_{1a}R$ in both chronic as well as acute emotional stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Data will be made available on request.

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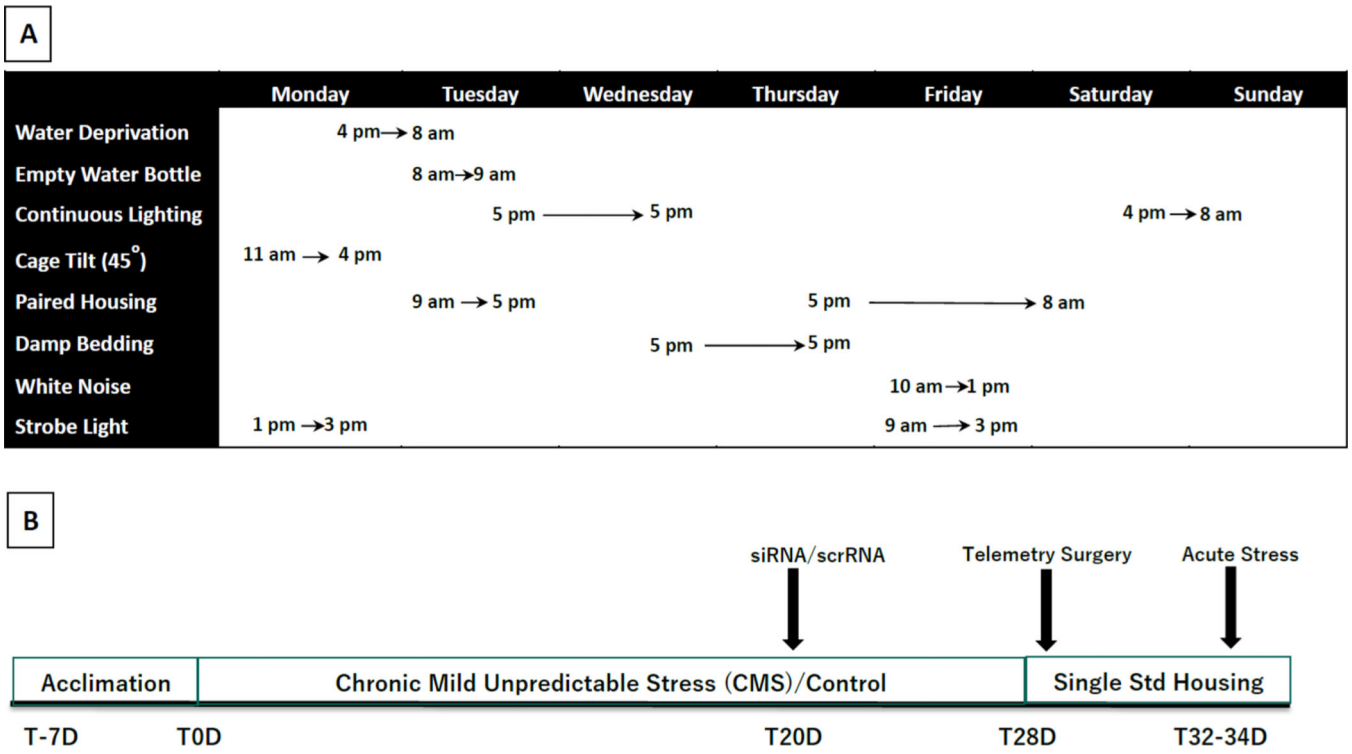

Fig. 1.

(A) Scheme for chronic unpredictable mild stress as described by Komnenov [27] modified from Grippo et al [36]. (B) Timeline for CMS and control protocols, surgical interventions and acute stress testing.

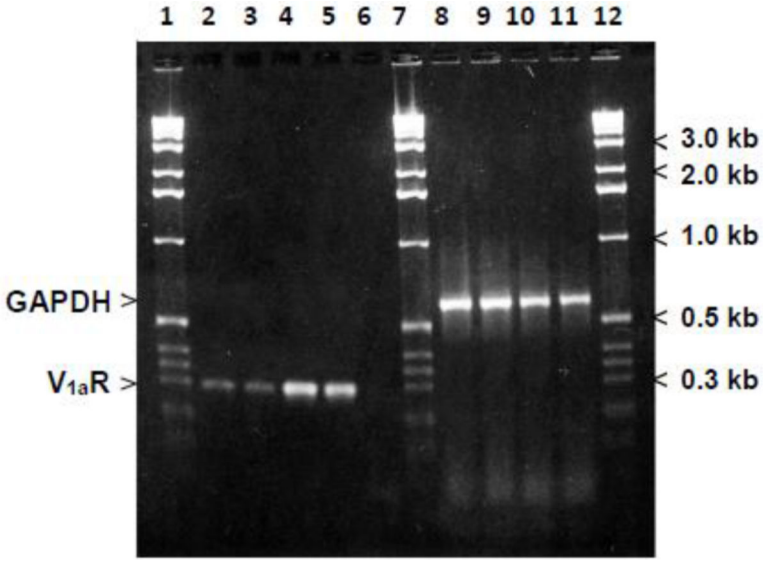

Fig. 2.

RT-PCR analysis of PVNs extracted from rats 14 days after injection with either siRNA targeted to V1aR or scrambled RNA (scrRNA). Lanes 2, 8 (rat 1) and lanes 3, 9 (rat 2): V1aR siRNA; lanes 4, 10 (rat 3) and lanes 5, 11 (rat 4): scrRNA; lane 6: V1aR negative control (all "nested" reaction components minus template); lanes 1,7, and12: molecular weight markers.

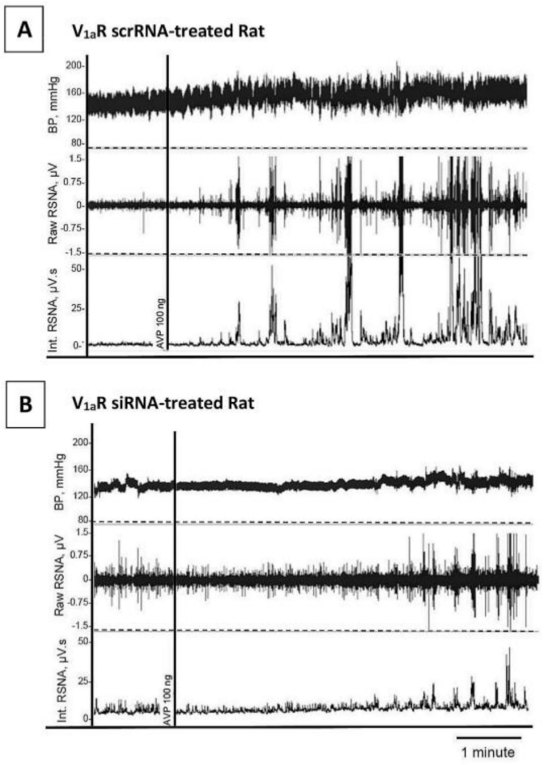

Fig. 3.

Representative recordings of blood pressure (BP), raw renal sympathetic nerve activity (RSNA), and integrated RSNA (int RSNA) uncorrected for noise after injection of 100 ng AVP (shown at marker) from a CMS rat treated 14 days earlier with (A) scrRNA or a separate CMS rat treated with (B) siRNA directed against the $V_{1a}R$ in the PVN.

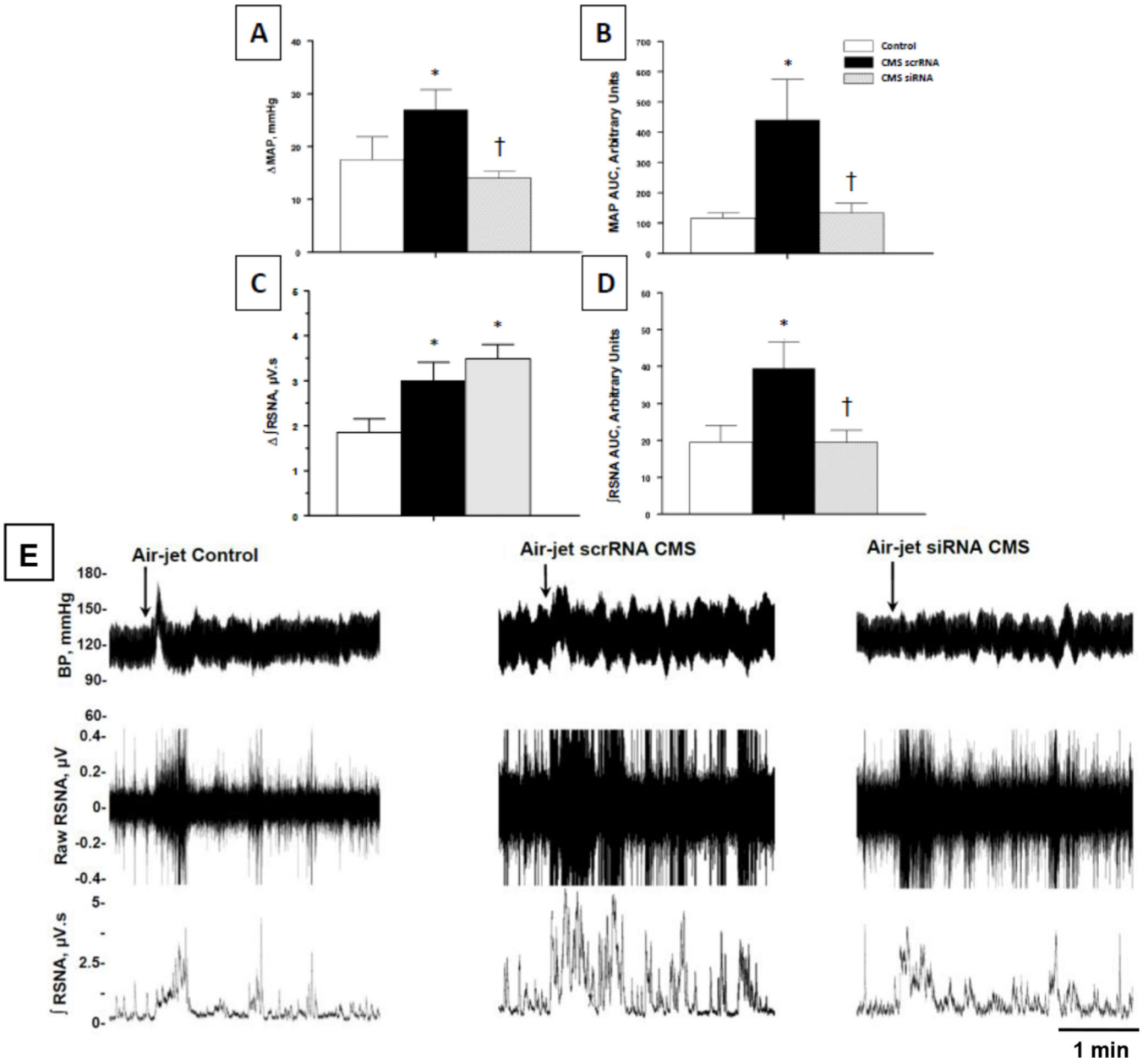

Fig. 4.

The MAP and integrated RSNA after 15-sec air-jet stress expressed as either the maximum change from baseline (peak change; A, C) or expressed as area under the curve, AUC (B, D) in control (n = 8), scrRNA-treated CMS rats (n = 8), or siRNA-treated CMS rats (n = 7). Representative telemetric recordings of blood pressure (BP), raw renal sympathetic nerve activity (RSNA), and integrated renal sympathetic nerve activity (∫RNSA) prior to correction for noise from control (E). CMS, chronic unpredictable stress; scrRNA, scrambled RNA; siRNA, siRNA directed against V1aR. Values are mean ±SE. *$P < 0.05$ vs control; †$P < 0.05$ vs CMS scrRNA.

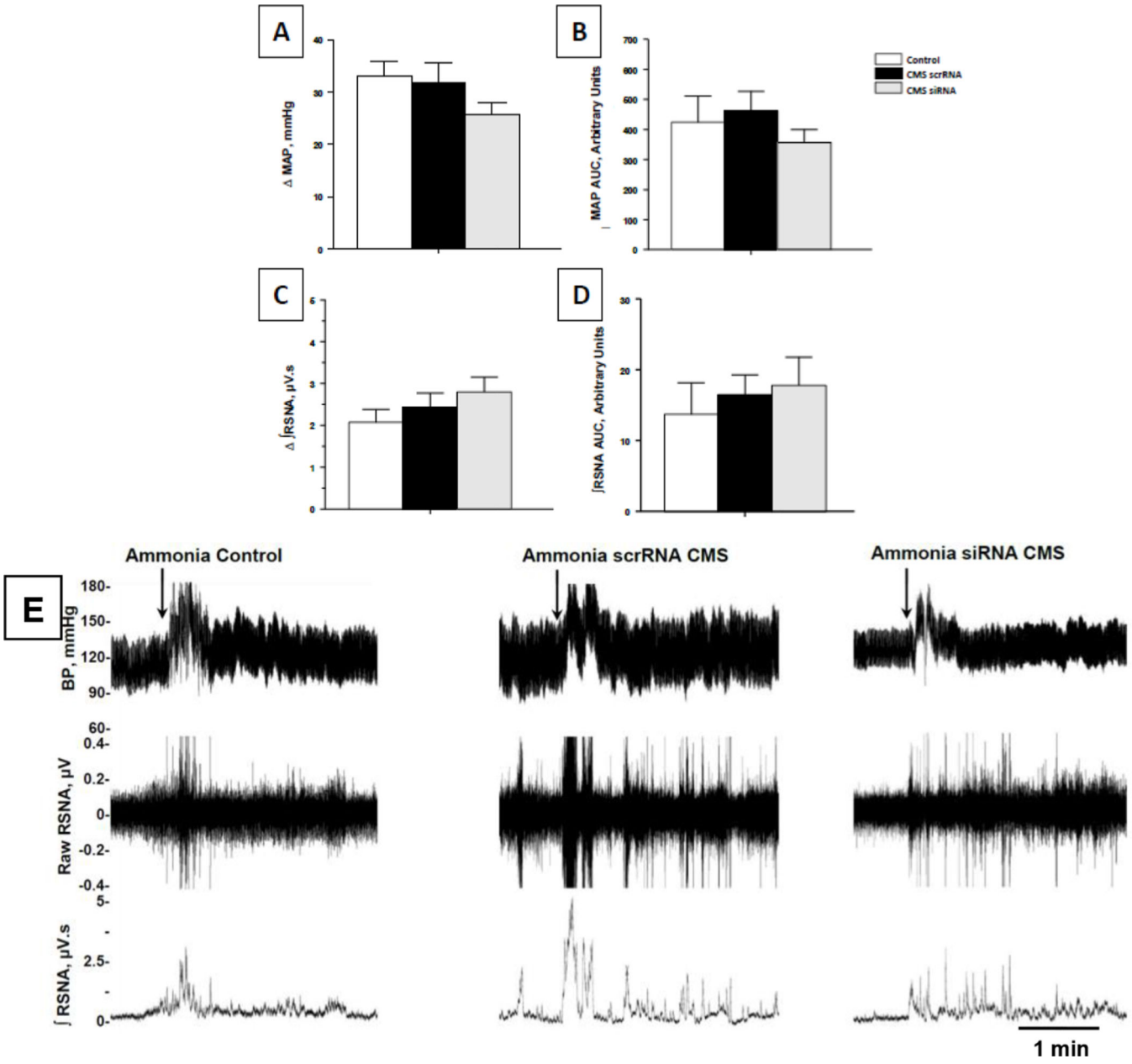

Fig. 5.

MAP and integrated RSNA after 15-sec nasopharyngeal reflex testing with ammonia expressed as either the maximum change from baseline (peak change; A, C) or as area under the curve, AUC (B, D) in control (n = 8), scrRNA-treated CMS rats (n = 8), or siRNA-treated CMS rats (n = 7). Representative telemetric recordings of blood pressure (BP), raw renal sympathetic nerve activity (RSNA), and integrated renal sympathetic nerve activity (∫ RNSA) prior to correction for noise from control (E). CMS, chronic unpredictable stress; scrRNA, scrambled RNA; siRNA, siRNA directed against V1aR. Values are mean ± SE.

Table 1

Change in MAP after 100 ng AVP injected into PVN of Control Rats 14 Days after PVN Treatment with Vehicle, scrRNA, or siRNA directed to $V_{1a}R$

Group	n	ΔMAP, mmHg
Vehicle	3	17.2 ± 0.3
scrRNA	3	16.2 ± 0.1
siRNA	3	9.0 ± 0.9 *†

Values are mean ± SE.

*$P < 0.05$ vs vehicle;

†$P < 0.05$ vs scrRNA

Table 2

24-hr Averaged Hemodynamics and Integrated RSNA in Control and CMS Male Rats Treated with Scrambled or siRNA Directed against V_{1a} Receptors

Group1.22	n	Heart Rate, bpm	MAP, mmHg	∫RSNA, μV.s
Control	8	401 ± 10	107.4 ± 1.9	0.99 ± 0.08
CMS scrRNA	8	410 ± 13	115.3 ± 1.6 *	1.22 ± 0.16
CMS siRNA	7	388 ± 17	110.0 ± 2.7	1.93 ± 0.28 *†

MAP, mean arterial pressure; ∫RSNA, integrated renal sympathetic nerve activity.

Values are mean ± SE.

* $P < 0.05$ vs control

† $P < 0.05$ vs CMS scrRNA

Table 3

MAP, HR, and Integrated RSNA Responses to Acute Stressors in Control and CMS Rats

	n	Pre-test MAP (mmHg)	Peak MAP (mmHg)	Pre-test HR (bpm)	Peak HR (bpm)	Pre-test ∫RSNA (μV.s)	Peak ∫RSNA (μV.S)	Duration (sec)
Air-jet Stress								
Control	8	111.4 ± 1.4	127.5 ± 4.2§	388 ± 10	436 ± 11§	0.90 ± 0.14	2.84 ± 0.26§	30.4 ± 13.5
CMS scrRNA	8	118.1 ± 1.7*	143.8 ± 3.1*§	357 ± 15	400 ± 23§	1.09 ± 0.17	4.20 ± 0.44§	86.0 ± 21.9*
CMS siRNA	7	113.5 ± 1.6	125.5 ± 1.8†§	393 ± 21	432 ± 18§	1.83 ± 0.38*	5.40 ± 0.55*§	37.1 ± 7.5
Nasopharyngeal Reflex								
Control	8	109.6 ± 1.5	146.2 ± 4.2§	419 ± 12	359 ± 12§	0.92 ± 0.07	1.78 ± 0.43§	42.7 ± 9.4
CMS scrRNA	8	117.0 ± 1.7*	151.5 ± 3.6§	377 ± 15	251 ± 53§	1.08 ± 0.11	3.52 ± 0.39*§	77.8 ± 11.6*
CMS siRNA	7	110.4 ± 1.6†	148.4 ± 4.6§	397 ± 17	298 ± 16§	2.30 ± 0.32*†	5.10 ± 0.56*†§	49.3 ± 8.7

MAP, mean arterial pressure; HR, heart rate; ∫RSNA, integrated renal sympathetic nerve activity Values are mean ± SE

$^{*}P < 0.05$ vs control;

$^{\dagger}P < 0.05$ vs CMS scrRNA by ANOVA

$^{\S}P < 0.01$ vs pre-test value by paired t-test