ORIGINAL RESEARCH

Remote Limb Ischemic Preconditioning Attenuates Cerebrovascular Depression During Sinusoidal Galvanic Vestibular Stimulation via α_1-Adrenoceptor–Protein Kinase Cε–Endothelial NO Synthase Pathway in Rats

Devin W. McBride, PhD; Cesar Reis, MD; John H. Zhang, MD, PhD; Richard Applegate II MD; Jiping Tang, MD

***Background*—**Vasovagal syncope (VVS) is characterized by hypotension and bradycardia followed by lowering of cerebral blood flow. Remote limb ischemic preconditioning (RIPC) is well documented to provide cardio- and neuroprotection as well as to improve cerebral blood flow. We hypothesized that RIPC will provide protection against VVS-induced hypotension, bradycardia, and cerebral hypoperfusion. Second, because endothelial nitric oxide synthase has been reported as a mediator of cerebral blood flow control, we hypothesized that the mechanism by which RIPC primes the vasculature against VVS is via the α_1-adrenoceptor–protein kinase Cε–endothelial nitric oxide synthase pathway.

***Methods and Results*—**We utilized sinusoidal galvanic vestibular stimulation in rats as a model of VVS. RIPC attenuated the lowerings of mean arterial pressure, heart rate, and cerebral blood flow caused by sinusoidal galvanic vestibular stimulation, as well as improving behavior during, and recovery after, stimulation. RIPC induced elevated serum norepinephrine, increased expression of brain α_1-adrenoceptors, and reduced brain expression of norepinephrine transporter 1. Antagonizing adrenoceptors and norepinephrine transporter 1 prevented RIPC protection of cerebral perfusion during sinusoidal galvanic vestibular stimulation.

***Conclusions*—**Taken together, this study indicates that RIPC may be a potential therapy that can prevent VVS pathophysiology, decrease syncopal episodes, and reduce the injuries associated with syncopal falls. Furthermore, the α_1-adrenoceptor–protein kinase Cε–endothelial nitric oxide synthase pathway may be a therapeutic target for regulating changes in cerebral blood flow. **(*J Am Heart Assoc*. 2018;7:e007105. DOI: 10.1161/JAHA.117.007105.)**

Key Words: catecholamine • ischemia • preconditioning • syncope

Vasovagal syncope (VVS) is the transient loss of consciousness that involves loss of postural tone, collapse, and spontaneous recovery.[1] VVS, the most common type of syncope, affects between 25% and 40% of individuals[2] and has a 30% chance of recurrence.[3] Annually, ≈400 000 individuals are diagnosed with VVS, of whom 2% to 5% require emergency room visits, leading to an annual burden of about $2.4 billion on the US healthcare system.[4]

Although the mechanism of VVS is not fully understood, the current paradigm is that decreased venous return to the heart induces vigorous contraction of the myocardium against inadequately filled atria, thereby triggering the Bezold-Jarisch reflex, which causes paradoxical hypotension and bradycardia,[1,5] leading to decreased cerebral perfusion and precipitating a loss of consciousness.[5] With the use of the head-up tilt test, the physiological changes occurring in VVS patients have led to better insight into potential mechanisms of VVS. Head-up tilt testing in humans has shown that sympathetic nerve activity and myocardial contractility are reduced preceding syncope onset, followed by hypotension.[6] Furthermore, serum catecholamines, namely norepinephrine and epinephrine, have been reported to be elevated at the onset of syncope, suggesting that sympathoadrenal activation may play a role in the pathophysiology of VVS.[6]

From the Departments of Physiology & Pharmacology (D.W.M., J.H.Z., J.T.), Anesthesiology (C.R., R.A.), and Neurosurgery (J.H.Z.), Loma Linda University, Loma Linda, CA.

Accompanying Data S1, Tables S1 through S10, and Figures S1 through S11 are available at http://jaha.ahajournals.org/content/7/7/e007105/DC1/embed/inline-supplementary-material-1.pdf

Correspondence to: Jiping Tang, MD, Department of Physiology & Pharmacology, Loma Linda University, 11041 Campus St, Risley Hall Room 219, Loma Linda, CA 92350. E-mail: jtang@llu.edu

Received July 20, 2017; accepted January 24, 2018.

Clinical Perspective

What Is New?

- Remote limb ischemic preconditioning is used to prevent the cardio- and cerebrovascular depressions induced by sinusoidal galvanic vestibular stimulation (model for vasovagal syncope).
- The mechanism of remote limb ischemic preconditioning protection of the cerebrovascular depression is via norepinephrine activation of the α_1-adrenoceptor–protein kinase Cε–endothelial nitric oxide synthase pathway.

What Are the Clinical Implications?

- Remote limb ischemic preconditioning may be a preconditioning strategy that can be used to reduce the severity and frequency of vasovagal syncope episodes.
- Additionally, the α_1-adrenoceptor–protein kinase Cε–endothelial nitric oxide synthase pathway may be a therapeutic target for preventing vasovagal syncope.

Remote limb ischemic preconditioning (RIPC) is well known to provide cardioprotection[7]; RIPC may protect the heart against myocardial infarction,[8] tachycardia,[9] and bradycardia[10] and improves cardiac function.[8] RIPC is also neuroprotective,[11] and of particular relevance to VVS are the effects RIPC has on cerebral blood flow. RIPC has been shown to increase cerebral blood flow in both experimental and clinical studies.[11] Therefore, RIPC may be a therapeutic option to provide benefit against both the cardio- and neuro-vascular depressions of VVS.

Adrenoceptors are documented to play a role in the regulation of cerebral blood flow. In brief, α_1-adrenoceptors are responsible for vasoconstriction, and thus, stimulation of α_1-adrenoceptors causes decreased cerebral blood flow. In direct opposition, β-adrenoceptors lead to vasodilation and higher cerebral blood flow. The latter observations may be linked to β-adrenoceptor activation of endothelial nitric oxide synthase (eNOS) and nitric oxide production. Interestingly, α_1-adrenoceptors have also been shown to induce eNOS activation downstream of vasoconstriction to cause delayed vasodilation.[12] Furthermore, of the utmost relevance to the current study, Gürdal et al found that prolonged stimulation of α_1-adrenoceptors can decrease α_1-adrenoceptor-mediated vasoconstriction as well as increase eNOS expression and activity.[13]

Based on the cardio- and neuro-protective attributes of RIPC, in particular the ability of RIPC to affect cerebral blood flow, our primary hypothesis was that RIPC will provide protection against VVS-induced hypotension, bradycardia, and reduced cerebral blood flow in rats subjected to sinusoidal galvanic vestibular stimulation. Second, because nitric oxide has been reported as a key mediator of the cerebral blood flow control observed in models of ischemia-reperfusion and may be linked with both RIPC and adrenoceptors, we also hypothesized that the mechanism by which RIPC confers tolerance of the vasculature against VVS is via desensitization of α_1-adrenoceptors (reduced vasoconstriction) and increased protein kinase Cε (PKCε) and eNOS expressions.

Material and Methods

A total of 126 adult male Sprague-Dawley rats (3 months old, Envigo), 24 aged male Sprague-Dawley rats (12 months old, Envigo), and 24 female Sprague-Dawley rats (3 months old, Envigo) were used. Rats were housed in a humidity- and temperature-controlled environment with a 12-hour light-dark cycle, and rats were given food and water ad libitum. During all surgical procedures and methods, body temperature was maintained at 37±0.5°C using a heating pad controlled by a rectal probe. Sinusoidal galvanic vestibular stimulation (sGVS) in rats is used as the model of VVS. All experiments were approved by and conducted according to the Institutional Animal Care and Use Committee at Loma Linda University, conducted in compliance with the *NIH Guidelines for the Use of Animals in Neuroscience Research*, and reported according to the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines. The data, methods, and materials are available to other researchers for purposes of reproducing the results or replicating the procedure (contact corresponding author).

Animals were simply randomized using an electronic generator. Experiment 1 investigated the effect of RIPC on mean arterial pressure, heart rate, and cerebral blood flow during sGVS (groups: sham, vehicle [isoflurane] preconditioning then sGVS, RIPC [5 days] then sGVS, and RIPC [10 days] then sGVS; n=8/group). In a separate cohort, female rats were randomly assigned to 1 of 3 groups to study potential sex differences in response to sGVS and RIPC protection against sGVS (groups: sham, vehicle preconditioning then sGVS, and RIPC [10 days] then sGVS; n=8/group). In another cohort, aged male rats (12 months old) were randomly assigned to 1 of 3 groups to study potential age differences in response to sGVS and RIPC protection against sGVS (groups: sham, vehicle preconditioning then sGVS, and RIPC [10 days] then sGVS; n=8/group). Experiment 2 investigated the response of awake rats to sGVS after preconditioning (groups: sham, vehicle preconditioning then sGVS, RIPC then sGVS; n=8/group). Experiment 3 examined the effect of RIPC on catecholamines and adrenoceptor expression (groups: vehicle preconditioning and RIPC; n=7/group). Experiment 4 studied the role of adrenoceptors in RIPC protection against sGVS (groups: sham [n=16], vehicle preconditioning [with IV normal saline] then sGVS, vehicle preconditioning [with intranasal normal saline] then sGVS, RIPC [with IV normal

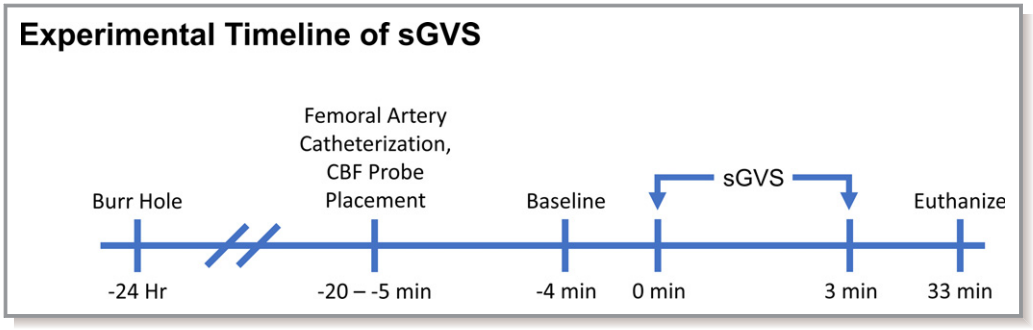


Figure 1. Schematic of the experimental timeline of sinusoidal galvanic vestibular stimulation (sGVS). Twenty-four hours before sGVS, a burr hole was made in the skull. On the day of sGVS, rats are first given a femoral artery catheter, followed by reopening of the burr hole in the skull for cerebral blood flow probe placement. Rats are then subjected to sGVS for 3 minutes (after a 4-minute baseline of mean arterial pressure, heart rate, and cerebral blood flow) and euthanized 30 minutes after stopping stimulation.

saline] then sGVS, RIPC [with intranasal normal saline] then sGVS, RIPC with labetalol then sGVS, RIPC with doxazosin then sGVS, RIPC with atenolol then sGVS, and RIPC with desipramine then sGVS; n=8/group). Figures 1 through 6 show the study design and timeline for each experiment. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

The sample size required for mean arterial pressure, heart rate, and cerebral blood flow was based on a power analysis (SigmaPlot 11.0, Systat, San Jose, CA) of previous data from our laboratory (minimum detectable difference in means=6.0, standard deviation=3.25, power=0.80, α=0.05, groups=5-6), which indicated that 8 animals per group would be sufficient to test for statistical significance. The sample size required for ELISA (minimum detectable difference in means=250, standard deviation=150, power=0.80, α=0.05, groups=2) and Western blot data (minimum detectable difference in means=1.0, standard deviation=0.4, power=0.80, α=0.05, groups=6), based on a power analysis of previous data in our laboratory, indicated that 7 and 6 animals per group, respectively, would be sufficient to test for statistically significant differences.

Sinusoidal Galvanic Vestibular

One day before sGVS, rats were anesthetized using isoflurane (4% induction, 2.5% sustained, delivered in a mixture of oxygen [0.3 L/min] and medical gas [0.7 L/min]) and placed into a rodent stereotaxic frame. The scalp was shaved and disinfected (isopropanol prep pads). A midline incision was made through the skin and connective tissue, and the periosteum was separated from the skull to expose bregma and the sagittal and coronal sutures. Using a microdrill, a burr hole (3 mm in diameter) was created with the center located 5 mm proximal to the coronal suture and 4 mm right lateral to the sagittal suture. The bone flap was gently removed without damaging the underlying dura or brain tissue. After completing the burr hole, bone wax was applied to seal the burr hole, and

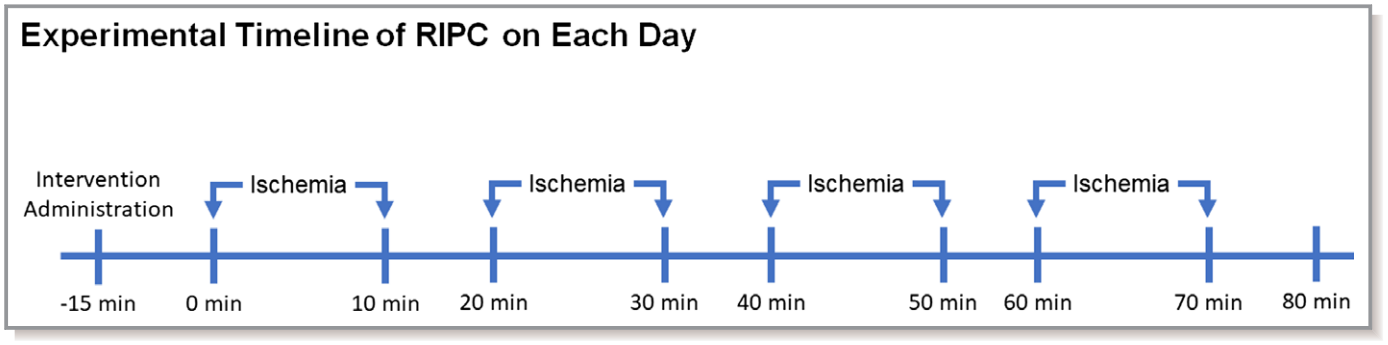


Figure 2. Schematic of the experimental timeline of the remote limb ischemic preconditioning (RIPC) procedure on each day of preconditioning. Rats in experiments 1 to 3 are not given anything at the "intervention administration" time. Rats in experiment 4 are given an intervention at the "intervention administration" time according to the group each animal was distributed into. RIPC was performed using 4 cycles of 10 minutes of ischemia followed by 10 minutes of reperfusion. After the 4 cycles were completed, animals were allowed to recover before being returned to their home cages.

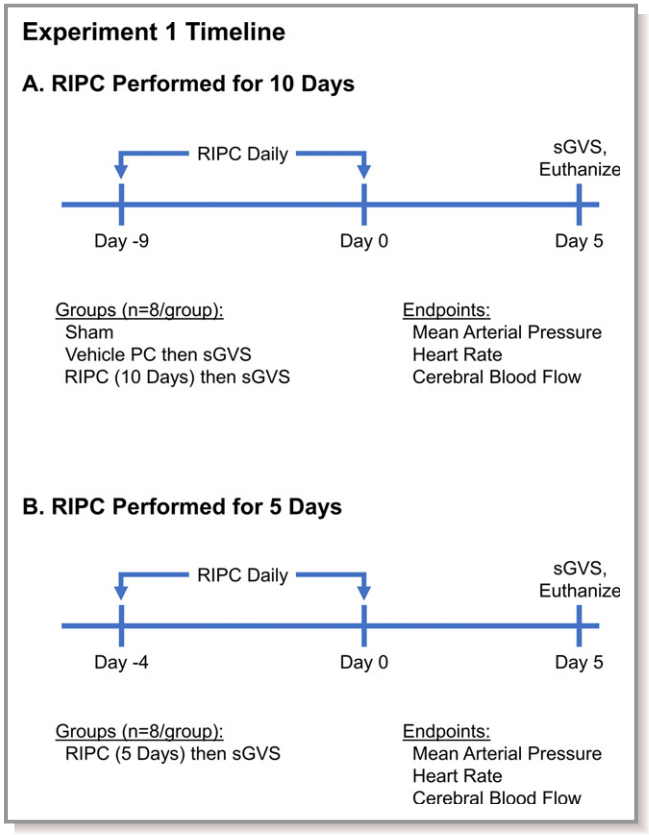


Figure 3. Schematic of the experimental timeline of experiment 1. A, Remote limb ischemic preconditioning (RIPC) for 10 days. B, RIPC for 5 days. Animals were subjected to nothing (sham), isoflurane (vehicle preconditioning [PC]), or RIPC with the last day of the regimen completed on day 0 (5 days before sinusoidal galvanic vestibular stimulation [sGVS]). Mean arterial pressure, heart rate, and cerebral blood flow were monitored on the day of sGVS (day 5).

the skin was sutured. Buprenorphine was administered subcutaneously (0.01 mg/kg), and the animal was allowed to recover before being returned to its home cage.

On the day of sGVS, animals were anesthetized using isoflurane (4% induction, 2.5% sustained) and placed supine. The skin over the femoral artery was shaved and disinfected. An incision was made, and tissue was dissected to expose the femoral artery. Blood flow was momentarily halted using a suture. An incision was made in the femoral artery, and a PE50 catheter was inserted and advanced 1 to 2 cm into the femoral artery. The catheter was connected to a transducer for measurement of blood pressure and heart rate (Digi-Med BPA 400a, Micro-Med Inc, Louisville, KY). Blood pressure and heart rate were monitored for 4 minutes before sGVS, during sGVS (3 minutes), and for 30 minutes post-sGVS.

After placement of the femoral catheter, the animal was gently moved and placed prone into a rodent stereotaxic frame, and its head was secured. The sutures on the scalp were removed, the wound reopened, and the bone wax removed, exposing the dura and brain tissue. A laser Doppler probe (OxyFlo probe, MNP100XP, AdInstruments Inc, Colorado Springs, CO) was placed above the exposed brain tissue and used for measurement of cerebral blood flow (PowerLab PL3504 and LabChart Pro, AdInstruments Inc, Colorado Springs, CO). Cerebral blood flow was monitored for 4 minutes before sGVS, during sGVS (3 minutes), and for 30 minutes post-sGVS.

sGVS was induced as previously described.[14] Briefly, after laser Doppler probe placement, 2 Ag/AgCl needle electrodes were inserted into the skin over the mastoids, behind the auditory meati. sGVS was created using a computer-controlled stimulator (Grass Technologies, West Warwick, RI), which generated sinusoidal currents (4 mA current at 0.025 Hz) binaurally. sGVS was induced for 3 minutes. Thirty minutes after sGVS was stopped, animals were deeply anesthetized and then underwent cardiac perfusion of ice-cold 1× PBS. Brains and hearts were removed and snap-frozen, then stored at −20°C. Figure 1 displays the experimental timeline of sGVS.

Remote Limb Ischemic Preconditioning

RIPC was performed for either 5 or 10 consecutive days. The RIPC was stopped 5 days before the animals were subjected to sGVS. Each day, anesthetized rats (2.5% isoflurane) underwent bilateral hindlimb ischemia-reperfusion for 4 cycles of 10 minutes of ischemia followed by 10 minutes of reperfusion. Each hindlimb was encircled with a padded rubber tourniquet with the ends threaded through a rubber tube to form a reversible snare. Ischemia was induced by making the snare as tight as possible using hemostatic forceps. Reperfusion was begun by releasing the hemostatic forceps. Vehicle (isoflurane) preconditioning followed all procedures except the snare was never tightened. Figure 2 displays the timeline of RIPC.

Experiment 1: Effect of RIPC on Blood Pressure, Heart Rate, and Cerebral Perfusion During sGVS

Thirty-two 3-month-old male animals were randomly assigned to sham, vehicle preconditioning then sGVS, RIPC for 5 days then sGVS, or RIPC for 10 days then sGVS (n=8/group). In a separate cohort, 24 female animals were randomly assigned to sham, vehicle preconditioning then sGVS, or RIPC for 10 days then sGVS (n=8/group). In another cohort, 24 12-month-old male rats were randomly assigned to sham, vehicle preconditioning then sGVS, or RIPC for 10 days then sGVS (n=8/group). Sham animals were rats that underwent all surgical procedures (burr hole, femoral artery catheterization), monitoring of mean arterial pressure, heart rate, and cerebral

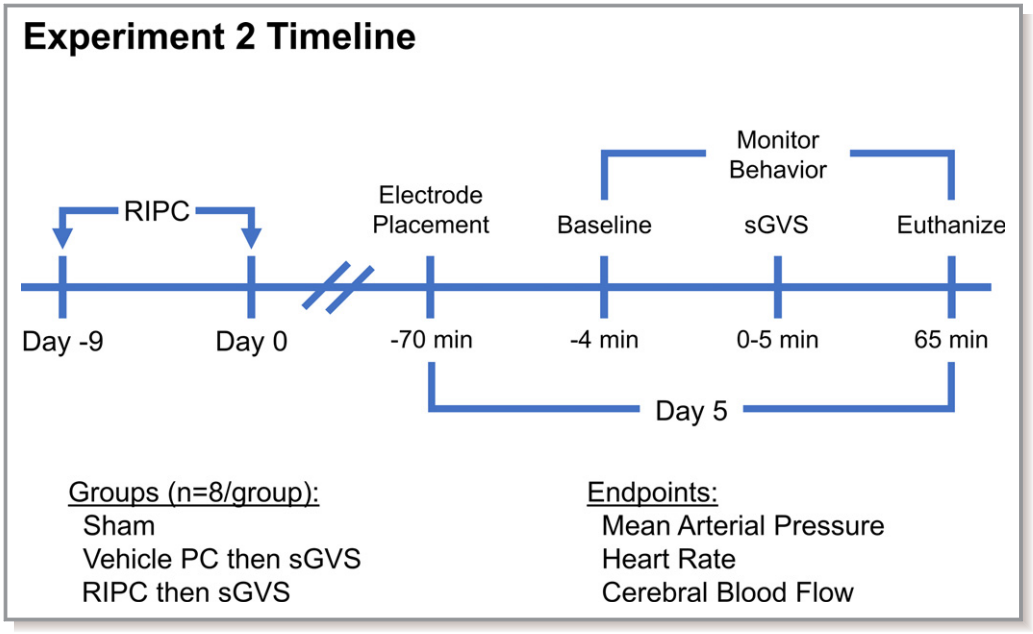


Figure 4. Schematic of the experimental timeline of experiment 2. Animals were subjected to nothing (sham), isoflurane (vehicle preconditioning [PC]), or remote limb ischemic preconditioning (RIPC) for 10 days. On day 5, animals were subjected to awake sinusoidal galvanic vestibular stimulation (sGVS); behavior was monitored for 5 minutes before sGVS, during sGVS (5 minutes long), and for 60 minutes after stopping sGVS.

blood flow, and electrode placement but without electrical stimulation (ie, sGVS was not induced). Vehicle-preconditioned (PC) animals underwent all RIPC procedures without tightening of the hindlimb snares. Animals were subjected to sGVS 5 days after completing the preconditioning regimen (Figure 3).

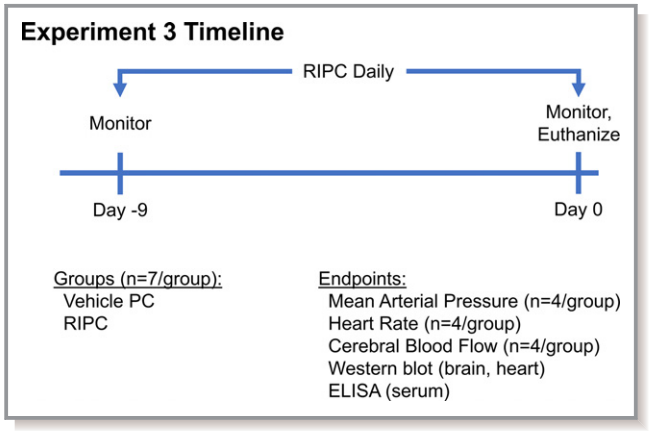


Figure 5. Schematic of the experimental timeline of experiment 3. Animals were subjected to either isoflurane (vehicle preconditioning [PC]) or remote limb ischemic preconditioning (RIPC) for 10 days. Mean arterial pressure, heart rate, and cerebral blood flow were monitored on the first and last days of the regimen (day −9 and day 0, respectively). Blood was also collected on the first and last days of the regimen before beginning the preconditioning and at 0, 30, and 60 minutes postpreconditioning for measurement of serum catecholamines via ELISA. On the final day of preconditioning (day 0), animals were euthanatized, and the brains and hearts collected for Western blot.

Experiment 2: Effect of RIPC on sGVS Behavior in Awake Rats

Twenty-four 3-month-old male Sprague-Dawley rats were randomly assigned to sham, vehicle preconditioning then sGVS, or RIPC then sGVS (n=8/group). Vehicle preconditioning and RIPC were performed as described above (for 10 consecutive days). Animals did not undergo femoral artery catheterization or burr hole surgery. On the day of sGVS, animals were briefly anesthetized with isoflurane for electrode placement (less than 10 minutes of isoflurane exposure). The animals recovered for 60 minutes, and then behavior was recorded for baseline characteristics. Then rats were subjected to sGVS for 5 minutes and then observed for 60 minutes poststimulation (Figure 4).

Experiment 3: Effect of RIPC on Catecholamine Release and Expression of Adrenoceptors

Fourteen 3-month-old male Sprague-Dawley rats were randomly assigned to either vehicle (isoflurane) preconditioning or RIPC for 10 days (n=7/group). Vehicle preconditioning and RIPC were performed as described above, but femoral artery catheterization was performed on right femoral artery on the first day of RIPC and on the left femoral artery on the last day

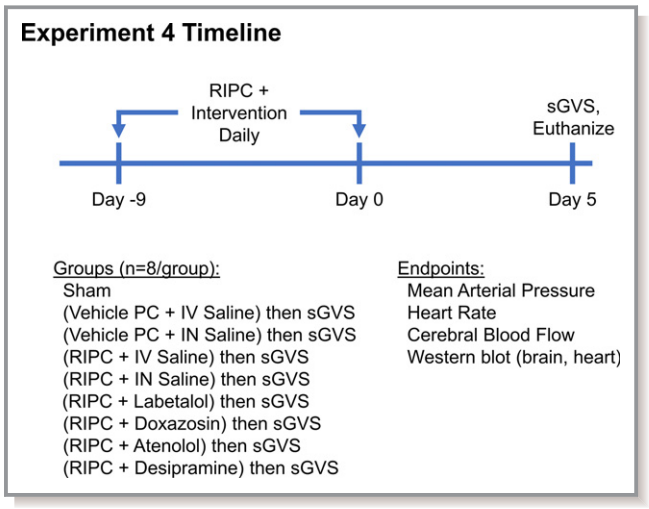


Figure 6. Schematic of the experimental timeline of experiment 4. Animals were subjected to nothing (sham), isoflurane (vehicle preconditioning [PC]), or remote limb ischemic preconditioning (RIPC) with the last day of the regimen completed 5 days before sinusoidal galvanic vestibular stimulation (sGVS). Rats in the vehicle-PC groups were given either intravenous (IV) or intranasal (IN) normal saline 15 minutes before beginning preconditioning on each day. Rats in the RIPC groups were given labetalol, doxazosin, or atenolol intravenously or desipramine intranasally 15 minutes before beginning preconditioning on each day. Mean arterial pressure, heart rate, and cerebral blood flow were monitored on the day of sGVS.

of RIPC. Femoral artery catheterization was performed as described above. On the first and last day of RIPC, blood (450 μL) was collected (into 50 μL of solution containing 11 mmol EDTA and 44 nmol sodium metabisulfite) from the femoral artery catheter before beginning RIPC, at then at 0, 30, and 60 minutes post-RIPC. The blood was centrifuged at 3000*g* for 20 minutes at 4°C. Serum was collected, snap-frozen, and then stored at −20°C. Catecholamines (epinephrine and norepinephrine) were measured in serum samples using ELISA (BA E-5400, LDN, Nordhorn, Germany) following the manufacturer's guidelines. On the last day of RIPC, 60 minutes after the end of the final ischemia-reperfusion cycle, animals were deeply anesthetized and then underwent cardiac perfusion of ice cold 1× PBS. The brains and hearts were removed and snap-frozen and stored at −20°C. Figure 5 displays the experimental timeline of experiment 3.

Experiment 4: Study the Role of Adrenoceptors in RIPC Protection Against sGVS

Seventy-two 3-month-old male Sprague-Dawley rats were randomly assigned to sham, isoflurane preconditioning (with IV normal saline) then sGVS, isoflurane preconditioning (with intranasal normal saline) then sGVS, RIPC (with IV normal saline) then sGVS, RIPC (with intranasal normal saline) then sGVS, RIPC+labetalol then sGVS, RIPC+doxazosin then sGVS, RIPC+atenolol then sGVS, or RIPC+desipramine then sGVS (n=8/group). Vehicle (isoflurane) preconditioning and RIPC were performed, as described above, for 10 days. Labetalol (antagonist of α- and β-adrenoceptors), doxazosin (α_1-adrenoceptor antagonist), atenolol (β_1-adrenoceptor antagonist), and desipramine (norepinephrine transporter 1 [NET1] antagonist) were administered 15 minutes before beginning RIPC on each day of RIPC. Labetalol (3 mg/kg), doxazosin (6 mg/kg), and atenolol (5 mg/kg) were dissolved in normal saline and administered via tail vein injection (200 μL). Desipramine (0.02 mg/kg) was dissolved in normal saline and administered via intranasal injection (10 μL in the left nostril, and then 1 minute later, 10 μL in the right nostril). All animals were subjected to sGVS 5 days after completing the preconditioning regimens (Figure 6).

Data Collection, Data Processing, and Statistical Analysis

All raw data were collected, processed, and analyzed by a blinded investigator. Data are presented as the mean and the standard deviation. Normality was confirmed for all data presented, all tests were 2-sided, and no further adjustment for multiple comparisons was done for the overall number of tests. GraphPad Prism 6 (La Jolla, CA) was used for statistical analysis. $P<0.05$ was considered statistically significant.

Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow

The raw data for mean arterial pressure, heart rate, and cerebral blood flow were separated into 3 experimental sections for experiments 1 and 4: baseline (minutes −4 to 0), stimulation (minutes 0–3), and poststimulation (minutes 3–13). Within each section, the raw data were averaged, and the standard deviation was computed. The data were then converted into the percentage change from baseline and analyzed using repeated-measures 2-way ANOVA with Tukey or Sidak post hoc tests. Additionally, the minimum values during sGVS stimulation of the mean arterial pressure, heart rate, and cerebral blood flow were determined and analyzed using 1-way ANOVA with Tukey post hoc tests.

Behavior in Awake Rats

During stimulation, the following measures were recorded: breathing rate, number of stumbles/falls, coordination/balance, and responsiveness. Poststimulation, rats were monitored, and the time until recovery from sGVS behavior was recorded. The average breathing rate and time to recovery were analyzed using 1-way ANOVA with Tukey

Table 1. Scoring Criteria for the Syncope Score Test During Sinusoidal Galvanic Vestibular Stimulation in Awake Rats

	Score			
	0	1	2	3
Breathing	Normal (75-95 BPM)	Rapid (>95 BPM)	Shallow, normal rate (75-95 BPM)	Shallow, low rate (<75 BPM)
Coordination	Normal	Slight dyscoordination	Swaying during walking	Severe dyscoordination: swaying during standing, falling
Responsiveness	Rapid	Slow	No response but awake	No response, fainted
Falls	No falls	Stumbles	Fall	Faint (fall with >3 s recovery)

BPM indicates breaths per minute.

post hoc tests. The number of falls and syncope score (Table 1) were analyzed with Kruskal-Wallis tests with Dunn post hoc.

ELISA and Western Blot

The raw data from ELISA (absorbance at 450 nm) was converted into concentration (pg/mL) using a standard curve generated from the standards included in the ELISA kit. ELISA data were analyzed using repeated-measures 2-way ANOVA with Sidak post hoc tests. For the Western blot data, first the band densities for every target protein were divided by the band density of β-actin for each lane (target protein density/β-actin density) (save for PKCε). For each gel, 2 to 3 lanes were used for samples of sham (for experiment 4) or vehicle-PC (for experiment 3) animals. The density ratios of these "control" lanes were averaged, and the density ratios of every lane were normalized to the average density ratio of the control. PKCε particulate and cytosolic fractions were loaded with 60 ng of protein each. Equal loadings were confirmed with Ponceau staining. The ratios of PKCε in the particulate and cytosolic fractions are reported. Western blot data were analyzed using 1-way ANOVA with Tukey post hoc tests.

Results

No mortality was observed in this study, and no animals were excluded from analysis. All statistical reports (ie, exact *P*-values) are provided in Tables S1 through S10. Additional experimental methods and results are included in Data S1. In preliminary experiments the effect of bilateral versus unilateral hindlimb RIPC for protection against sGVS-induced cardiovascular depression indicated that both unilateral and bilateral hindlimb RIPC were sufficient in providing protection against drops in mean arterial pressure, heart rate, and cerebral blood flow (Figure S1). The effects of the different cycles of RIPC were also investigated for any effect on mean arterial pressure, heart rate, and cerebral blood flow in preliminary experiments. Taken together, the various cycles of RIPC have limited effects on these 3 physiological parameters with the RIPC protocol described above (ie, 4 cycles of 10 minutes of ischemia/10 minutes of reperfusion) (Figures S2 and S3).

Experiment 1: RIPC Attenuates sGVS-Induced Lowerings of Blood Pressure, Heart Rate, and Cerebral Blood Flow

sGVS caused marked drops in mean arterial pressure, heart rate, and cerebral blood flow in vehicle-PC rats compared to those of sham animals (stimulation: $P<0.05$ sham versus vehicle PC then sGVS for all 3 physiological parameters). After stimulation is stopped, the mean arterial pressure and heart rate for vehicle-PC sGVS animals return to values statistically similar to those of the sham group. However, cerebral blood flow remained significantly lower than sham values (post-stimulation cerebral blood flow: $P<0.05$ sham versus vehicle PC then sGVS) (Figure 7, Table 2).

RIPC prevented the lowerings of mean arterial pressure, heart rate, and cerebral blood flow during sGVS such that these physiological parameters were significantly higher than vehicle-PC rats (stimulation mean arterial pressure: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS, $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (stimulation heart rate: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS, $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (stimulation cerebral blood flow: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS, $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS), and indistinguishable from those values of sham. After stimulation, the cerebral blood flow of RIPC sGVS rats remained significantly higher than that of vehicle-PC animals (poststimulation cerebral blood flow: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS, $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS).

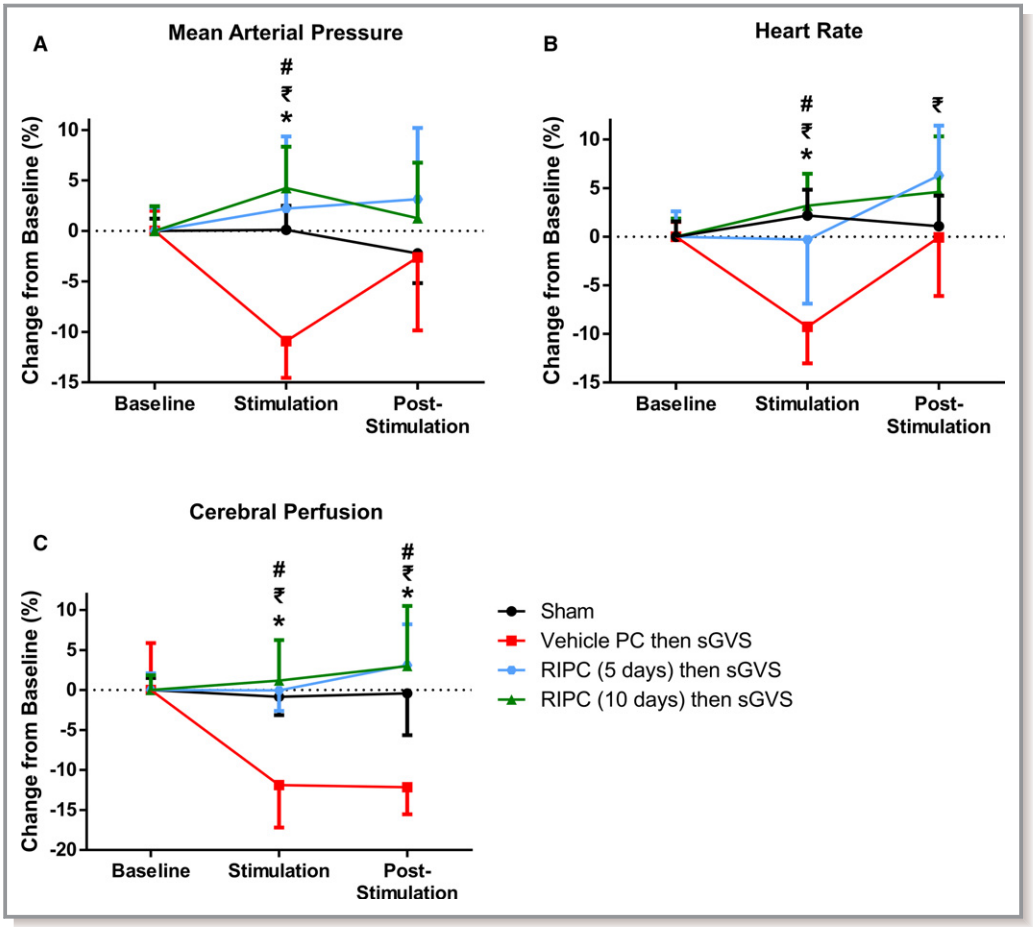


Figure 7. RIPC prevents sGVS-induced decreases in mean arterial pressure (A), heart rate (B), and cerebral blood flow (C). **P*<0.05 sham vs vehicle PC then sGVS, ₹*P*<0.05 vehicle PC then sGVS vs RIPC (5 days) then sGVS, #*P*<0.05 vehicle PC then sGVS vs RIPC (10 days) then sGVS. n=8/group. Repeated-measures 2-way ANOVA with Tukey post hoc. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

RIPC Protection Against sGVS in Female Rats

Female rats subjected to vehicle preconditioning then sGVS had significant drops in mean arterial pressure, heart rate, and cerebral blood flow compared to sham females (*P*<0.05 for all 3 physiological parameters during stimulation). RIPC in female rats attenuated the decreases in heart rate and cerebral blood flow caused by sGVS in vehicle-PC females (stimulation heart rate: *P*<0.05 vehicle PC then sGVS versus RIPC then sGVS) (stimulation cerebral blood flow: *P*<0.05 vehicle PC then sGVS versus RIPC then sGVS) but had only a marginal effect on the decreased mean arterial pressure (stimulation mean arterial pressure: *P*>0.05 sham versus RIPC then sGVS, *P*>0.05 vehicle PC then sGVS versus RIPC then sGVS) (Figure 8A through 8C, Table 2).

Sex Differences

Vehicle-PC female rats subjected to sGVS had significantly attenuated mean arterial pressure drop during stimulation compared to their male counterparts (Figure 9A, Table 2). No difference was observed between male and female rats for the heart rate drop during stimulation (Figure 9B). The response to cerebrovascular depression was significantly greater in female vehicle-PC than in male vehicle-PC rats (ie, vehicle-PC female rats had a greater drop in cerebral perfusion than vehicle-PC male rats) (Figure 9C).

Following 10 days of bilateral hindlimb RIPC, male rats subjected to sGVS have significantly higher mean arterial pressures (during stimulation) and heart rates (during and poststimulation) compared with their female counterparts (Figure 9D and 9E); however, no statistical difference was observed between the cerebral blood flows of male and female rats PC with RIPC before sGVS (Figure 9F).

RIPC Protection Against sGVS in Aged Male Rats

In aged male rats with vehicle PC then subjected to sGVS, statistically significant drops in mean arterial pressure, heart

Table 2. Mean (Standard Deviation) Reported Percentage Change From Baseline for the Physiological Parameters in Experiment 1

	Mean Arterial Pressure		Heart Rate		Cerebral Blood Flow	
	Stimulation	Poststimulation	Stimulation	Poststimulation	Stimulation	Poststimulation
Sham	0.1 (2.39)	−2.2 (2.96)	2.2 (2.65)	1.1 (3.16)	−0.8 (2.30)	−0.4 (5.23)
Vehicle PC then sGVS	−10.9 (3.64)	−2.6 (7.25)	−9.3 (3.76)	−0.1 (6.02)	−11.9 (5.32)	−12.1 (3.38)
RIPC (5 d) then sGVS	2.2 (7.14)	1.3 (5.50)	−0.3 (6.60)	6.3 (5.13)	0.0 (2.57)	3.1 (5.13)
RIPC (10 d) then sGVS	4.3 (4.08)	3.1 (7.07)	3.2 (3.29)	4.6 (5.71)	1.2 (5.07)	3.0 (7.51)
Female						
Sham	2.2 (1.4)	8.2 (2.24)	2.3 (2.57)	16.2 (7.83)	4.6 (7.89)	5.8 (6.68)
Vehicle PC then sGVS	−4.0 (1.50)	0.9 (5.79)	−8.6 (4.80)	5.4 (5.02)	−25.9 (7.98)	−19.5 (7.95)
RIPC then sGVS	−1.1 (5.30)	1.5 (3.23)	−1.8 (2.74)	−1.9 (2.74)	2.5 (7.15)	7.0 (10.43)
Aged male						
Sham	−0.3 (0.41)	−2.7 (4.03)	−0.3 (1.04)	−2.1 (5.58)	0.3 (1.70)	0.8 (5.94)
Vehicle PC then sGVS	−6.0 (3.36)	−0.4 (2.77)	−10.2 (2.97)	−8.1 (3.12)	−34.1 (6.18)	−27.1 (21.0)
RIPC then sGVS	−3.9 (3.89)	−3.2 (2.92)	−1.8 (2.84)	−2.2 (2.05)	0.2 (6.15)	1.9 (13.78)

Statistical analysis for all intergroup comparisons for the mean values are not reported here because they are reported in Figures 1 and 2. Exact *P*-values for the intergroup comparisons are reported in Tables S1 and S2. n=8/group. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

rate, and cerebral blood flow occur ($P<0.05$ for all 3 physiological parameters during stimulation). RIPC in aged males significantly attenuates the sGVS-induced lowering of heart rate and cerebral blood flow to values indistinguishable from those of sham (stimulation heart rate: $P<0.05$ vehicle PC then sGVS versus RIPC then sGVS) (stimulation cerebral blood flow: $P<0.05$ vehicle PC then sGVS versus RIPC then sGVS). RIPC had a marginal effect on the mean arterial pressure drop during stimulation (stimulation mean arterial pressure: $P>0.05$ sham versus RIPC then sGVS, $P>0.05$ vehicle PC then sGVS versus RIPC then sGVS) (Figure 8D through 8F, Table 2).

Age Differences

sGVS in aged male rats (receiving vehicle PC) leads to significantly less mean arterial pressure depression compared to that of young male rats (Figure 10A, Table 2). No difference was observed in the heart rate between young and aged rats (subjected to vehicle PC) during sGVS (Figure 10B). Despite no difference in the heart rate lowering and less mean arterial pressure drop during sGVS, aged male rats (receiving vehicle PC) had a greater drop in cerebral blood flow during stimulation than young males (Figure 10C). When subjected to RIPC, young male rats had significantly higher mean arterial pressures and heart rates than aged male rats (Figure 10D and 10E) but no difference in cerebral blood flow (Figure 10F).

Lasting Protection by RIPC Against sGVS

When animals are subjected to sGVS 10 days after completing preconditioning (Figure S4), a 10-day period of RIPC continues to provide protection against the reductions in mean arterial pressure, heart rate, and cerebral blood flow during sGVS compared to vehicle-PC animals (stimulation mean arterial pressure: $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (stimulation heart rate: $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (stimulation cerebral blood flow: $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS). Post-sGVS, the mean arterial pressure, heart rate, and cerebral blood flow remained significantly different between vehicle-PC animals and rats receiving 10 days of RIPC (stimulation mean arterial pressure: $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (stimulation heart rate: $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (stimulation cerebral blood flow: $P<0.05$ vehicle PC then sGVS versus RIPC [10 days] then sGVS) (Figure S5).

A 5-day period of RIPC continues to provide protection against sGVS-induced lowerings of mean arterial pressure and heart rate (stimulation mean arterial pressure: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS; poststimulation mean arterial pressure: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS) (stimulation heart rate: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS; poststimulation heart rate: $P<0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS) but does not prevent sGVS-induced lowering of cerebral blood flow (stimulation cerebral blood flow: $P>0.05$ vehicle PC then sGVS versus RIPC [5 days] then sGVS).

Experiment 2: RIPC Protects Against sGVS in Awake Rats

Rats receiving vehicle PC before sGVS exhibit behavioral changes during sGVS that are similar to those observed in VVS

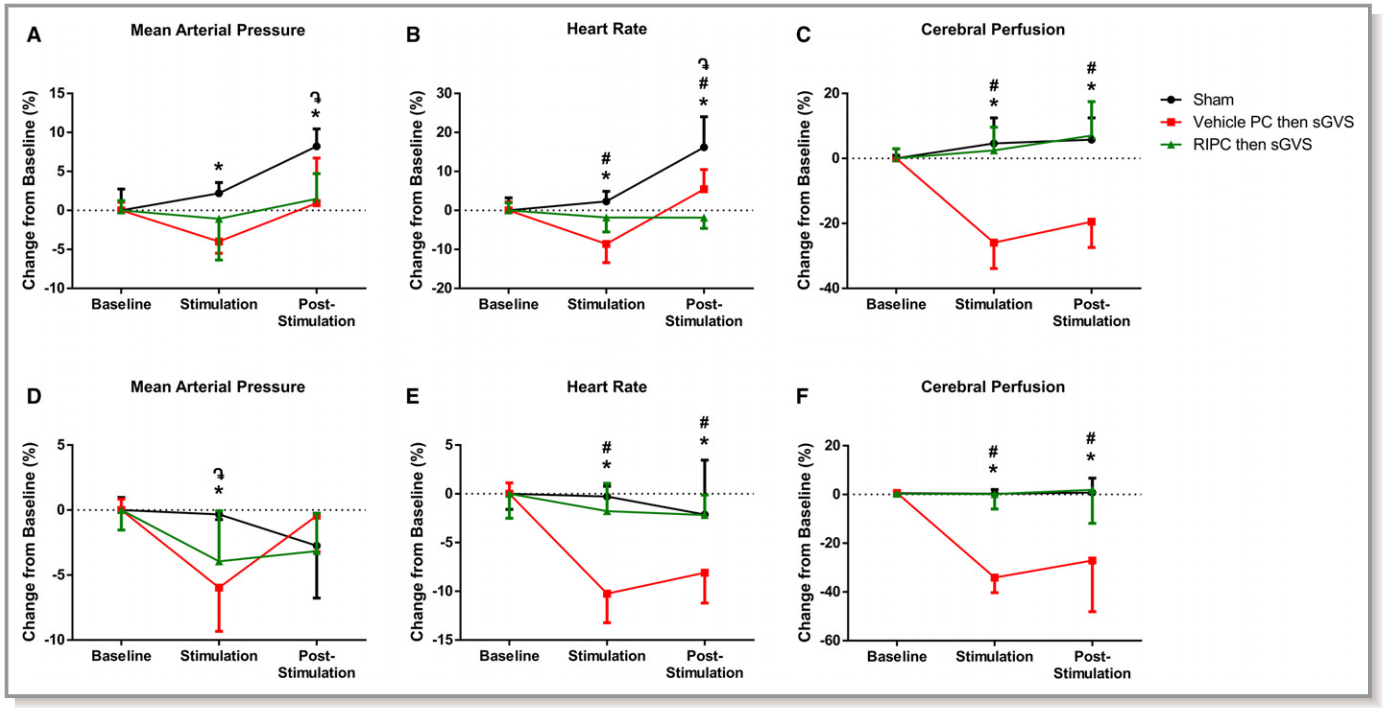


Figure 8. RIPC affords protection to females (A through C) and aged males (D through F) against sGVS. **P*<0.05 sham vs vehicle PC then sGVS, ϡ*P*<0.05 sham vs RIPC then sGVS, #*P*<0.05 vehicle PC then sGVS vs RIPC then sGVS. n=8/group. Repeated-measures 2-way ANOVA with Tukey post hoc. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

patients; sGVS causes a marked decrease in breathing rate and significant increases in the number of falls and syncope score, as well as longer time to recover from sGVS (Figure 11, Table 3). RIPC before sGVS in awake animals attenuates sGVS-induced behavioral changes such that the behavior of RIPC rats is not statistically different from that of sham animals.

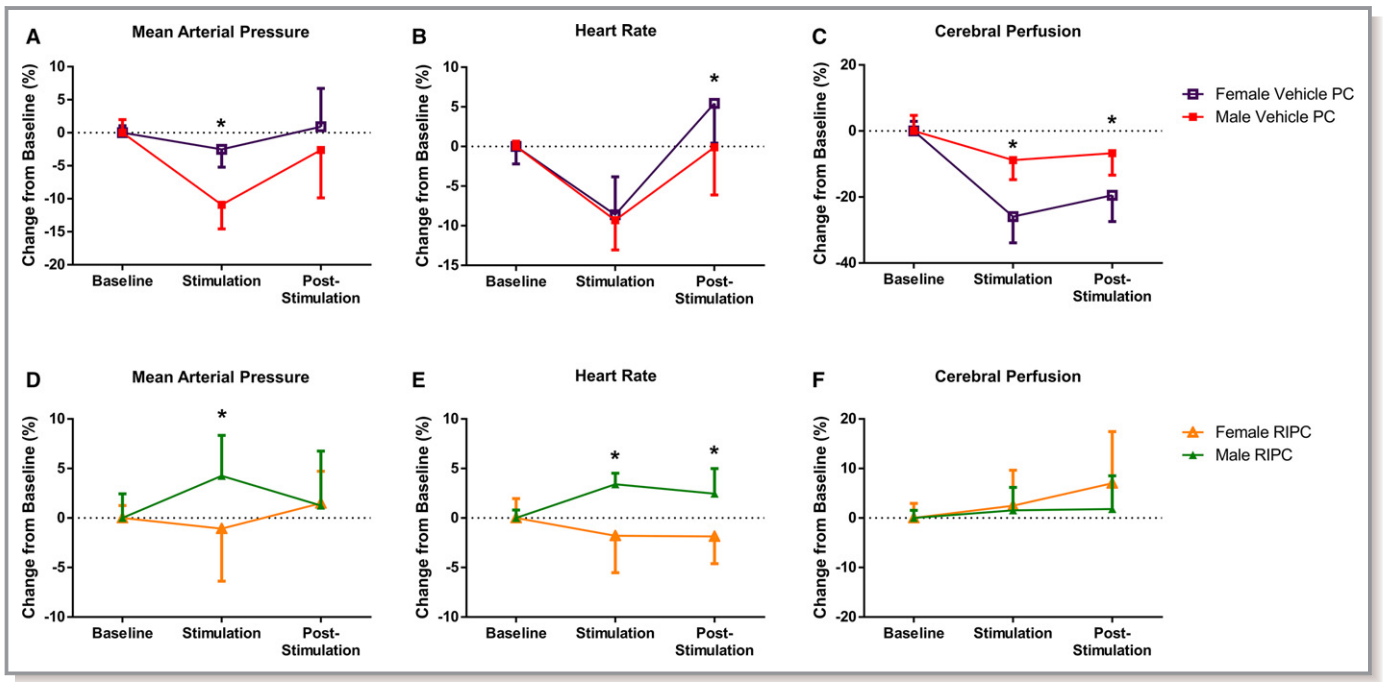


Figure 9. Sex differences in response to sGVS. sGVS was performed after completing vehicle preconditioning (A through C) or remote limb ischemic preconditioning (RIPC) (D through F). **P*<0.05 between the 2 groups at the same time point. n=8/group. Repeated-measures 2-way ANOVA with Sidak post hoc. PC indicates preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

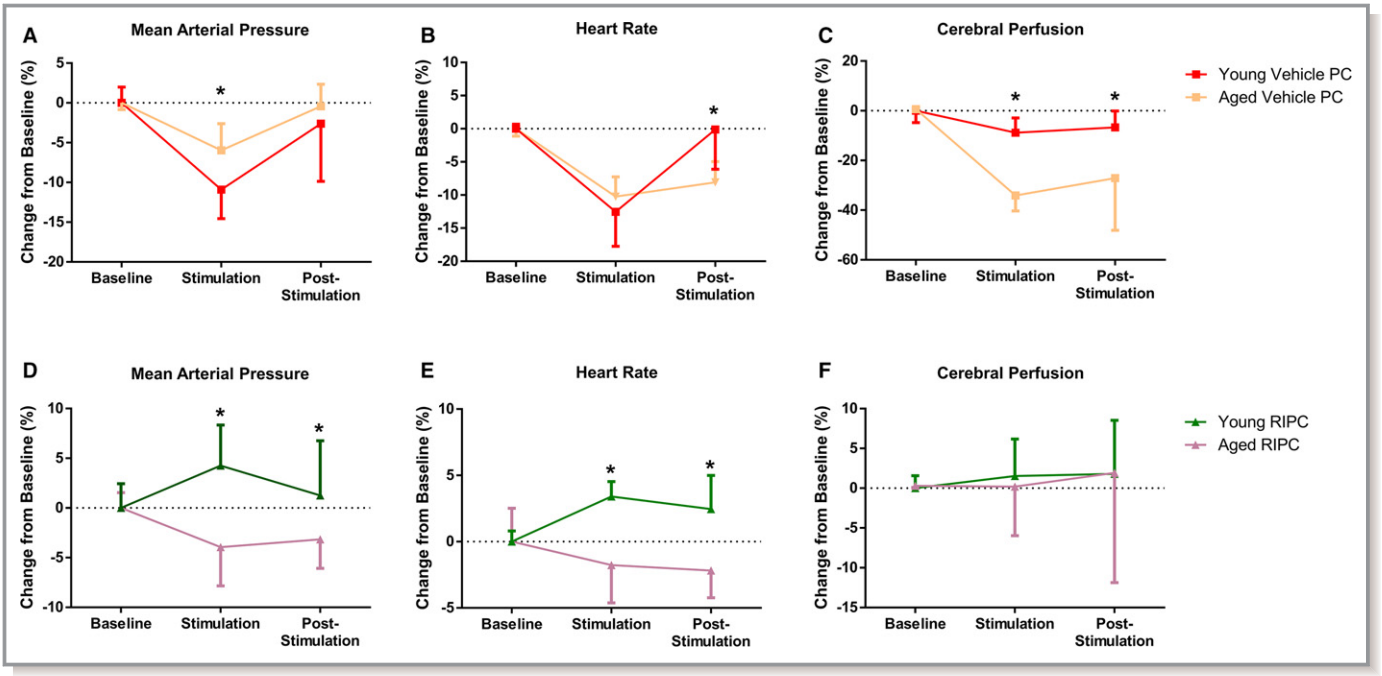


Figure 10. Age differences in response to sGVS. sGVS was performed after completing vehicle preconditioning (A through C) or remote limb ischemic preconditioning (RIPC) (D through F). *P<0.05 between the 2 groups at the same time point. n=8/group. Repeated-measures 2-way ANOVA with Sidak post hoc. PC indicates preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

Experiment 3: RIPC Causes a Surge in Serum Norepinephrine, Leading to Upregulated α_1-Adrenoceptor and Reduced NET1 in the Brain

Serum collected on the first day of PC indicated elevated norepinephrine levels in RIPC rats compared to vehicle-PC rats (P<0.05 for all time-points post-PC). Serum epinephrine was also higher in RIPC rats compared with vehicle-PC animals (P<0.05 for 0 and 60 minutes post-PC) (Figure 12A and 12B).

On the final (10th) day of preconditioning, serum norepinephrine levels were statistically different between the RIPC and vehicle-PC rats at 60 minutes post-PC (P<0.05 vehicle PC versus RIPC). Serum epinephrine was not significantly different between RIPC rats and vehicle-PC rats (Figure 12C and 12D).

The pan-adrenoceptor antagonist labetalol given before RIPC did not significantly attenuate the elevation of serum norepinephrine caused by RIPC on the first day of preconditioning. Labetalol led to increased serum epinephrine compared to RIPC alone on the first day. No changes were observed for labetalol administration with respect to either catecholamine on the last day of PC (Figure S6).

On the final day of PC, compared to vehicle-PC rats, animals subjected to RIPC had a significantly higher brain expression of α_1-adrenoceptor (P<0.05), no change in the brain expression of β_1-adrenoceptor (P>0.05), and a significantly lower brain expression of NET1 (P<0.05) (Figure 13, Figure S7). Labetalol significantly attenuated α_1-adrenoceptor and NET1 brain expressions after RIPC (Figure S8).

Experiment 4: Antagonizing Adrenoceptors and NET1 Reverses RIPC Protection Against sGVS

Effects of Adrenoceptor Antagonism on sGVS-Induced Cardio- and Cerebrovascular Depression

The pan-adrenoceptor antagonist labetalol, administered during RIPC, did not reverse RIPC's protection against mean arterial pressure nor heart rate sGVS-induced depressions (stimulation mean arterial pressure: P<0.05 [vehicle PC+saline] then sGVS versus [RIPC+labetalol] then sGVS) (stimulation heart rate: P<0.05 [vehicle PC+saline] then sGVS versus [RIPC+labetalol] then sGVS). However, labetalol given during RIPC completely reversed the cerebral blood flow protection by RIPC (stimulation cerebral blood flow: P<0.05 sham versus [RIPC+labetalol] then sGVS, P>0.05 [vehicle PC+saline] then sGVS versus [RIPC+labetalol] then sGVS, P<0.05 [RIPC+saline] then sGVS versus [RIPC+labetalol] then sGVS) (Figure 14A through 14C, Tables 4 through 6).

Antagonism of α_1-adrenoceptor during RIPC partially reversed RIPC protection of mean arterial pressure sGVS-induced depression (stimulation mean arterial pressure: P>0.05 [vehicle PC+saline] then sGVS versus [RIPC+doxazosin] then sGVS, P<0.05 [RIPC+saline] then sGVS versus [RIPC+doxazosin] then sGVS) and completely reverses the

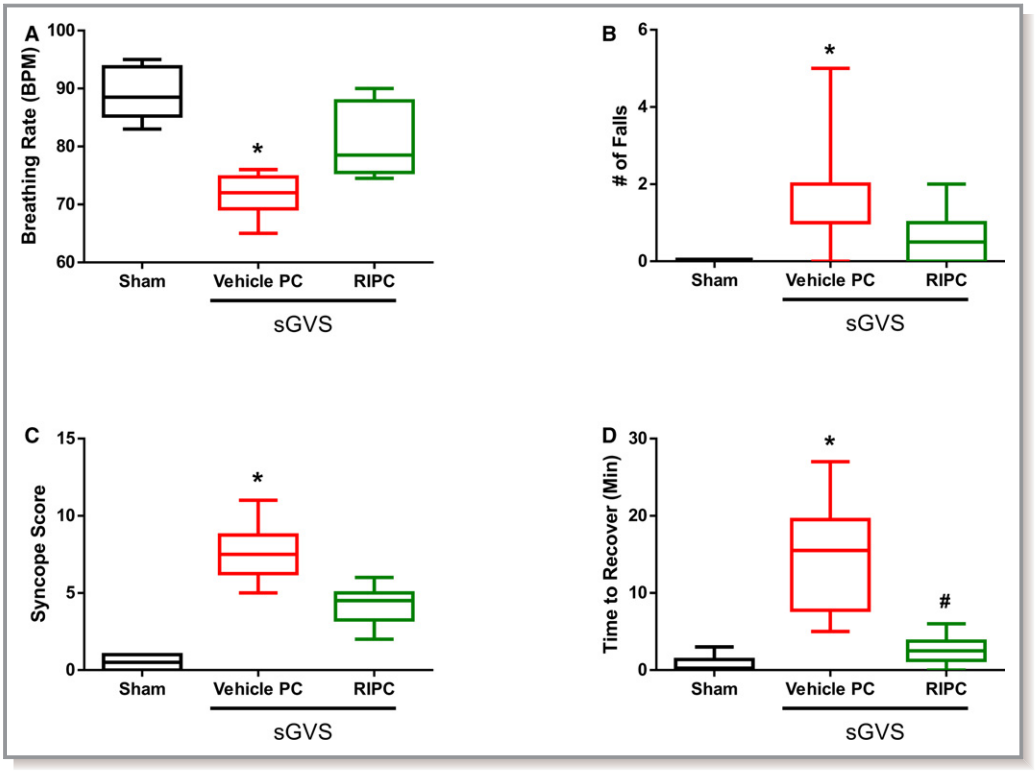


Figure 11. RIPC affords protection against sGVS in awake rats. A, Rate of breathing (breathes per minute, BPM) during sGVS. Kruskal-Wallis test with Dunn post hoc. B, Number of falls/stumbles during sGVS. Kruskal-Wallis test with Dunn post hoc. C, Syncope score during sGVS (Table 1 for scoring criteria). Kruskal-Wallis test with Dunn post hoc. D, Time to recover (minutes) after stopping sGVS. One-way ANOVA with Tukey post hoc. *P<0.05 sham vs vehicle PC then sGVS, $^{\#}P$<0.05 vehicle PC then sGVS vs RIPC then sGVS. n=8/group. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

protection by RIPC on heart rate and cerebral blood flow lowerings during sGVS (stimulation heart rate: P<0.05 sham versus [RIPC+doxazosin] then sGVS, P>0.05 [vehicle PC+saline] then sGVS versus [RIPC+doxazosin] then sGVS, P<0.05 [RIPC+saline] then sGVS versus [RIPC+doxazosin] then sGVS) (stimulation cerebral blood flow: P<0.05 sham versus [RIPC+doxazosin] then sGVS, P>0.05 [vehicle PC+saline] then sGVS versus [RIPC+doxazosin] then sGVS, P<0.05 [RIPC+saline] then sGVS versus [RIPC+doxazosin] then sGVS) (Figure 14A through 14C).

Table 3. Mean (Standard Deviation) Results for the Behavioral Tests in Experiment 3

	Breathing Rate	Number of Falls	Syncope Score	Time to Recovery
Sham	89 (4.5)	0 (0.0)	0.5 (0.53)	0.7 (1.07)
Vehicle PC then sGVS	72 (3.6)	1.9 (1.46)	7.6 (1.85)	14 (7.2)
RIPC then sGVS	81 (6.2)	0.6 (0.74)	4.3 (1.28)	2.6 (1.85)

Exact P-values for the intergroup comparisons are reported in Table S3. n=8/group. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

When a β_1-adrenoceptor antagonist is given during RIPC, the protection afforded by RIPC against sGVS-induced mean arterial pressure and heart rate drops is reversed (stimulation mean arterial pressure: P>0.05 [vehicle PC+saline] then sGVS versus [RIPC+atenolol] then sGVS, P<0.05 [RIPC+saline] then sGVS versus [RIPC+atenolol] then sGVS) (stimulation heart rate: P<0.05 sham versus [RIPC+atenolol] then sGVS), P>0.05 [vehicle PC+saline] then sGVS versus [RIPC+atenolol] then sGVS, P<0.05 [RIPC+saline] then sGVS versus [RIPC+atenolol] then sGVS). However, antagonism of β_1-adrenoceptors during RIPC does not prevent RIPC protection of cerebral blood flow depression caused by sGVS (stimulation cerebral blood flow: P>0.05 sham versus [RIPC+atenolol] then sGVS, P>0.05 [RIPC+saline] then sGVS versus [RIPC+atenolol] then sGVS) (Figure 14A through 14C).

Effects of NET1 Antagonism on sGVS-Induced Cardio- and Cerebro-vascular Depressions

Intranasal administration of a NET1 antagonist during RIPC prevented RIPC protection against sGVS-induced lowerings of mean arterial pressure, heart rate, and cerebral blood flow

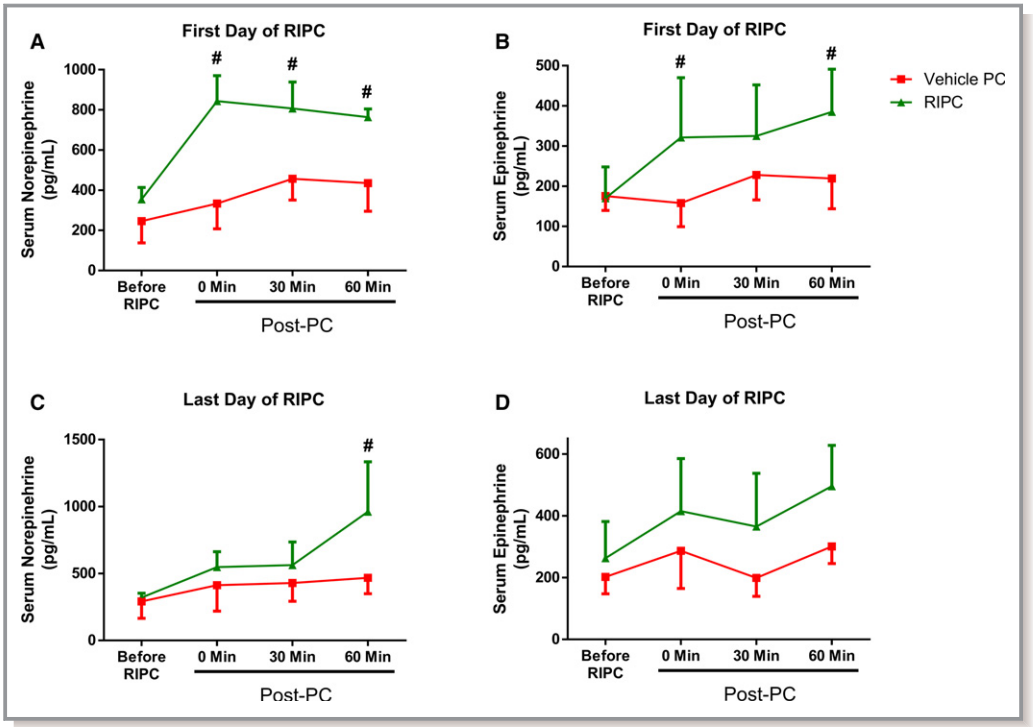


Figure 12. Serum catecholamine response after RIPC. A and B, On the first day of preconditioning, norepinephrine and epinephrine are elevated after RIPC. C and D, Last (10th) day of preconditioning. #$P<0.05$ vehicle PC then sGVS vs RIPC then sGVS. n=7/group. Repeated-measures 2-way ANOVA with Sidak post hoc. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

(stimulation mean arterial pressure: $P>0.05$ [vehicle PC+saline] then sGVS versus [RIPC+desipramine] then sGVS, $P<0.05$ [RIPC+saline] then sGVS versus [RIPC+desipramine] then sGVS) (stimulation heart rate: $P>0.05$ [vehicle PC+saline] then sGVS versus [RIPC+desipramine] then sGVS, $P<0.05$ [RIPC+saline] then sGVS versus [RIPC+desipramine] then sGVS) (stimulation cerebral blood flow: $P>0.05$ [vehicle PC+saline] then sGVS versus [RIPC+desipramine] then sGVS,

Table 4. Mean (Standard Deviation) Reported Percentage Change From Baseline for the Mean Arterial Pressure in Experiment 4

	Stimulation	Poststimulation
Sham	−0.3 (3.55)	−1.9 (3.30)
(Vehicle PC+IV saline) then sGVS	−8.9 (4.29)	−3.7 (6.78)
(RIPC+IV saline) then sGVS	6.0 (4.71)	4.5 (5.15)
(RIPC+labetalol) then sGVS	3.7 (3.24)	0.9 (7.05)
(RIPC+doxazosin) then sGVS	−2.9 (6.11)	0.7 (6.29)
(RIPC+atenolol) then sGVS	−5.5 (2.58)	1.4 (8.27)
Sham	0.0 (3.66)	−0.5 (3.13)
(Vehicle PC+IN saline) then sGVS	−9.7 (4.85)	−3.2 (6.11)
(RIPC+IN saline) then sGVS	3.2 (5.13)	3.2 (4.16)
(RIPC+desipramine) then sGVS	−12.4 (13.83)	6.7 (5.42)

Exact *P*-values for the intergroup comparisons are reported in Table S5. n=8/group. IN indicates intranasal; IV, intravenous; PC, preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

Table 5. Mean (Standard Deviation) Reported Percentage Change From Baseline for Heart Rate in Experiment 4

	Stimulation	Poststimulation
Sham	2.9 (4.11)	1.7 (4.73)
(Vehicle PC+IV saline) then sGVS	−10.0 (4.65)	−3.3 (6.95)
(RIPC+IV saline) then sGVS	2.8 (1.80)	3.1 (2.05)
(RIPC+labetalol) then sGVS	−0.3 (0.62)	−1.1 (3.54)
(RIPC+doxazosin) then sGVS	−3.4 (1.29)	−6.4 (5.38)
(RIPC+atenolol) then sGVS	−5.7 (2.34)	0.3 (4.16)
Sham	1.2 (2.75)	2.0 (4.15)
(Vehicle PC+IN saline) then sGVS	−12.5 (5.21)	−4.8 (5.41)
(RIPC+IN saline) then sGVS	3.4 (1.12)	2.5 (2.53)
(RIPC+desipramine) then sGVS	−8.9 (4.27)	−1.5 (8.68)

Exact *P*-values for the intergroup comparisons are reported in Table S5. n=8/group. IN indicates intranasal; IV, intravenous; PC, preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

Table 6. Mean (Standard Deviation) Reported Percentage Change From Baseline for Cerebral Blood Flow in Experiment 4

	Stimulation	Poststimulation
Sham	0.8 (2.42)	2.0 (3.89)
(Vehicle PC+IV saline) then sGVS	−10.2 (5.23)	−10.8 (4.38)
(RIPC+IV saline) then sGVS	1.9 (4.23)	0.6 (5.95)
(RIPC+labetalol) then sGVS	−9.7 (9.64)	−9.8 (15.19)
(RIPC+doxazosin) then sGVS	−18.4 (8.80)	−10.9 (16.29)
(RIPC+atenolol) then sGVS	−2.0 (5.83)	−0.9 (8.14)
Sham	0.0 (2.36)	0.8 (4.56)
(Vehicle PC+IN saline) then sGVS	−8.8 (5.89)	−6.7 (6.63)
(RIPC+IN saline) then sGVS	1.5 (4.65)	1.8 (6.73)
(RIPC+desipramine) then sGVS	−14.9 (5.89)	−7.4 (5.54)

Exact *P*-values for the intergroup comparisons are reported in Table S6. n=8/group. IN indicates intranasal; IV, intravenous; PC, preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

P<0.05 [RIPC+saline] then sGVS versus [RIPC+desipramine] then sGVS) (Figure 14D through 14F).

Brain Expression of Adrenoceptors, NET1, PKCε, and eNOS After sGVS

After sGVS, rats subjected to RIPC have a significantly higher level of α_1-adrenoceptor, particulate PKCε/cytosolic PKCε, and phospho-eNOS/eNOS in the brain compared to sham and vehicle-PC animals (α_1-adrenoceptor: *P*<0.05, particulate PKCε/cytosolic PKCε: *P*<0.05, phospho-eNOS/eNOS: *P*<0.05). No difference in the brain expressions of β_1-adrenoceptor or NET1 were observed among the sham, vehicle-PC, and RIPC animals (Figure 15, Figure S9). The same expressions are observed in Figure 16 and Figure S10.

When an α_1-adrenoceptor antagonist is administered during RIPC, the brain expressions of α_1-adrenoceptor, particulate PKCε/cytosolic PKCε, and phospho-eNOS/eNOS are returned to sham values (α_1-adrenoceptor: *P*<0.05 [RIPC+saline] then sGVS versus [RIPC+doxazosin] then sGVS, particulate PKCε/cytosolic PKCε: *P*<0.05 [RIPC+saline] then sGVS versus [RIPC+doxazosin] then sGVS, phospho-eNOS/eNOS: *P*<0.05 [RIPC+saline] then sGVS versus [RIPC+doxazosin] then sGVS). No effect on the brain expression of β_1-adrenoceptor was observed between RIPC with doxazosin and sham, vehicle-PC, or RIPC animals. Brain expression of NET1 is elevated in animals subjected to RIPC with doxazosin compared to that of sham, vehicle-PC, and RIPC animals (*P*<0.05) (Figure 15).

Antagonism of β_1-adrenoceptor during RIPC causes the brain expressions of α_1-adrenoceptor, particulate PKCε/cytosolic PKCε, and phospho-eNOS/eNOS to be returned to sham values (α_1-adrenoceptor: *P*<0.05 [RIPC+saline] then sGVS versus [RIPC+atenolol] then sGVS, particulate PKCε/cytosolic PKCε: *P*<0.05 [RIPC+saline] then sGVS versus [RIPC+atenolol] then sGVS, phospho-eNOS/eNOS: *P*<0.05 [RIPC+saline] then sGVS versus [RIPC+atenolol] then sGVS). No effect on β_1-adrenoceptor brain expression was observed between rats subjected to RIPC with atenolol and either sham, vehicle-PC, or RIPC animals. NET1 brain expression is increased in RIPC with atenolol animals compared to that of sham, vehicle-PC, and RIPC animals (*P*<0.05) (Figure 15).

Intranasal administration of a NET1 inhibitor during RIPC causes decreased brain expressions of α_1-adrenoceptor,

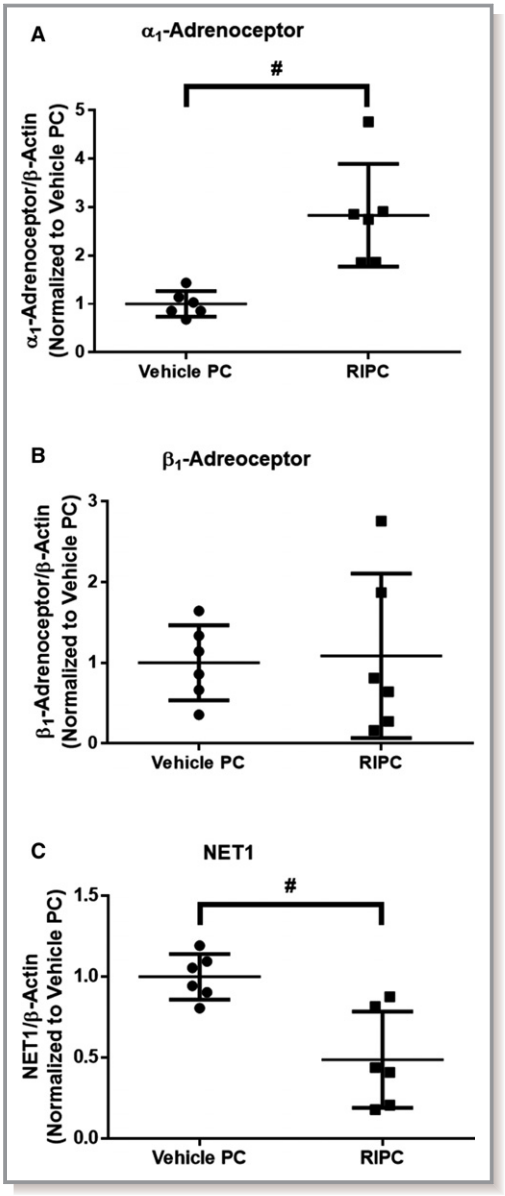


Figure 13. RIPC elevates brain α_1-adrenoceptor and reduces NET1. A, α_1-Adrenoceptor expression. B, β_1-Adrenoceptor expression. C, NET1 expression. #*P*<0.05. n=6/group. Unpaired t-test. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning.

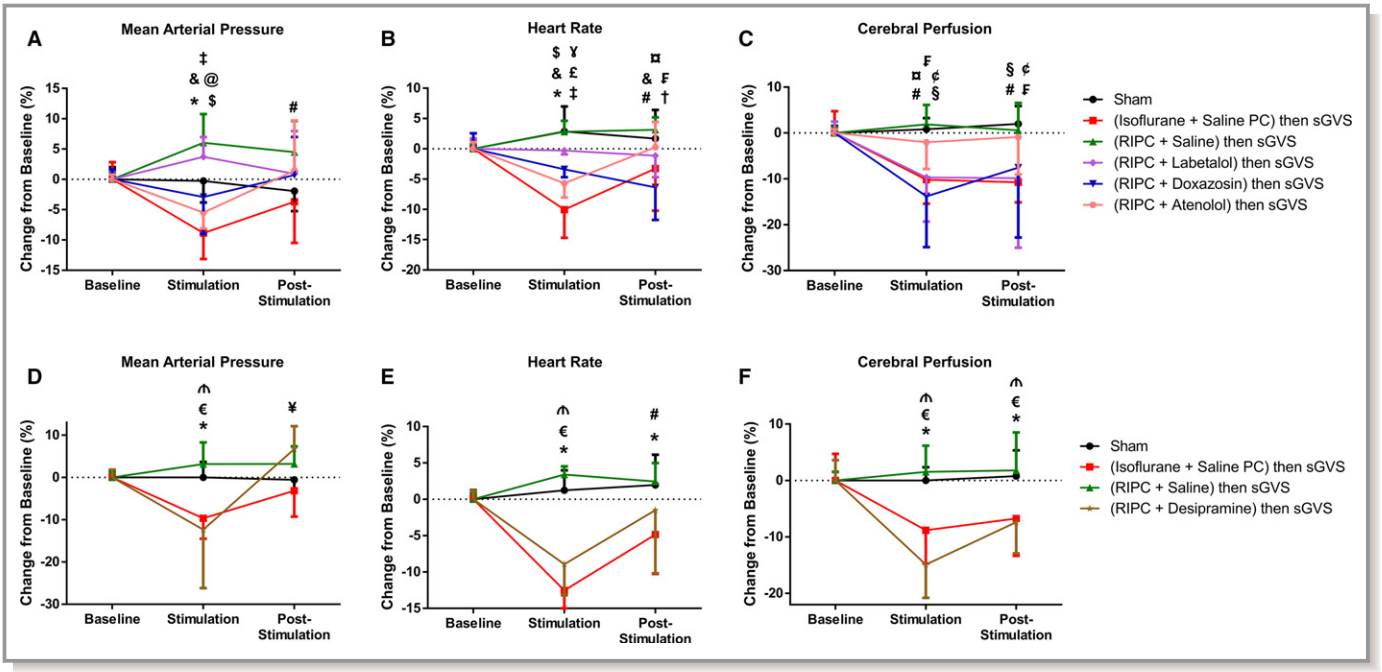


Figure 14. Antagonizing adrenoceptors and NET1 reverses RIPC protection against sGVS. A through C, Antagonism of α_1-adrenoceptor prevents RIPC protection against heart rate and cerebral blood flow drops and partially reverses RIPC benefits on mean arterial pressure. Inhibition of β_1-adrenoceptor reverses RIPC protection of decreased heart rate, partially attenuates RIPC benefits on mean arterial pressure, and has no effect on RIPC benefits on cerebral blood flow. D through F, NET1 antagonism reverses RIPC therapeutic effects of sGVS cardio- and cerebro-vascular depressions. $^{*}P<0.05$ sham vs (vehicle PC+saline) then sGVS, $^{£}P<0.05$ sham vs (RIPC+doxazosin) then sGVS and (RIPC+atenolol) then sGVS, $^{¥}P<0.05$ sham vs (RIPC+doxazosin) then sGVS, $^{§}P<0.05$ sham vs (vehicle PC+saline) then sGVS and (RIPC+labetalol) then sGVS, $^{€}P<0.05$ sham vs (RIPC+desipramine) then sGVS, $^{\#}P<0.05$ (vehicle PC+saline) then sGVS vs (RIPC+saline) then sGVS, $^{@}P<0.05$ (vehicle PC+saline) then sGVS vs (RIPC+saline) then sGVS and (RIPC+labetalol) then sGVS, $^{¥}P<0.05$ (vehicle PC+Saline) then sGVS vs (RIPC+saline) then sGVS and (RIPC+labetalol) then sGVS and (RIPC+doxazosin) then sGVS, $^{¥}P<0.05$ (vehicle PC+saline) then sGVS vs (RIPC+desipramine) then sGVS, $^{\&}P<0.05$ (RIPC+saline) then sGVS vs (RIPC+doxazosin) then sGVS, $^{\$}P<0.05$ (RIPC+saline) then sGVS vs (RIPC+atenolol) then sGVS, $^{¢}P<0.05$ (RIPC+saline) then sGVS vs (RIPC+labetalol) then sGVS and (RIPC+doxazosin) then sGVS, $^{ṁ}P>0.05$ (RIPC+saline) then sGVS vs (vehicle PC+saline) then sGVS and (RIPC+desipramine) then sGVS, $^{\dagger}P<0.05$ (RIPC+labetalol) then sGVS vs (RIPC+doxazosin) then sGVS, $^{‡}P<0.05$ (RIPC+labetalol) then sGVS vs (RIPC+atenolol) then sGVS, and $^{¤}P<0.05$ (RIPC+doxazosin) then sGVS vs (RIPC+atenolol) then sGVS. n=8/group. Repeated-measures 2-way ANOVA with Tukey post hoc. PC indicates preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

particulate PKCε/cytosolic PKCε, and phospho-eNOS/eNOS compared to RIPC animals (α_1-adrenoceptor: $P<0.05$ [RIPC+saline] then sGVS versus [RIPC+desipramine] then sGVS, particulate PKCε/cytosolic PKCε: $P<0.05$ [RIPC+saline] then sGVS versus [RIPC+desipramine] then sGVS, phospho-eNOS/eNOS: $P<0.05$ [RIPC+saline] then sGVS versus [RIPC+desipramine] then sGVS). No effect on β_1-adrenoceptor brain expression was observed in RIPC rats receiving desipramine. NET1 inhibition during RIPC caused reduced expression of NET1 compared to sham and vehicle-PC animals ($P<0.05$) (Figure 16).

Discussion

VVS is the transient loss of consciousness caused by depressed blood pressure, heart rate, and cerebral perfusion.[1] The rat model of VVS using sGVS mimics the primary characteristics of VVS in humans.[14] To date, the treatments available for VVS have only targeted a single facet of the VVS pathophysiology, namely the cardiovascular depression. Here we hypothesized that RIPC would not only attenuate the cardiovascular depression observed during VVS but also prevent cerebral hypoperfusion. The data provided within support our primary hypothesis. Several key observations were found in this study that have not, to our knowledge, been reported in literature: (1) RIPC affords protection against the lowering of mean arterial pressure, heart rate, and cerebral blood flow in rats subjected to sGVS; (2) norepinephrine increases in response to RIPC, which leads to increased α_1-adrenoceptor and decreased NET1 in the brain; (3) norepinephrine is a critical mediator for RIPC protection against sGVS; and (4) adrenoceptors are responsible for brain and cardioprotection against sGVS.

In our first experiment, RIPC was found to protect the heart against bradycardia, protect against hypotension, and also prevent cerebral blood flow lowering. We also observed RIPC

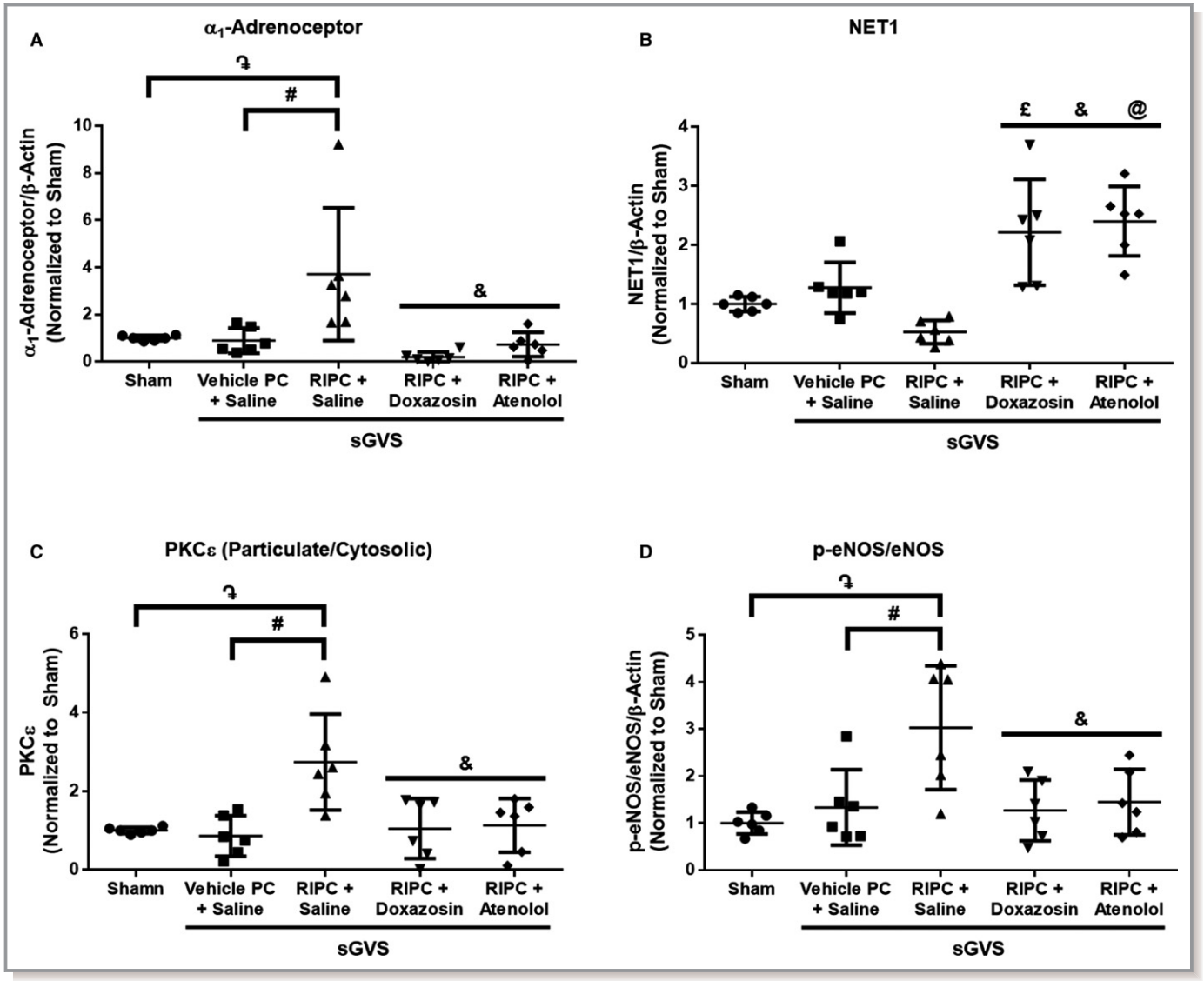


Figure 15. Adrenoceptor antagonism prevents RIPC-induced changes in brain expression of α_1-arenoceptor (A), NET1 (B), particulate PKCε/cytosolic PKCε (C), and p-eNOS/eNOS (D). $^{\wr}P<0.05$, $^{\#}P<0.05$, $^{£}P<0.05$ vs sham, $^{@}P<0.05$ vs (vehicle PC+saline) then sGVS, $^{\&}P<0.05$ vs (RIPC+saline) then sGVS. n=6/group. One-way ANOVA with Tukey post hoc. PC indicates preconditioning; p-eNOS, phospho-eNOS; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

protection against sGVS in young and aged males, as well as in young females. Furthermore, in awake rats subjected to sGVS, RIPC was found to reduce the behavioral changes associated with sGVS. Interestingly, animals receiving vehicle PC then subjected to sGVS exhibit vasovagal syncope-like behavior for about 15 minutes poststimulation (Figure 11D). This observation follows a similar timing as that which it takes for cerebral blood flow to begin to return to baseline values. In our previous study we found that cerebral begins to recover between 15 to 30 minutes poststimulation.[14] Clinically, vasovagal patients (up to 75% of patients) experience postsyncopal behavioral changes, including fatigue and lethargy, light-headedness, disorientation, nausea, confusion, palpitations, and altered mental status.[15,16] In humans the post-ictal behavioral changes typically recovery within a couple minutes from the faint, however, recovery is dependent on the length of the syncopal episode.[16]

Sex Differences in Response to sGVS

Vehicle-PC female rats subjected to sGVS had significantly attenuated mean arterial pressure and heart rate drops during stimulation compared to their male counterparts (Figure 9A and 9B). The reduced responsiveness to mean arterial pressure and heart rate lowerings may be due to sex differences in the peripheral and cardiac localization, density, and/or sensitivity of adrenergic receptors. These differences have been reported in rats[17,18] and rabbits,[19] as well as in

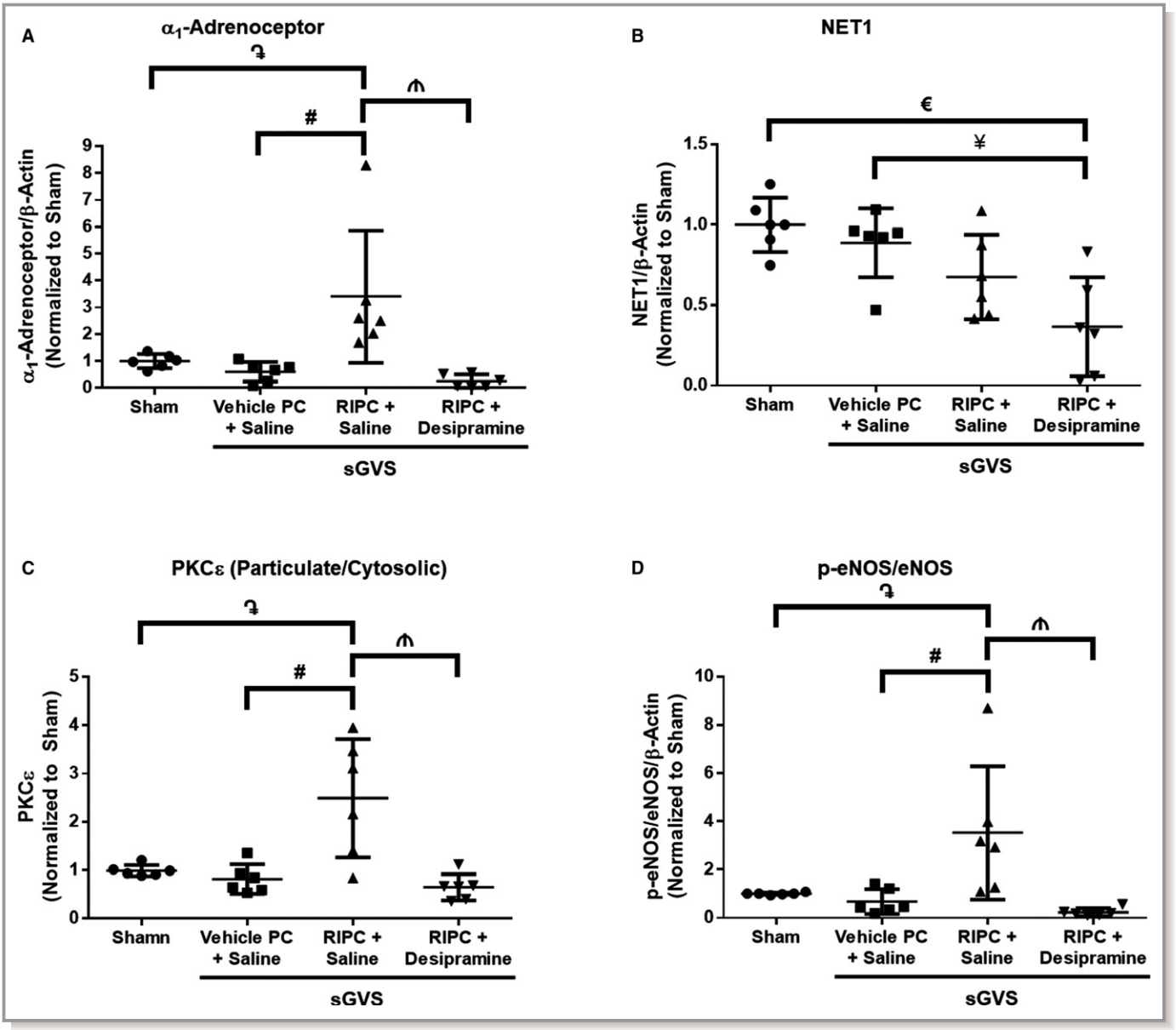


Figure 16. NET1 antagonism prevents RIPC-induced changes in brain expression of α_1-adrenoceptor (A), NET1 (B), particulate PKCε/cytosolic PKCε (C), and p-eNOS/eNOS (D). $^{\text{ϡ}}P<0.05$, $^{\#}P<0.05$, $^{\text{ⓜ}}P<0.05$, $^{€}P<0.05$, $^{¥}P<0.05$. n=6/group. One-way ANOVA with Tukey post hoc. PC indicates preconditioning; p-eNOS, phospho-eNOS; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

humans.[20,21] Another possible reason for the sex differences in the mean arterial pressure and heart rate lowerings is that female rat hearts have higher (by ≈2-fold) PKCε expression than males,[22] and PKCε is well documented to form complexes with both Akt and eNOS, as well as mitogen-activated protein kinases (such as ERKs, JNKs, p38MAPK) and components of the mitochondrial permeability transition pore (ie, VDAC, ANT, HKII).[23] The former complex (PKCε/Akt/eNOS) may signal to the mKATP channel to confer cardioprotection.[24] The latter complex in the mitrochondria likely suppresses generation of reactive oxygen species generation by α-ketoglutarate dehydrogenase and removal of aldehydes by aldehyde dehydrogenase 2.[22] However, the specific roles PKCε plays in the sex differences for cardiovascular depression caused by sGVS remains to be studied.

Interestingly, although female rats had less cardiovascular depression due to sGVS, the response to cerebrovascular depression was significantly greater than the vehicle-PC male rats (ie, vehicle-PC female rats had a greater drop in cerebral perfusion than vehicle-PC male rats) (Figure 9C). Previous studies have reported little to no difference in the brain affinity of β-adrenoceptors in rats, but the response of brain α_2-adrenoceptors is different between the sexes.[25] Additionally, there is still a debate on whether sex differences exist or

Table 7. Minimum Values of Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow During Stimulation

	Mean Arterial Pressure	Heart Rate	Cerebral Blood Flow
Experiment 1			
Young Male			
Sham	−1.8 (4.42)	0.3 (2.72)	−2.6 (4.44)
Vehicle PC then sGVS	−22.6 (3.2)*	−23.4 (4.66)*	−20.8 (11.72)*
RIPC (5 d) then sGVS	−5.0 (12.50)†	−3.5 (10.04)†	−3.5 (2.82)†
RIPC (10 d) then sGVS	2.0 (4.19)†	1.9 (3.15)†	−2.0 (2.69)†
Female			
Sham	0.0 (1.92)	−2.8 (1.13)	2.0 (7.76)
Vehicle PC then sGVS	−22.1 (21.58)*	−46.3 (21.39)*	−49.2 (12.18)*
RIPC then sGVS	−17.2 (12.67)	−23.3 (14.76)†	−11.1 (12.4)†
Aged male			
Sham	−0.83 (0.54)	−1.9 (1.21)	−4.4 (3.39)
Vehicle PC then sGVS	−20.1 (11.16)*	−16.3 (5.12)*	−66.6 (8.35)*
RIPC then sGVS	−11.8 (11.57)	−8.9 (11.93)	−5.1 (7.72)†
Experiment 3			
Sham	−1.5 (2.90)	1.4 (2.63)	−1.6 (2.09)
(Vehicle PC+IV saline) then sGVS	−16.3 (8.77)*	−18.4 (12.27)*	−26.0 (14.28)*
(RIPC+IV saline) then sGVS	3.1 (3.55)†	1.6 (0.88)†	−4.6 (7.19)
(RIPC+labetalol) then sGVS	−7.6 (3.43)‡	−15.9 (11.45)*,‡	−18.9 (16.62)
(RIPC+doxazosin) then sGVS	−19.1 (7.80)*,‡,§	−22.4 (7.66)*,‡	−44.9 (22.35)*,‡,§
(RIPC+atenolol) then sGVS	−11.4 (6.48)*,‡	−17.0 (5.26)*,‡	−27.9 (22.83)*
Sham	−0.1 (3.16)	−0.1 (3.50)	−3.1 (4.29)
(Vehicle PC+IN saline) then sGVS	−18.5 (4.63)*	−15.8 (8.92)*	−27.7 (10.94)*
(RIPC+IN saline) then sGVS	1.8 (3.67)†	2.1 (1.61)†	−1.9 (2.90)†
(RIPC+desipramine) then sGVS	−39.5 (20.64)*,†,‡	−42.6 (10.80)*,†,‡	−35.1 (24.91)*,‡

n=8/group. Exact *P*-values for the intergroup comparisons are reported in Table S7. Mean (standard deviation). IN indicates intranasal; IV, intravenous; PC, preconditioning; RIPC, remote limb ischemic preconditioning; sGVS, sinusoidal galvanic vestibular stimulation.

*$P<0.05$ vs sham.

†$P<0.05$ vs (vehicle PC+saline) then sGVS.

‡$P<0.05$ vs (RIPC+saline) then sGVS.

§$P<0.05$ vs (RIPC+labetalol) then sGVS.

not in regulation of cerebral blood flow,[26] yet it seems more likely that there is a sex difference in response of the cerebral blood flow.[27] In either case, additional experiments are needed to better understand the observed differences in the cardio- and cerebrovascular responses between female and male rats.

RIPC Neuroprotection Against sGVS Is Via the α_1-Adrenoceptor-PKCε-eNOS Pathway

Based on the success of RIPC against sGVS, we further hypothesized that the mechanism by which RIPC is therapeutically beneficial for VVS is via priming the α_1-adrenoceptor-PKCε-eNOS pathway in the vasculature by norepinephrine.

RIPC caused release of norepinephrine into the serum, from which it was transported by NET1 and activated α_1-adrenoceptors. Over the course of RIPC, chronic activation of α_1-adrenoceptor by norepinephrine led to increased expression of α_1-adrenoceptors in the brain and decreased the brain expression of NET1. When α_1-adrenoceptor agonism or norepinephrine transport by NET1 is inhibited during RIPC, the protective effects of RIPC against sGVS are lost, suggesting that norepinephrine, α_1-adrenoceptor, and NET1 are critical in RIPC protection of the cerebrovasculature during sGVS (ie, maintaining cerebral blood flow) and play a role in the cardiovascular benefits of RIPC (ie, maintaining blood pressure and heart rate). The study by Oxman et al. showed that norepinephrine given prophylactically protects against tachyarrhythmia in isolated rat hearts, mimicking the

effects of RIPC,[9] and showing that norepinephrine may be involved in RIPC cardioprotection. Interestingly, a study by Gürdal et al. indicated that chronic activation of α_1-adrenoceptor can decrease α_1-adrenoceptor-mediated vasoconstriction, as well as increase eNOS expression and activity,[13] suggesting that preconditioning of the α_1-adrenoceptor can provide cardioprotection via changes to eNOS. Clinical trials for NET1 antagonism as a treatment of VVS indicate that targeting the NET1 and/or downstream signaling may be therapeutically beneficial. Our work here further supports the roles of norepinephrine, α_1-adrenoceptor, and NET1 in cardioprotection. This work also argues for the role of norepinephrine, α_1-adrenoceptor, and NET1 in regulation and/or protection of cerebral blood flow.

Another effect of RIPC that is particularly pertinent to VVS pathophysiology is the effect RIPC has on cerebral blood flow; experimentally and clinically, RIPC increases cerebral blood flow. In a mouse model of vascular cognitive impairment, Khan et al. observed a sustained increase in cerebral blood flow perfusion in mice subjected to RIPC that may be dependent on increased eNOS/nitric oxide/nitrite.[28] Our findings also strengthen the downstream signaling of RIPC converging on the eNOS pathway and the critical role of eNOS in RIPC protection of cerebral blood flow.[25]

Preconditioning for VVS

VVS is predictable because the rate of recurrence in humans is up to 40%[2]; therefore, pretreatment or preconditioning therapies are potential options for preventing syncopal episodes. Currently VVS is treated prophylactically with several therapies. β-Adrenoceptor antagonists have been widely used and were the first choice for many years; however, the Prevention of Syncope Trial (POST) found that β-blockers provide no benefit and may even worsen VVS outcome and thus are now contraindicated. Yet, metoprolol is being examined for aged patients in an ongoing clinical trial. Fludrocortisone, a corticosteroid, has shown mixed success and is currently limited to younger, nonhypertensive patients. α_1-Adrenoceptor agonists have shown some success, and midodrine is being tested in the POST IV trial with the results expected soon. However, midodrine has several side effects, which reduce its enthusiasm. Additionally, NET inhibitors are also being studied for preventing VVS. A small clinical study found that severely symptomatic VVS patients benefited from NET1 antagonism; however, the trial included only 7 patients.[29] A NET1 inhibitor is currently being evaluated in the POST 6 trial.

RIPC as a Preconditioning Therapy for VVS

Although many cardio- and cerebrovascular diseases for which RIPC has been reported to be beneficial are spontaneously occurring, VVS offers the potential for preconditioning due to its high prevalence and recurrence. Thus, RIPC seems to be well suited for preventing VVS pathophysiology and occurrence. In this regard, RIPC was found to prevent sGVS via preconditioning the heart, systemic circulation, and cerebrovasculature in rats. Because RIPC is currently being tested in clinical trials for many cardio- and cerebrovascular diseases/injuries/surgeries, RIPC can be fast-tracked into clinical trials for preventing VVS. Furthermore, RIPC has been shown to be involved in activation of several targets that have been pharmacologically investigated for VVS: β-adrenoceptors,[30] α_1-adrenoceptor,[31] and NET1,[29] (and being investigated in POST 6). Thus, RIPC seems to be superior to the current pharmacological treatments being used/investigated due to is pleiotropic effects.

Limitations and Future Studies

The main limitation of this study is that the rat model of VVS, which uses sGVS, may not exactly mimic VVS pathophysiology. Nonetheless, sGVS in rats causes a number of similarities to human VVS, including hypotension, bradycardia, reduced cerebral perfusion, and fainting-like behavior.[14] Indeed, if we compare the minimum values of mean arterial pressure and heart rate during sGVS in rats (Table 7), the values are strikingly similar to those values observed during human VVS. The data herein suggest that RIPC is a potential therapeutic option for VVS. Future studies will be undertaken to examine RIPC in patients with VVS; clinical translation of RIPC will be rapid because RIPC is safe, easy to perform at home or in the hospital, has no reported side effects, and is currently used in the clinic.[32]

An additional limitation of the sGVS rat model is the recovery time of cerebral blood flow for vehicle-PC animals. One would expect cerebral blood flow to return to baseline values immediately after stopping stimulation. However, we found that cerebral blood flow in vehicle PC animals takes 15 to 30 minutes to recover. The mechanism of the sustained cerebral blood flow depression after stimulation needs to be investigated in future studies but may be related to adrenoceptor-mediated signaling.

Another limitation of this study is the choice of the RIPC regimen. As far as we know, no studies have utilized repeated RIPC to study neurocardiogenic response. The number of cycles (4) and ischemia-reperfusion durations (10 minutes) have been reported to provide cardio- and neuro-protection,[7,11] but the length (number of days) of RIPC was chosen arbitrarily. Within this study, 5 days of RIPC provided protection against sGVS in rats on day 5 but not day 10, whereas the protection afforded by 10 days of RIPC was beneficial on both days 5 and 10. Yet the length of protection

against VVS by RIPC is not yet known. Future studies will be performed in an attempt to identify the optimal RIPC regimen for preventing VVS and provide lasting protection.

This study examined adrenoceptors as the major players for RIPC protection against VVS, yet a myriad of receptors and downstream signaling pathways have been reported to be involved in RIPC cardio- and neuro-protection.[7,11] Therefore, although the data within suggest that α_1-adrenoceptor and NET1 have roles in RIPC protection against VVS-induced cerebral hypoperfusion, additional mechanisms may exist. Indeed, adenosine has been shown to be a major factor responsible for RIPC protection, yet interestingly, there is crosstalk between adenosine-mediated signaling and α_1-adrenoceptor signaling.[33] Our results show that use of an antagonist for either α_1- or β_1-adrenoceptor during RIPC prevents RIPC cardioprotection, but no change in heart expressions of these receptors or downstream signaling was observed (Figure S11). However, it may be that the sensitivity of these receptors (or other adrenoceptor subtypes) may be the cause of these findings. Additionally, α_1-adrenoceptor was investigated but not the roles of the individual α_1-adrenoceptor subtypes; α_{1B}-adrenoceptor has been shown to be dominant in cardioprotection.[34] Furthermore, the mechanism of RIPC cardioprotection against sGVS only partially involved an α_1-adrenoceptor-mediated signaling pathway and thus needs to be explored in future studies.

Interestingly, both doxazosin (α_1-adrenoceptor antagonist) and atenolol (β_1-adrenoceptor antagonist) partially inhibited RIPC protection against blood pressure and heart rate drops, whereas labetalol did not. Given that α_1- and β_1-adrenoceptor antagonists prevented RIPC protection, one would think that a combined α- and β-adrenoceptor antagonist (eg, labetalol) would also attenuate RIPC protection. However, this may be explained by the specificity of the antagonists used. Doxazosin and atenolol are specific for α_1- and β_1-adrenoceptors, whereas labetalol is a nonspecific adrenoceptor antagonist affecting α_1-, β_1-, β_2-, and β_3-adrenoceptors. In the brain, the primary adrenoceptors are α_1- and β_1-, however, the heart and periphery contain all of the various subtypes. Therefore, labetalol may affect the brain and heart/periphery differently. Future studies will be conducted to uncouple the role each adrenoceptor subtype plays in heart rate and mean arterial pressure depressions caused by sGVS and their role in RIPC protection.

The effects of despiramine (NET1 antagonist), which inhibits the reuptake of norepinephrine in the presynapse causing increased intersynaptic levels of norepinephrine, were expected to stimulate α_1-adrenoceptors, providing protection against sGVS. However, despiramine effects were similar to the effects of α_1-adrenoceptor antagonism (doxazosin). Two potential reasons for the effects of despiramine mimicking doxazosin are that despiramine may also inhibit α-adrenoceptors or may reduce α-adrenoceptor sensitivity to norepinephrine.[35–37] The former side effect will mimic doxazosin, whereas the latter may affect the action of norepinephrine during either RIPC or sGVS. The exact reason for the observed effects needs to be examined in future studies in which we monitor the uptake of norepinephrine and measure adrenoceptor sensitivity to norepinephrine, as well as investigate additional groups in which we use antagonists for NET1 and α_1-adrenoceptor in the same animal.

RIPC has many reported mechanisms of action for cardio- and cerebro-vascular diseases. There are 3 primary routes by which RIPC may confer its protection: neural, humoral, and systemic avenues.[38] While the data presented within suggest that norepinephrine (neural pathway) is a key mediator for RIPC protection against cerebrovascular depression induced by sGVS, it is possible that other molecules, such as adenosine and bradykinin, are also important. We also found that the cardioprotection by RIPC against sGVS was not solely mediated by adrenoceptors, and there are other factors that may play a greater role in the cardioprotection. Determining the role each route of RIPC protection (ie, neural, humoral, systemic) plays in preventing sGVS cardio- and cerebrovascular depressions will be the focus of a future study.

Aged males subjected to RIPC did not have attenuated mean arterial pressure during sGVS as expected. The mechanism for this lack of mean arterial pressure response is unknown, but it may be related to variations in peripheral adrenoceptor density/sensitivity. This, too, needs to be explored in future studies.

Finally, in this study, no adjustment was made for multiple testing when conducting statistical analyses. Thus, the statistical significance reported within may have occurred by chance alone due to the large number of hypothesis tests.

Conclusion

Within this study we investigated the hypothesis that RIPC is protective against VVS-induced hypotension, bradycardia, and reduced cerebral blood flow in rats subjected to sGVS. The findings support our hypothesis and suggest that RIPC may be a therapeutic option for attenuating the physiological and behavioral changes caused by VVS and may even prevent VVS episodes. We identified the α_1-adrenoceptor/PKCε/eNOS pathway as playing a role in RIPC protection against sGVS-induced cerebrovascular changes.

Acknowledgments

The authors would like to thank Ethan Frank and Mary Yurashevich for help with the experiments.

Sources of Funding

This work was supported by a Loma Linda University Seed grant (GCAT) to Drs Applegate and Tang. Dr McBride was supported by a NIH R01 (NS081740, Zhang).

Disclosures

None.

References

1. da Silva RM. Syncope: epidemiology, etiology, and prognosis. *Front Physiol*. 2014;5:471.
2. Klein KM, Berkovic SF. Genetics of vasovagal syncope. *Auton Neurosci*. 2014;184:60–65.
3. Soteriades ES, Evans JC, Larson MG, Chen MH, Chen L, Benjamin EJ, Levy D. Incidence and prognosis of syncope. *N Eng J Med*. 2002;347:878–885.
4. Grubb BP, Olshansky B. *Syncope: Mechanisms and Management*. Oxford, UK: Wiley-Blackwell; 2005.
5. Jardine DL. Vasovagal syncope: new physiologic insights. *Cardiol Clin*. 2013;31:75–87.
6. Mitro P, Rybarova E, Zemberova E, Tkac I. Enhanced plasma catecholamine and cAMP response during the head-up tilt test in patients with vasovagal syncope. *Wien Klin Wochenschr*. 2005;117:353–358.
7. Hausenloy DJ, Yellon DM. Ischaemic conditioning and reperfusion injury. *Nat Rev Cardiol*. 2016;13:193–209.
8. Xie JJ, Liao XL, Chen WG, Huang DD, Chang FJ, Chen W, Luo ZL, Wang ZP, Ou JS. Remote ischaemic preconditioning reduces myocardial injury in patients undergoing heart valve surgery: randomised controlled trial. *Heart*. 2012;98:384–388.
9. Oxman T, Arad M, Klein R, Avazov N, Rabinowitz B. Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. *Am J Physiol*. 1997;273:H1707–H1712.
10. Abdul-Ghani S, Fleishman AN, Khaliulin I, Meloni M, Angelini GD, Suleiman MS. Remote ischemic preconditioning triggers changes in autonomic nervous system activity: implications for cardioprotection. *Physiol Rep*. 2017;5:e13085.
11. Hess DC, Blauenfeldt RA, Andersen G, Hougaard KD, Hoda MN, Ding Y, Ji X. Remote ischaemic conditioning—a new paradigm of self-protection in the brain. *Nat Rev Neurol*. 2015;11:698–710.
12. Looft-Wilson RC, Todd SE, Araj CA, Mutchler SM, Goodell CA. Alpha(1)-adrenergic-mediated eNOS phosphorylation in intact arteries. *Vasc Pharmacol*. 2013;58:112–117.
13. Gürdal H, Can A, Uğur M. The role of nitric oxide synthase in reduced vascocontractile responsiveness induced by prolonged α_1-adrenergic receptor stimulation in rat thoracic aorta. *Br J Pharmacol*. 2005;145:203–210.
14. McBride DW, Reis C, Frank E, Klebe DW, Zhang JH, Applegate R II, Tang J. An experimental model of vasovagal syncope induces cerebral hypoperfusion and fainting-like behavior in awake rats. *PLoS One*. 2016;11:e0163280.
15. Graham LA, Kenny RA. Clinical characteristics of patients with vasovagal reactions presenting as unexplained syncope. *Europace*. 2001;3:141–146.
16. Wieling W, Thijs RD, van Dijk N, Wilde AA, Benditt DG, van Dijk JG. Symptoms and signs of syncope: a review of the link between physiology and clinical clues. *Brain*. 2009;132:2630–2642.
17. Du XJ, Dart AM, Riemersma RA, Oliver MF. Sex difference in presynaptic adrenergic inhibition of norepinephrine release during normoxia and ischemia in the rat heart. *Circ Res*. 1991;68:827–835.
18. Page GG, Fennelly AM, Littleton-Kearney MT, Ben-Eliyahu S. Male-female differences in the impact of β-adrenoceptor stimulation on resistance to experimental metastasis: exploring the effects of age and gonadal hormone involvement. *J Neuroimmunol*. 2008;193:113–119.
19. Hoeker GS, Hood AR, Katra RP, Poelzing S, Pogwizd SM. Sex differences in β-adrenergic responsiveness of action potentials and intracellular calcium handling in isolated rabbit hearts. *PLoS One*. 2014;9:e111411.
20. Freedman RR, Moten M. Gender differences in modulation of peripheral vascular adrenoceptors. *Ann Behav Med*. 1995;17:15–18.
21. Freedman RR, Sabharwal SC, Desai N. Sex differences in peripheral vascular adrenergic receptors. *Circ Res*. 1987;61:581–585.
22. Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, Murphy E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res*. 2010;106:1681–1691.
23. Miura T, Tanno M, Sato T. Mitochondrial kinase signalling pathways in myocardial protection from ischaemia/reperfusion-induced necrosis. *Cardiovasc Res*. 2010;88:7–15.
24. Zhang J, Baines CP, Zong C, Cardwell EM, Wang G, Vondriska TM, Ping P. Functional proteomic analysis of a three-tier PKCε-Akt-eNOS signaling module in cardiac protection. *Am J Physiol Heart Circ Physiol*. 2005;288:H954–H961.
25. Heal DJ, Bristow LM, Hurst EM, Elliott JM, Buckett WR. Sex-related differences in central adrenergic function and responsiveness to repeated administration of desipramine or electroconvulsive shock. *Br J Pharmacol*. 1989;97:111–118.
26. Esposito G, Van Horn JD, Weinberger DR, Berman KF. Gender differences in cerebral blood flow as a function of cognitive state with PET. *J Nucl Med*. 1996;37:559–564.
27. Xing CY, Tarumi T, Meijers RL, Turner M, Repshas J, Xiong L, Ding K, Vongpatanasin W, Yuan LJ, Zhang R. Arterial pressure, heart rate, and cerebral hemodynamics across the adult life span. *Hypertension*. 2017;69:712–720.
28. Khan MB, Hoda MN, Vaibhav K, Giri S, Wang P, Waller JL, Ergul A, Dhandapani KM, Fagan SC, Hess DC. Remote ischemic postconditioning: harnessing endogenous protection in a murine model of vascular cognitive impairment. *Transl Stroke Res*. 2015;6:69–77.
29. Coffin ST, Raj SR. Non-invasive management of vasovagal syncope. *Auton Neurosci*. 2014;184:27–32.
30. Aimo A, Borrelli C, Giannoni A, Pastormerlo LE, Barison A, Mirizzi G, Emdin M, Passino C. Cardioprotection by remote ischemic conditioning: mechanisms and clinical evidences. *World J Cardiol*. 2015;7:621–632.
31. Taliyan R, Singh M, Sharma PL, Yadav HN, Sidhu KS. Possible involvement of α_1-adrenergic receptor and K(ATP) channels in cardioprotective effect of remote aortic preconditioning in isolated rat heart. *J Cardiovasc Dis Res*. 2010;1:145–151.
32. Meng R, Asmaro K, Meng L, Liu Y, Ma C, Xi C, Li G, Ren C, Luo Y, Ling F, Jia J, Hua Y, Wang X, Ding Y, Lo EH, Ji X. Upper limb ischemic preconditioning prevents recurrent stroke in intracranial arterial stenosis. *Neurology*. 2012;79:1853–1861.
33. Preston A, Frydenberg M, Haynes JM. A_1 and A_{2A} adenosine receptor modulation of α_1-adrenoceptor-mediated contractility in human cultured prostatic stromal cells. *Br J Pharmacol*. 2004;141:302–310.
34. Tsuchida A, Liu Y, Liu GS, Cohen MV, Downey JM. Alpha 1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res*. 1994;75:576–585.
35. Charney DS, Heninger GR, Sternberg DE, Redmond DE, Leckman JF, Maas JW, Roth RH. Presynaptic adrenergic receptor sensitivity in depression. The effect of long-term desipramine treatment. *Arch Gen Psychiatry*. 1981;38:1334–1340.
36. Ebadi M. *CRC Desk Reference of Clinical Pharmacology*. Boca Raton, FL: CRC Press; 1997.
37. Schafer W. How do antidepressants work? Prospects for genetic analysis of drug mechanisms. *Cell*. 1999;98:551–554.
38. Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovasc Res*. 2008;79:377–386.

SUPPLEMENTAL MATERIAL

Remote Limb Ischemic Preconditioning Attenuates Cerebrovascular Depressions During Sinusoidal Galvanic Vestibular Stimulation via the α_1-Adrenoceptor–Protein Kinase Cε–Endothelial NO Synthase Pathway in Rats

Devin W. McBride, Ph.D.,[1] Cesar Reis, M.D.,[2] John H. Zhang, M.D., Ph.D.,[1, 3] Richard Applegate II, M.D.,[2] and Jiping Tang, M.D.[1]

[1]Department of Physiology & Pharmacology, [2]Department of Anesthesiology, [3]Department of Neurosurgery, Loma Linda University, Loma Linda, California, USA

Supplemental Methods

In addition to the 174 rats reported in the manuscript, 60 additional SD rats were used to obtain the supplemental/supporting data. Eight 3-month old male rats were used in the unilateral versus bilateral RIPC study. These 8 rats were subjected to 5 days of unilateral hindlimb RIPC, followed by sGVS 5 and 10 days after stopping preconditioning. The cardio- and cerebro-vascular responses of these rats during sGVS were compared to rats subjected to 5 days of bilateral hindlimb RIPC (reported in the manuscript).

Another 32 three-month old male SD rats were randomly assigned to either sham, vehicle preconditioning then sGVS, RIPC for 5 days then sGVS, or RIPC for 10 days then sGVS (n=8/group). Sham animals were normal rats which underwent all surgical procedures (burr hole, femoral artery catheterization), monitoring of mean arterial pressure, heart rate, and cerebral blood flow, and electrode placement but without electrical stimulation (*i.e.* sGVS was not induced). Vehicle preconditioned animals underwent all RIPC procedures without tightening of the hindlimb snares. RIPC was performed as described in the manuscript (4 cycles of 10 min ischemia/10 min reperfusion, bilateral hindlimb, while under isoflurane). Animals were subjected to sGVS 10 days after completing the RIPC regimen (Figure S4).

An additional 8 three-month old male SD rats were subjected to 10 days of vehicle preconditioning with labetalol administration (3 mg/kg, 200 μL, IV). These animals were used to monitor mean arterial pressure, heart rate, and cerebral blood flow during the first day of preconditioning (Day -9) and on the last day of preconditioning (Day 0) before being euthanized (cardiac perfusion of PBS, brains removed and snap frozen) immediately following completion of the preconditioning regimen (*i.e.* euthanized on the last day of preconditioning) (followed the same experimental protocol as Experiment 3 in the manuscript (Figure 5)).

Twelve 3-month old male SD rats were used to quantify the brain expressions of α_1- and β_1-adrenoceptors, and NET1 immediately after completing RIPC. Six animals were subjected to 10 days of RIPC with labetalol and six rats were subjected to 10 days of vehicle preconditioning with labetalol and euthanized immediately after completing RIPC on the last day. Labetalol (3 mg/kg, 200 μL, IV) was administered prior to preconditioning on each day of preconditioning. This was performed following the protocol in Experiment 4 in the manuscript (Figure 6).

Data S1

Effect of Unilateral Hindlimb versus Bilateral Hindlimb RIPC on sGVS-Induced Cardio- and Cerebro-Vascular Depressions

Few studies on RIPC investigate whether any differences are observed on the therapeutic effect of RIPC for unilateral hindlimb versus bilateral hindlimb ischemia. We performed RIPC for 5 days to examine the effect of unilateral and bilateral ischemia-reperfusion. One group (n=8) underwent unilateral hindlimb RIPC for 5 days and another group (n=8) underwent bilateral hindlimb RIPC for 5 days (bilateral RIPC group data shared from manuscript). Both groups were subjected to sGVS as described in Experiment 1. The effects of unilateral RIPC was indistinguishable from that of bilateral RIPC for mean arterial pressure and cerebral blood flow before, during, and after sGVS on Day 5 (Figures S1A and S1C) and Day 10 (Figures S1D and S1F). Interestingly, bilateral RIPC lead to a statistically higher heart rate than unilateral RIPC during sGVS on Day 10 (Figure S1E), as well as post-stimulation on Days 5 and 10 (Figures S1B and S1E). The cause of the elevated heart rate during sGVS on Day 10 and post-stimulation on Days 5 and 10 is unknown and will need to be further investigated.

Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow During RIPC

Twelve male SD rats (3-month old) from the vehicle preconditioned (n=4), RIPC (n=4), and RIPC + Labetalol (n=4) groups in Experiment 4 were subjected to femoral artery catheterization on the first and the last day of preconditioning for measurement of heart rate and mean arterial pressure, as well as for collection of blood (before PC, immediately after completing PC (0 Min), and then 30 and 60 min post-PC).

During the course of RIPC (*i.e.* during the ischemia-reperfusion cycles of a single day of RIPC), minor, and likely insignificant, changes in mean arterial pressure, heart rate, and cerebral blood flow occur on the first day of RIPC (Figure S2), as well as on the tenth (last) day of RIPC (Figure S3). The significant differences in the mean arterial pressure (during the ischemia and reperfusion cycles (Figure S2A, Left Panel)) between the two groups is likely due to the ischemia since the tourniquet used to cause ischemia is located adjacent to the femoral artery catheter. While small changes occur in the mean arterial pressure between the two groups, no significant difference is observed for the cerebral blood flow between rats undergoing isoflurane (vehicle) preconditioning and those undergoing RIPC (Figure S2C). These findings are similar to those by Zhao and Nowak, and Zhang *et al.* Zhao and Nowak observed no statistical difference between the mean arterial pressure post-preconditioning in spontaneously hypertensive rats using ischemia/reperfusion of the middle cerebral artery as the preconditioning stimulus.[2] Zhang *et al.* reported no significant difference in either mean arterial pressure or cerebral blood flow between the occlusion and opening segments of RIPC in rats using hindlimb ischemia/reperfusion preconditioning similar to our method of RIPC.[3]

Interestingly, the mean arterial pressure on the last day of RIPC is significantly higher in rats receiving RIPC compared to those receiving isoflurane (Figure S3A). Of note, is that the RIPC rats have higher mean arterial pressures compared to isoflurane preconditioned animals during ischemia, but not during reperfusion. This suggests that the RIPC animals have built up tolerance against reductions in mean arterial pressure, while the isoflurane preconditioned rats have not.

Another interesting point is that the cerebral blood flow for isoflurane preconditioning, as well as RIPC, rats is no longer decreasing overtime, suggesting that these animals may have built up

tolerance against isoflurane-induced lowering of cerebral blood flow. Prolonged use of isoflurane in animals has been documented to cause cerebral blood flow to decrease over time.[4-7] While rats on the first day of preconditioning adhere to this phenomenon, after 10 days of isoflurane exposures, there is no longer a time-dependent decrease in cerebral blood flow.

Lasting Protection by RIPC against sGVS

When animals subjected to sGVS 10 days after completing the preconditioning regimens, 10 days of RIPC continues to provide protection against the reductions in mean arterial pressure, heart rate, and cerebral blood flow during sGVS compared to vehicle preconditioned animals (Stimulation mean arterial pressure: $p<0.05$ Vehicle PC then sGVS vs RIPC (10 days) then sGVS, $p>0.05$ Sham vs RIPC (10 days) then sGVS) (Stimulation heart rate: $p<0.05$ Vehicle PC then sGVS vs RIPC (10 days) then sGVS, $p>0.05$ Sham vs RIPC (10 days) then sGVS) (Stimulation cerebral blood flow: $p<0.05$ Vehicle PC then sGVS vs RIPC (10 days) then sGVS, $p>0.05$ Sham vs RIPC (10 days) then sGVS). Post-sGVS, the mean arterial pressure, heart rate, and cerebral blood flow remained significantly different between vehicle preconditioned animals and rats receiving 10 days of RIPC (Stimulation mean arterial pressure: $p<0.05$ Vehicle PC then sGVS vs RIPC (10 days) then sGVS, $p>0.05$ Sham vs RIPC (10 days) then sGVS) (Stimulation heart rate: $p<0.05$ Vehicle PC then sGVS vs RIPC (10 days) then sGVS, $p>0.05$ Sham vs RIPC (10 days) then sGVS) (Stimulation cerebral blood flow: $p<0.05$ Vehicle PC then sGVS vs RIPC (10 days) then sGVS, $p>0.05$ Sham vs RIPC (10 days) then sGVS) (Figure S5).

Five days of RIPC continues to provide protection against sGVS-induced lowering of mean arterial pressure and heart rate (Stimulation mean arterial pressure: $p<0.05$ Vehicle PC then sGVS vs RIPC (5 days) then sGVS, $p>0.05$ Sham vs RIPC (5 days) then sGVS; Post-Stimulation mean arterial pressure: $p<0.05$ Vehicle PC then sGVS vs RIPC (5 days) then sGVS, $p>0.05$ Sham vs RIPC (5 days) then sGVS) (Stimulation heart rate: $p<0.05$ Vehicle PC then sGVS vs RIPC (5 days) then sGVS, $p>0.05$ Sham vs RIPC (5 days) then sGVS; Post-Stimulation heart rate: $p<0.05$ Vehicle PC then sGVS vs RIPC (5 days) then sGVS, $p>0.05$ Sham vs RIPC (5 days) then sGVS), but does not prevent sGVS-induced lowering of cerebral blood flow (Stimulation cerebral blood flow: $p>0.05$ Vehicle PC then sGVS vs RIPC (5 days) then sGVS, $p<0.05$ Sham vs RIPC (10 days) then sGVS; Post-Stimulation cerebral blood flow: $p>0.05$ Vehicle PC then sGVS vs RIPC (5 days) then sGVS, $p<0.05$ Sham vs RIPC (10 days) then sGVS).

Effect of Adrenoceptor Antagonism on Serum Catecholamines during RIPC

Serum norepinephrine is significantly elevated immediately following RIPC and remains elevated for up to 60 minutes post-preconditioning (Figures S6A and S6C), whereas RIPC has no effect on serum epinephrine after RIPC (Figures S6B and S6D). As one would expect, antagonizing adrenoceptors (in this case, with labetalol) does not have any effect on serum epinephrine on either the first or last days of RIPC. However, labetalol causes a marked increase in serum epinephrine on the first day of RIPC, but not the last day of RIPC.

Inhibiting Adrenoceptors Attenuates RIPC-Induced Upregulation of Brain α_1-Adrenoceptors and RIPC-Induced Reduction of Brain NET1 Expression

Representative Western blots for the data in Figure 13 is shown in Figure S7. Figure S7 shows representative Western blots of the brain expression of α_1- and β_1-adrenoceptors and NET1 after preconditioning which were quantified in Figure 13.

RIPC for 10 days leads to higher expression of α_1-adrenoceptors and lower expression of NET1

in the brain compared to vehicle preconditioning. When the pan-adrenoceptor inhibitor labetalol is given before RIPC each day, the upregulation of α_1-adrenoceptor in the brain by RIPC is prevented (Figure S8). This data suggests that blocking RIPC activation of adrenoceptors prevents elevated expression of α_1-adrenoceptor which has not been previously shown.

Labetalol given during RIPC or vehicle preconditioning did not have any statistically significant effect on the brain expression of β_1-adrenoceptor ($p>0.05$ for all group comparisons; $p=0.2121$ for Vehicle PC vs (Vehicle PC + Labetalol), $p=0.1443$ for RIPC vs (Vehicle PC + Labetalol), $p=0.1473$ for (RIPC + Labetalol) vs (Vehicle PC + Labetalol)) (Figure S8).

RIPC significantly reduces the expression of NET1 in the brain compared to vehicle preconditioning alone. This reduction by RIPC of NET1 is prevented by administering labetalol during RIPC; Labetalol given during RIPC returns brain NET1 levels back to that which is statistically indistinguishable from NET1 expression in vehicle preconditioned brains (Figure S8).

Representative Western Blots

Representative Western blots for the data in Figures 15 and 16 are shown in Figures S9 and S10. Figure S9 shows representative Western blot images of the brain expression for α_1-adrenoceptor, NET1, PKCε, and p-eNOS after sGVS which were quantified in Figure 15. Also shown in Figure S9 is the brain expression of β_1-adrenoceptor (representative Western blot and quantification) which showed no change between any of the group comparisons. Figure S10 shows representative Western blot images of the brain expression for α_1-adrenoceptor, NET1, PKCε, and p-eNOS after sGVS which were quantified in Figure 16. Also shown in Figure S10 is the brain expression of β_1-adrenoceptor (representative Western blot and quantification) which showed no change between any of the group comparisons.

Minimums of Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow During sGVS

A limitation of the sGVS rat model is that the drops in mean arterial pressure, heart rate, and cerebral blood flow are not as exaggerated in rats during sGVS as they are during human VVS. Indeed, the mean values of these three physiological parameters during stimulation do not drop as much during sGVS as they do during human VVS. However, this is likely due to the differences in presentation of the findings. In human VVS, the blood pressure and heart rate drops are presented as the minimum values, were as in the rodent sGVS model, we report these two values as the average value during stimulation (see Figures 7, 8, and 14, and Table S1). Tables S7-S9 shows the minimum values of these three physiological parameters during stimulation. Indeed, in comparing the minimum values of mean arterial pressure and heart rate in the rodent sGVS model and human VVS, the drops in mean arterial pressure and heart rate are now similar; the mean arterial pressure drop during sGVS in rats is approximately 22% while human blood pressure drop is around 25% during VVS;[1] the heart rate drop during sGVS in rats is about 20% while that during human VVS is around 22%.[1] Interestingly, the rat model of sGVS mimics the drops in blood pressure and heart rate strikingly well compared to those observed in humans during VVS.

Heart Expressions of Adrenoceptors and NET1 are Not Affected by RIPC

Following RIPC, no significant change in the expressions of α_1-adrenoceptor, β_1-adrenoceptor, nor NET1 was observed in the hearts of preconditioned animals (Figure S11). This suggests that the adrenoceptors may not play a significant role in cardio-protection of RIPC against sGVS. However, antagonism of α_1-adrenoceptor by doxazosin during RIPC partially reverses RIPC protection against lowering of the mean arterial pressure and heart rate, and inhibition of β_1-

adrenoceptor by atenolol during RIPC reverses RIPC-protection of mean arterial pressure and heart rate lowerings (Figure 14A and B). Therefore, both α_1- and β_1-adrenoceptors may play a role in RIPC cardioprotection against sGVS despite no change in their cardiac expressions. A possible explanation for cardioprotection by RIPC is a change in the sensitivity of the adrenoceptors (likely α_1-adrenoceptor). Specifically, the sensitivity of α_1-adrenoceptor may decrease due to prolonged stimulation by norepinephrine during RIPC. This requires further studies to validate, but the study by Gürdal *et al.* indicates this may be the case. Gürdal *et al.* showed that prolonged exposure of an α_1-adrenoceptor agonist can decrease α_1-adrenoceptor-mediated vasoconstriction in the rat aorta.[8]

Supplemental Tables

Table S1. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the percent change from baseline for the physiological parameters in Experiment 1 (Figures 7 and 8, Table 2). Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Mean Arterial Pressure		Heart Rate		Cerebral Blood Flow	
	Stimulation	Post-Stimulation	Stimulation	Post-Stimulation	Stimulation	Post-Stimulation
Sham vs Vehicle PC then sGVS	**<0.0001**	0.9983	**<0.0001**	0.9448	**<0.0001**	**<0.0001**
Sham vs RIPC (5 days) then sGVS	0.8147	0.4610	0.8835	0.0358	0.9884	0.4698
Sham vs RIPC (10 days) then sGVS	0.3073	0.4704	0.9673	0.3686	0.8410	0.4937
Vehicle PC then sGVS vs RIPC (5 days) then sGVS	**<0.0001**	0.3641	**<0.0001**	**0.0074**	**<0.0001**	**<0.0001**
Vehicle PC then sGVS vs RIPC (10 days) then sGVS	**<0.0001**	0.3727	**<0.0001**	0.1363	**<0.0001**	**<0.0001**
RIPC (5 days) then sGVS vs RIPC (10 days) then sGVS	0.8234	>0.9999	0.6324	0.6755	0.9573	>0.9999
Female						
Sham vs Vehicle PC then sGVS	**0.0016**	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Sham vs RIPC then sGVS	0.1369	**0.0006**	0.1328	**<0.0001**	0.9578	0.7407
Vehicle PC then sGVS vs RIPC then sGVS	0.2131	0.9341	**0.0100**	**0.0026**	**<0.0001**	**<0.0001**
Aged Male						
Sham vs Vehicle PC then sGVS	**0.0003**	0.2264	**<0.0001**	**0.0008**	**<0.0001**	**<0.0001**
Sham vs RIPC then sGVS	**0.0019**	0.5263	0.3430	0.8872	>0.9999	0.9336
Vehicle PC then sGVS vs RIPC then sGVS	0.8627	0.1208	**<0.0001**	**0.0033**	**<0.0001**	**<0.0001**

Table S2. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the percent change from baseline for the physiological parameters in Experiment 1 (Figure 9, Table 2). Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Mean Arterial Pressure		Heart Rate		Cerebral Blood Flow	
	Stimulation	Post-Stimulation	Stimulation	Post-Stimulation	Stimulation	Post-Stimulation
Male vs Female						
Vehicle PC then sGVS	**0.0190**	0.3915	0.9418	**0.0437**	**0.0003**	0.0809
RIPC then sGVS	**0.0452**	0.9993	**0.0229**	**0.0021**	0.8707	0.3291
Young vs Aged						
Vehicle PC then sGVS	0.0939	0.7055	0.9414	**0.0003**	**0.0003**	**0.0178**
RIPC then sGVS	**<0.0001**	**0.0185**	**0.0088**	**0.0003**	0.9946	0.9994

Table S3. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the percent change from baseline for the physiological parameters in Experiment 1 (Figure 11, Table 3). Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Breathing Rate	Number of Falls	Syncope Score	Time to Recovery
Sham vs Vehicle PC then sGVS	**0.0001**	**0.0012**	**<0.0001**	**<0.0001**
Sham vs RIPC then sGVS	0.2393	0.3313	0.0511	0.6708
Vehicle PC then sGVS vs RIPC then sGVS	0.0585	0.1548	0.1167	**<0.0001**

Table S4. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the percent change from baseline for the mean arterial pressure in Experiment 4 (Figure 14, Table 4). Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Stimulation	Post-Stimulation
Sham vs (Vehicle PC + IV Saline) then sGVS	**0.0065**	0.9754
Sham vs (RIPC + IV Saline) then sGVS	0.1009	0.0913
Sham vs (RIPC + Labetalol) then sGVS	0.5599	0.8535
Sham vs (RIPC + Doxazosin) then sGVS	0.8886	0.8886
Sham vs (RIPC + Atenolol) then sGVS	0.2643	0.7445
(Vehicle PC + IV Saline) then sGVS vs (RIPC + IV Saline) then sGVS	**<0.0001**	**0.0112**
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	**<0.0001**	0.4007
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	0.1344	0.4522
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	0.7197	0.2848
(RIPC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	0.9310	0.6681
(RIPC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	**0.0043**	0.6144
(RIPC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	**<0.0001**	0.7914
(RIPC + Labetalol) then sGVS vs (RIPC + Doxazosin) then sGVS	0.0744	>0.9999
(RIPC + Labetalol) then sGVS vs (RIPC + Atenolol) then sGVS	**0.0028**	>0.9999
(RIPC + Doxazosin) then sGVS vs (RIPC + Atenolol) then sGVS	0.8886	0.9997
Sham vs (Vehicle PC + IN Saline) then sGVS	**0.0072**	0.7920
Sham vs (RIPC + IN Saline) then sGVS	0.6933	0.5867
Sham vs (RIPC + Desipramine) then sGVS	**0.0003**	0.0731
(Vehicle PC + IN Saline) then sGVS vs (RIPC + IN Saline) then sGVS	**0.0002**	0.1342
(Vehicle PC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	0.7920	**0.0058**
(RIPC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	**<0.0001**	0.6298

Table S5. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the percent change from baseline for the heart rate in Experiment 4 (Figure 14, Table 5). Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Stimulation	Post-Stimulation
Sham vs (Vehicle PC + IV Saline) then sGVS	**<0.0001**	0.0628
Sham vs (RIPC + IV Saline) then sGVS	>0.9999	0.9694
Sham vs (RIPC + Labetalol) then sGVS	0.4715	0.6183
Sham vs (RIPC + Doxazosin) then sGVS	**0.0073**	**0.0002**
Sham vs (RIPC + Atenolol) then sGVS	**<0.0001**	0.9694
(Vehicle PC + IV Saline) then sGVS vs (RIPC + IV Saline) then sGVS	**<0.0001**	**0.0061**
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	**<0.0001**	0.8187
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	**0.0042**	0.5078
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	0.1591	0.3361
(RIPC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	0.5078	0.1792
(RIPC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	**0.0088**	**<0.0001**
(RIPC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	**<0.0001**	0.6183
(RIPC + Labetalol) then sGVS vs (RIPC + Doxazosin) then sGVS	0.5078	**0.0400**
(RIPC + Labetalol) then sGVS vs (RIPC + Atenolol) then sGVS	**0.0342**	0.9694
(RIPC + Doxazosin) then sGVS vs (RIPC + Atenolol) then sGVS	0.7892	**0.0034**
Sham vs (Vehicle PC + IN Saline) then sGVS	**<0.0001**	**0.0195**
Sham vs (RIPC + IN Saline) then sGVS	0.7705	0.9963
Sham vs (RIPC + Desipramine) then sGVS	**0.0002**	0.4227
(Vehicle PC + IN Saline) then sGVS vs (RIPC + IN Saline) then sGVS	**<0.0001**	**0.0104**
(Vehicle PC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	0.3974	0.4751
(RIPC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	**<0.0001**	0.3039

Table S6. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the percent change from baseline for the cerebral blood flow in Experiment 4 (Figure 14, Table 6). Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Stimulation	Post-Stimulation
Sham vs (Vehicle PC + IV Saline) then sGVS	**0.0407**	**0.0146**
Sham vs (RIPC + IV Saline) then sGVS	0.9997	0.9992
Sham vs (RIPC + Labetalol) then sGVS	**0.0493**	**0.0305**
Sham vs (RIPC + Doxazosin) then sGVS	**<0.0001**	**0.0135**
Sham vs (RIPC + Atenolol) then sGVS	0.9784	0.9748
(Vehicle PC + IV Saline) then sGVS vs (RIPC + IV Saline) then sGVS	**0.0252**	**0.0421**
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	>0.9999	0.9999
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	0.2802	>0.9999
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	0.2802	0.1129
(RIPC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	**0.0374**	0.0804
(RIPC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	**<0.0001**	**0.0392**
(RIPC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	0.9133	0.9988
(RIPC + Labetalol) then sGVS vs (RIPC + Doxazosin) then sGVS	0.2163	0.9998
(RIPC + Labetalol) then sGVS vs (RIPC + Atenolol) then sGVS	0.3543	0.1936
(RIPC + Doxazosin) then sGVS vs (RIPC + Atenolol) then sGVS	**0.0006**	0.1063
Sham vs (Vehicle PC + IN Saline) then sGVS	**0.0053**	**0.0234**
Sham vs (RIPC + IN Saline) then sGVS	0.9372	0.9800
Sham vs (RIPC + Desipramine) then sGVS	**<0.0001**	**0.0108**
(Vehicle PC + IN Saline) then sGVS vs (RIPC + IN Saline) then sGVS	**0.0008**	**0.0076**
(Vehicle PC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	0.0913	0.9929
(RIPC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	**<0.0001**	**0.0033**

Table S7. Minimum Values of the Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow During Stimulation. The p-values for the minimum value of the percent change from baseline (standard deviation) for each physiological parameter during stimulation (Table 7 Experiment 1) are reported. Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Mean Arterial Pressure	Heart Rate	Cerebral Blood Flow
Young Male			
Sham vs Vehicle PC then sGVS	**<0.0001**	**<0.0001**	**<0.0001**
Sham vs RIPC (5 days) then sGVS	0.8064	0.5795	0.9926
Sham vs RIPC (10 days) then sGVS	0.7130	0.9481	0.9978
Vehicle PC then sGVS vs RIPC (5 days) then sGVS	**0.0002**	**<0.0001**	**<0.0001**
Vehicle PC then sGVS vs RIPC (10 days) then sGVS	**<0.0001**	**<0.0001**	**<0.0001**
RIPC (5 days) then sGVS vs RIPC (10 days) then sGVS	0.2262	0.2825	0.9677
Female			
Sham vs Vehicle PC then sGVS	**0.0161**	**<0.0001**	**<0.0001**
Sham vs RIPC then sGVS	0.0673	**0.0323**	0.0659
Vehicle PC then sGVS vs RIPC then sGVS	0.7796	**0.0156**	**<0.0001**
Aged Male			
Sham vs Vehicle PC then sGVS	**0.0022**	**0.0038**	**<0.0001**
Sham vs RIPC then sGVS	0.1004	0.2099	0.9251
Vehicle PC then sGVS vs RIPC then sGVS	0.2237	0.1596	**<0.0001**

Table S8. Minimum Values of the Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow During Stimulation. The p-values for the minimum value of the percent change from baseline (standard deviation) for each physiological parameter during stimulation (Table 7 Experiment 4 IV Interventions) are reported. Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Mean Arterial Pressure	Heart Rate	Cerebral Blood Flow
Sham vs (Vehicle PC + IV Saline) then sGVS	**0.0002**	**0.0001**	**0.0449**
Sham vs (RIPC + IV Saline) then sGVS	0.6374	>0.9999	0.9990
Sham vs (RIPC + Labetalol) then sGVS	0.3327	**0.0011**	0.2835
Sham vs (RIPC + Doxazosin) then sGVS	**<0.0001**	**<0.0001**	**<0.0001**
Sham vs (RIPC + Atenolol) then sGVS	**0.0211**	**0.0004**	**0.0248**
(Vehicle PC + IV Saline) then sGVS vs (RIPC + IV Saline) then sGVS	**<0.0001**	**0.0001**	0.1060
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	0.0579	0.9879	0.9489
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	0.9334	0.9117	0.1989
(Vehicle PC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	0.5734	0.9992	0.9999
(RIPC + IV Saline) then sGVS vs (RIPC + Labetalol) then sGVS	**0.0102**	**0.0009**	0.4920
(RIPC + IV Saline) then sGVS vs (RIPC + Doxazosin) then sGVS	**<0.0001**	**<0.0001**	**0.0001**
(RIPC + IV Saline) then sGVS vs (RIPC + Atenolol) then sGVS	**0.0002**	**0.0004**	0.0623
(RIPC + Labetalol) then sGVS vs (RIPC + Doxazosin) then sGVS	**0.0048**	0.5758	**0.0273**
(RIPC + Labetaol) then sGVS vs (RIPC + Atenolol) then sGVS	0.7956	0.9998	0.8714
(RIPC + Doxazosin) then sGVS vs (RIPC + Atenolol) then sGVS	0.1229	0.7472	0.3017

Table S9. Minimum Values of the Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow During Stimulation. The p-values for the minimum value of the percent change from baseline (standard deviation) for each physiological parameter during stimulation (Table 7 Experiment 4 IN Interventions) are reported. Bold values indicate statistical significance (*i.e.* p<0.05). n=8/group.

	Mean Arterial Pressure	Heart Rate	Cerebral Blood Flow
Sham vs (Vehicle PC + IN Saline) then sGVS	**0.0106**	**0.0010**	**0.0071**
Sham vs (RIPC + IN Saline) then sGVS	0.9849	0.9294	0.9981
Sham vs (RIPC + Desipramine) then sGVS	**<0.0001**	**<0.0001**	**0.0004**
(Vehicle PC + IN Saline) then sGVS vs (RIPC + IN Saline) then sGVS	**0.0044**	**0.0002**	**0.0046**
(Vehicle PC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	**0.0031**	**<0.0001**	0.7109
(RIPC + IN Saline) then sGVS vs (RIPC + Desipramine) then sGVS	**<0.0001**	**<0.0001**	**0.0003**

Table S10. Exact p-values for the intergroup comparisons of the Mean (standard deviation) reported for the serum concentrations of norepinephrine and norepinephrine in Experiment 3 (Figure 12). Bold values indicate statistical significance (*i.e.* p<0.05). n=7/group.

	Before RIPC	Post-RIPC		
		0 Min	30 Min	60 Min
First Day of RIPC				
Norepinephrine Vehicle PC vs RIPC	0.2556	**<0.0001**	**<0.0001**	**<0.0001**
Epinephrine Vehicle PC vs RIPC	>0.9999	**0.0038**	0.1601	**0.0032**
Last (10th) Day of RIPC				
Norepinephrine Vehicle PC vs RIPC	0.9974	0.5766	0.6320	**0.0006**
Epinephrine Vehicle PC vs RIPC	0.8599	0.2617	0.1129	0.1000

Supplemental Figures

Figure S1. Effect of Unilateral Hindlimb and Bilateral Hindlimb RIPC on sGVS-Induced Cardio- and Cerebro-vascular Depressions. sGVS was performed 5 days after completing RIPC (Day 5, Top Row, A-C) or 10 days after RIPC (Day 10, Bottom Row, D-F). ***** $p<0.05$ between the two groups at the indicated time-point. n=8/group. Mean and SD are plotted. Repeated measures two-way ANOVA with Sidak post-hoc.

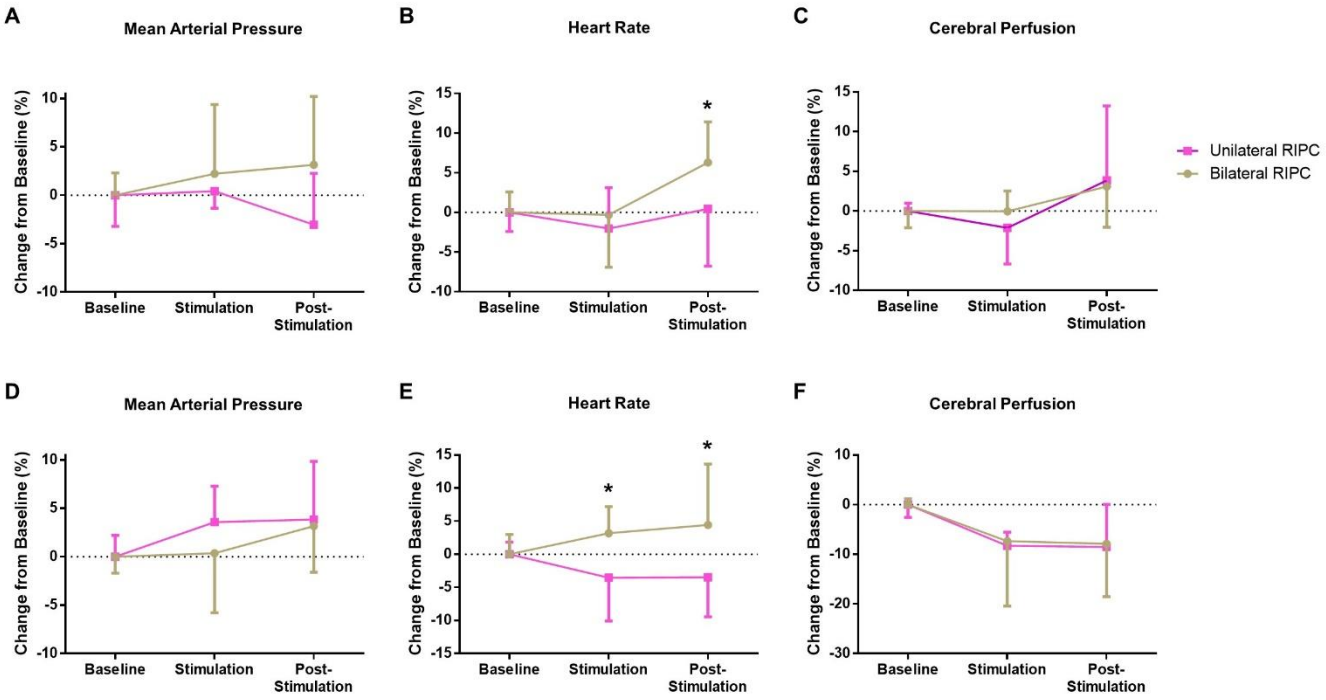


Figure S2. Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow on the First Day of RIPC (Day -10). Rats (n=4/group) from Experiment 3 were subjected to femoral artery catheterization for measurement of the mean arterial pressure (**A**), heart rate (**B**), and cerebral blood flow (**C**). Five minutes of Baseline was collected before beginning preconditioning. Left panels show the physiological parameters during Baseline, the combined ischemic cycles (cycles 1-4), the combined reperfusion cycles (cycles 1-4), and 5 minutes post-preconditioning. The middle panels show the physiological parameters during Baseline and for each 10-minute cycle of ischemia. The right panels show the physiological parameters during Baseline and for each 10-minute cycle of reperfusion. # p<0.05 between the two groups at the indicated timepoint. The p-values between the two groups for the Mean Arterial Pressure Reperfusion Cycles 3 and 4 (Top Left Panel) were p=0.0529 and p=0.0570, respectively. n=4/group. Mean and SD are plotted. Repeated measures two-way ANOVA with Sidak post-hoc.

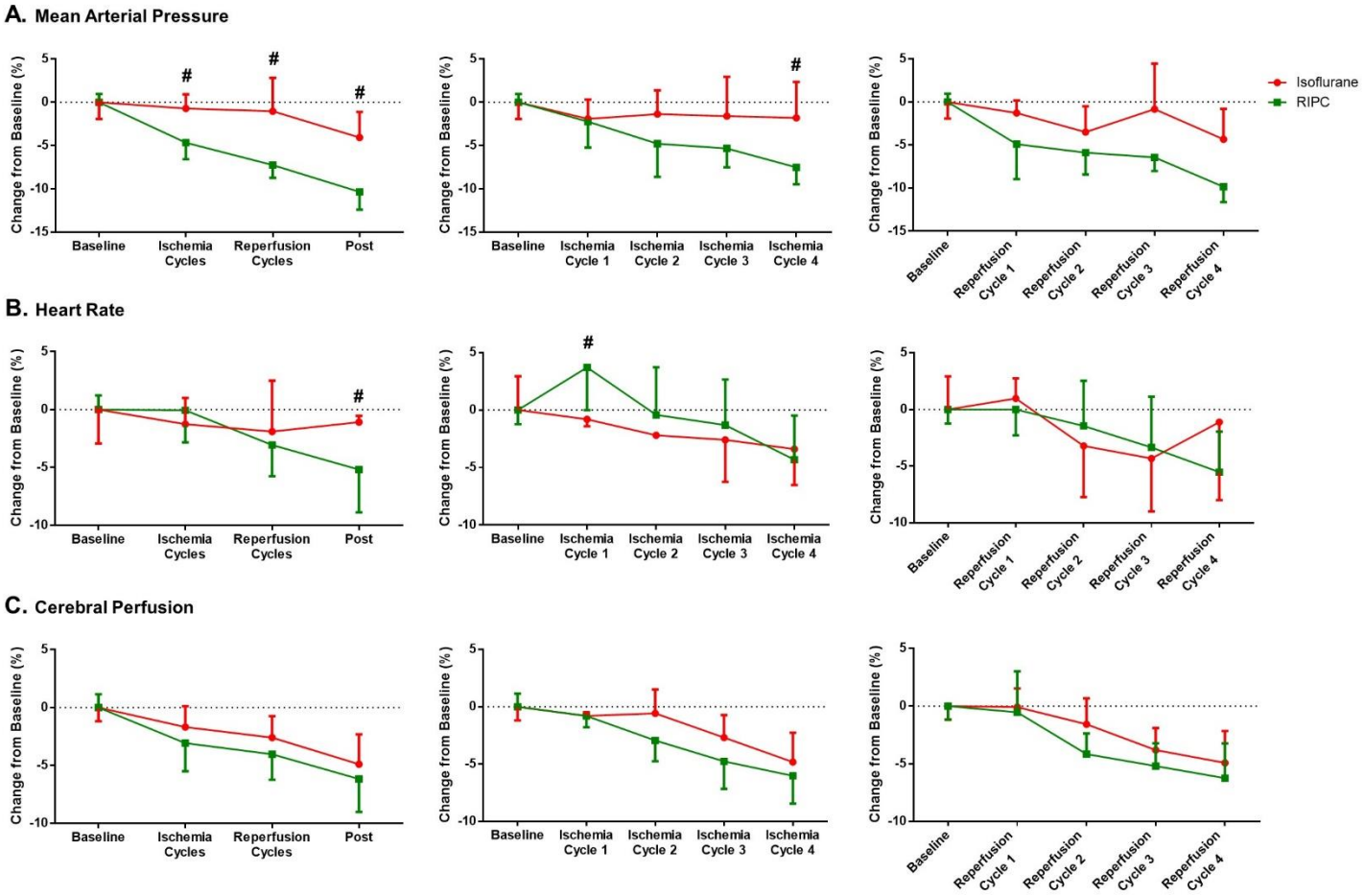


Figure S3. Mean Arterial Pressure, Heart Rate, and Cerebral Blood Flow on the Last Day of RIPC (Day 0). Rats (n=4/group) from Experiment 3 were subjected to femoral artery catheterization for measurement of the mean arterial pressure (**A**), heart rate (**B**), and cerebral blood flow (**C**). Five minutes of Baseline was collected before beginning preconditioning. Left panels show the physiological parameters during Baseline, the combined ischemic cycles (cycles 1-4), the combined reperfusion cycles (cycles 1-4), and 5 minutes post-preconditioning. The middle panels show the physiological parameters during Baseline and for each 10-minute cycle of ischemia. The right panels show the physiological parameters during Baseline and for each 10-minute cycle of reperfusion. # $p<0.05$ between the two groups at the indicated timepoint. n=4/group. Mean and SD are plotted. Repeated measures two-way ANOVA with Sidak post-hoc.

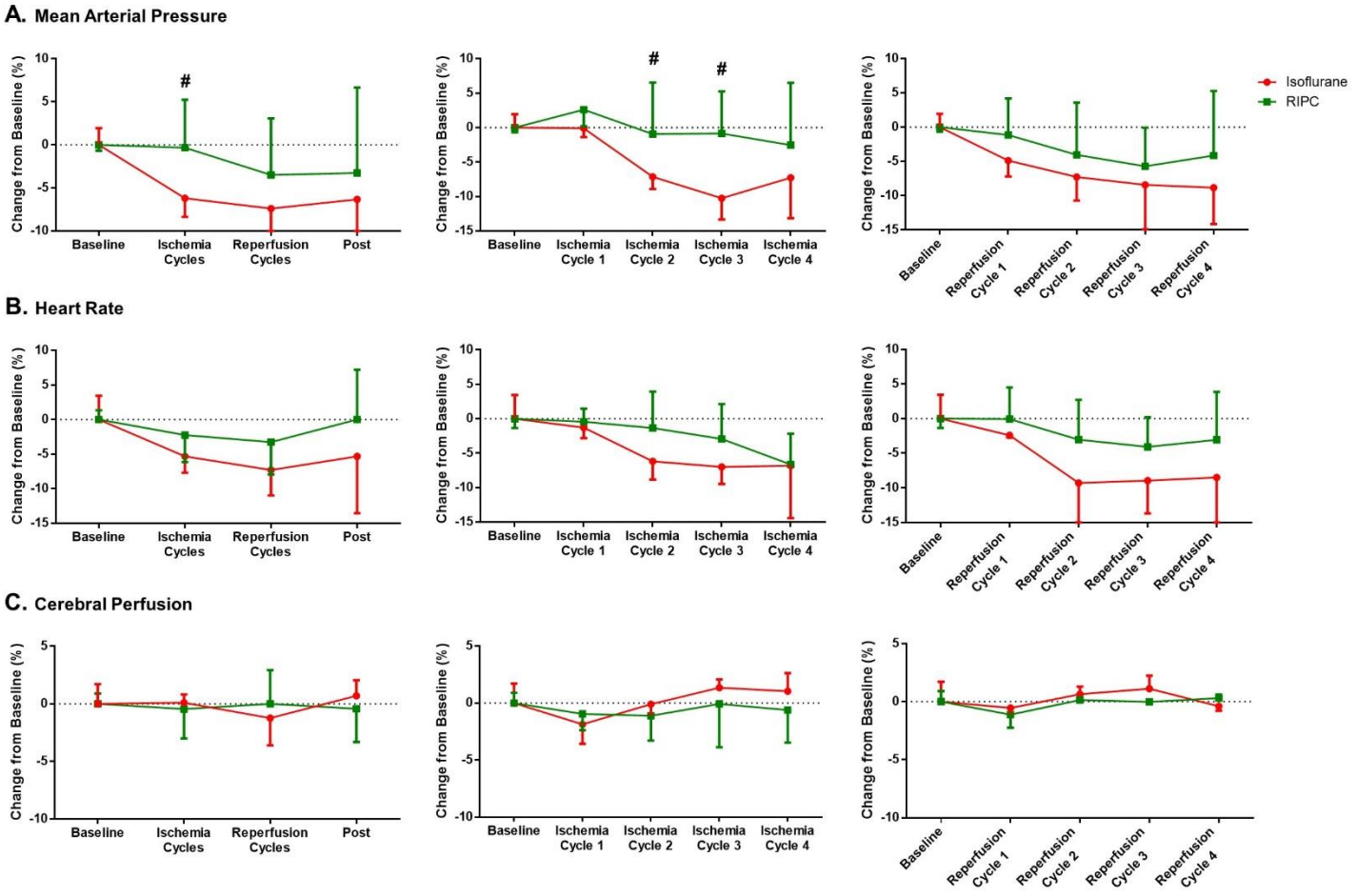


Figure S4. Schematic of the Experimental Timeline for Experiment 5. Animals were subjected to nothing (Sham), isoflurane (Vehicle PC), or RIPC with the last day of the regimen completed 10 days before sGVS. Two RIPC regimens were used: 5 days of RIPC (Day -4 to Day 0) and 10 days of RIPC (Day -9 to Day 0). Mean arterial pressure, heart rate, and cerebral blood flow were monitored on the day of sGVS (Day 10).

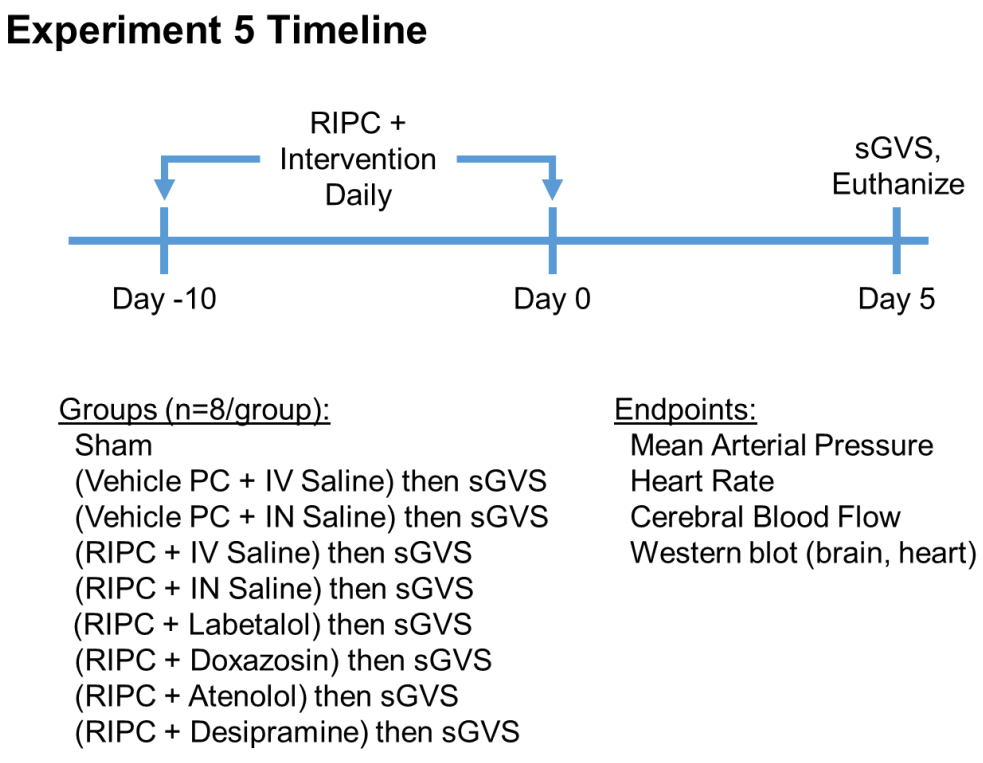


Figure S5. The Benefits of RIPC against sGVS-Induced Cardio-vascular Depression Extends for 10 Days after Stopping Preconditioning. ***** p<0.05 for Sham vs (Vehicle PC then sGVS), # p<0.05 for (Vehicle PC then sGVS) vs (RIPC (10 days) then sGVS), **₹** p<0.05 for (Vehicle PC then sGVS) vs (RIPC (5 days) then sGVS), **ɧ** p<0.05 for Sham vs (Vehicle PC then sGVS) and Sham vs (RIPC (5 days) then sGVS), **ɤ** p<0.05 for (RIPC (10 days) then sGVS) vs (Vehicle PC then sGVS) and (RIPC (10 days) then sGVS) vs (RIPC (5 days) then sGVS). n=8/group. Mean and SD are plotted. Repeated measures two-way ANOVA with Tukey post-hoc.

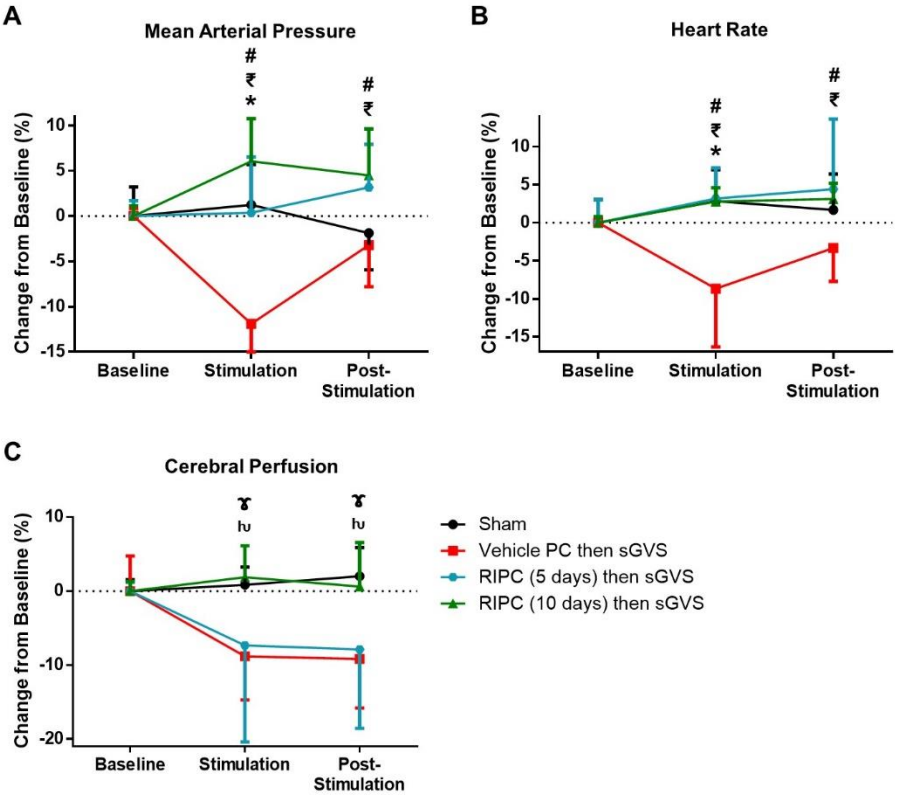


Figure S6. Serum Catecholamines During the First and Last Days of Preconditioning. # p<0.05 (Isoflurane + Saline) vs (RIPC + Saline), **&** p<0.05 for (Isoflurane + Saline) vs (RIPC + Saline) and (Isoflurane + Saline) vs (RIPC + Labetalol), @ p<0.05 for (RIPC + Labetalol) vs (Isoflurane + Saline) and (RIPC + Labetalol) vs (RIPC + Saline), **$** p<0.05 for (RIPC + Labetalol) vs (Isoflurane + Saline). n=7/group. Mean and SD are plotted. Repeated measures two-way ANOVA with Tukey post-hoc.

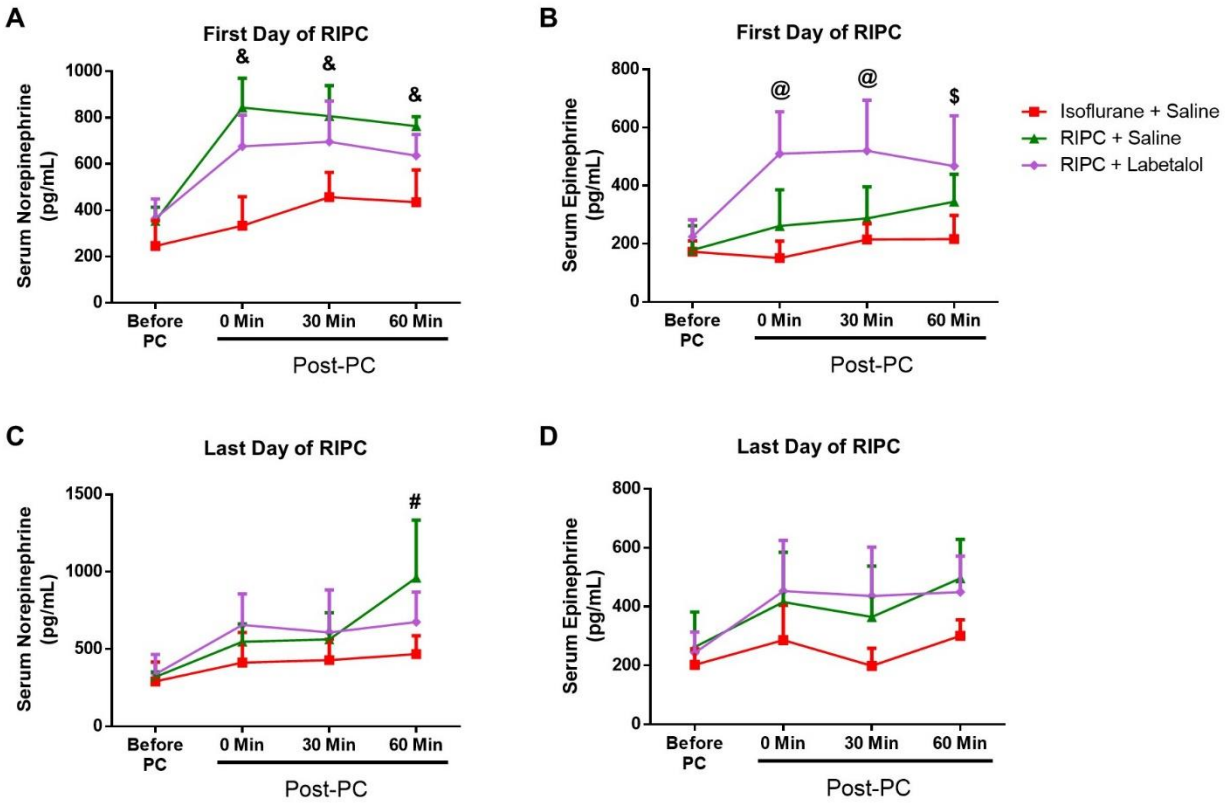


Figure S7. Representative Western blots (**A**) and Quantification (**B**-**D**) of α_1- and β_1-adrenoceptors, and NET 1 in the brain after preconditioning. Quantification is identical to the graphs in Figure 12. # $p<0.05$ vs Vehicle PC. n=6/group.

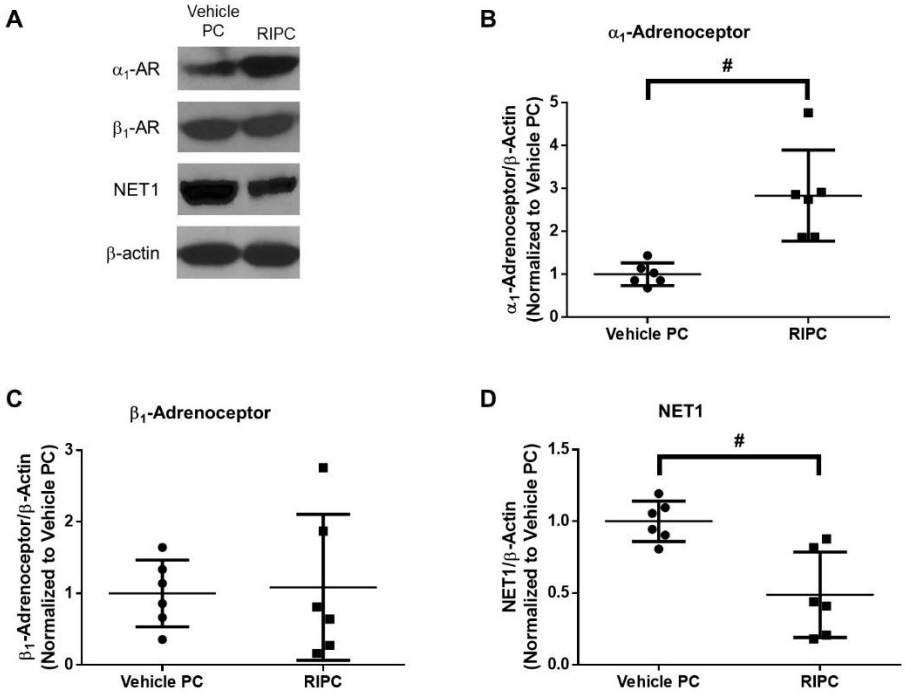


Figure S8. Brain Expressions of α_1- and β_1-Adrenoceptors, and NET1 after Preconditioning. **A.** Representative Western blots. **B-D.** Quantification of the Western blot films for α_1-adrenoceptor, β_1-adrenoceptor, and NET1. # p<0.05 for Vehicle PC vs RIPC, **&** p<0.05 for RIPC vs (RIPC + Labetalol) and (Vehicle PC + Saline) vs (RIPC + Labetalol). n=6/group. Dots indicate individual values. Mean and SD are plotted. One-way ANOVA with Tukey post-hoc.

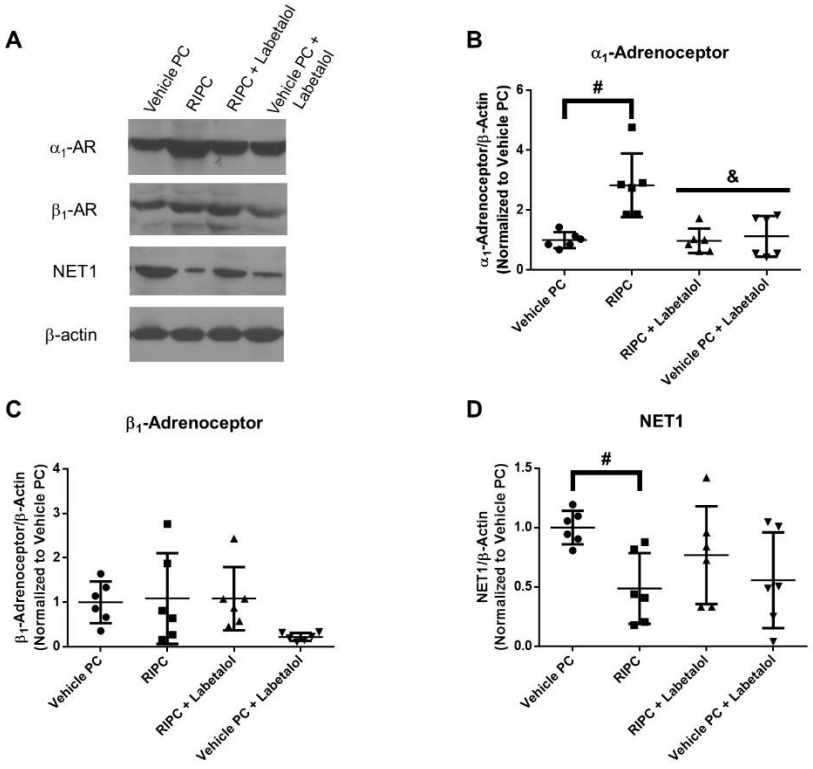


Figure S9. Representative Western blots (**A**) and Quantification (**B**-**F**) of α_1- and β_1-adrenoceptors, NET1, PKCε (Particulate/Cytosolic), and eNOS (p-eNOS/eNOS) in the brain after sGVS. Quantification is identical to the graphs in Figure 15. ↯ p<0.05 vs Sham, # p<0.05 vs (Vehicle PC + Saline) then sGVS, **&** p<0.05 vs (RIPC + Saline) then sGVS. n=6/group.

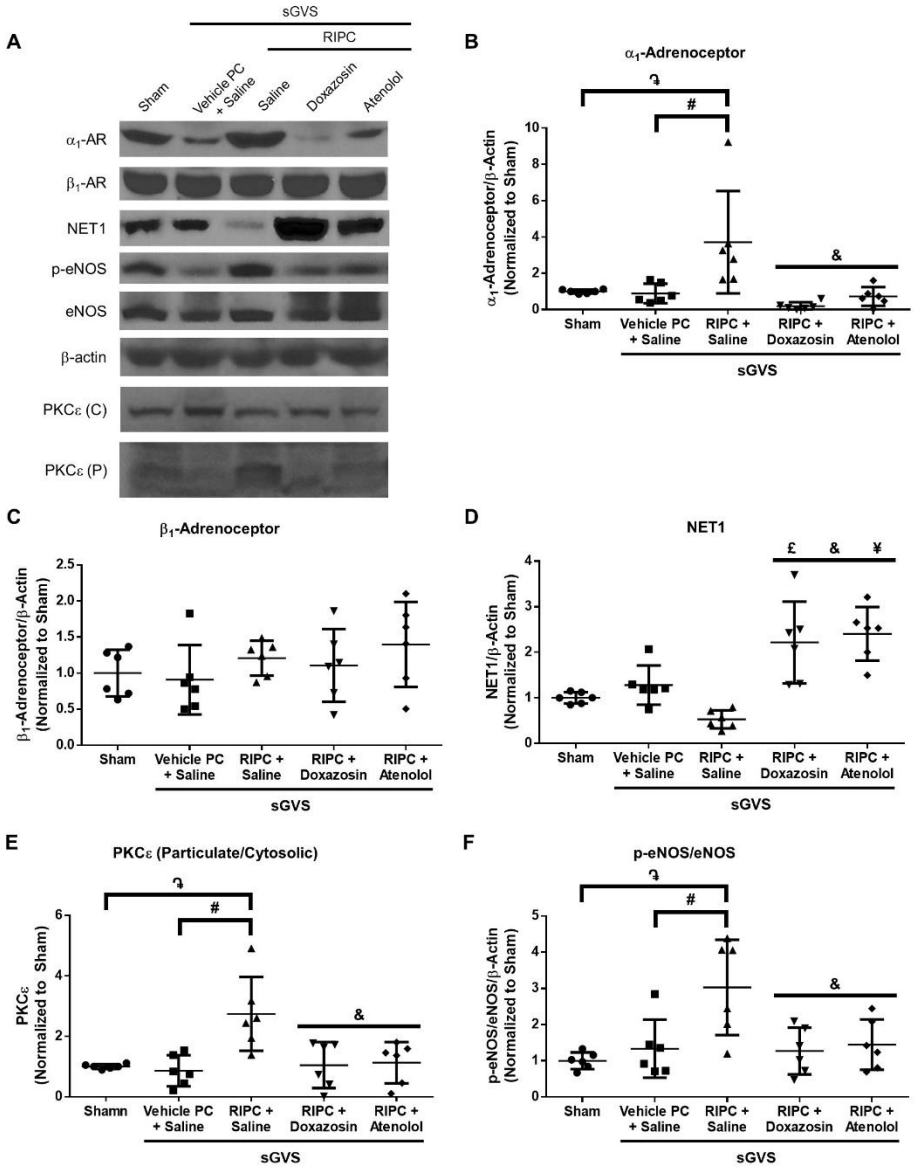


Figure S10. Representative Western blots (**A**) and Quantification (**B**-**D**) of α_1- and β_1-adrenoceptors, NET1, PKCε (Particulate/Cytosolic), and eNOS (p-eNOS/eNOS) in the brain after sGVS. Quantification is identical to the graphs in Figure 16. ☭ p<0.05 vs Sham, # p<0.05 vs (Vehicle PC + Saline) then sGVS, ⋔ p<0.05 vs (RIPC + Saline) then sGVS. n=6/group.

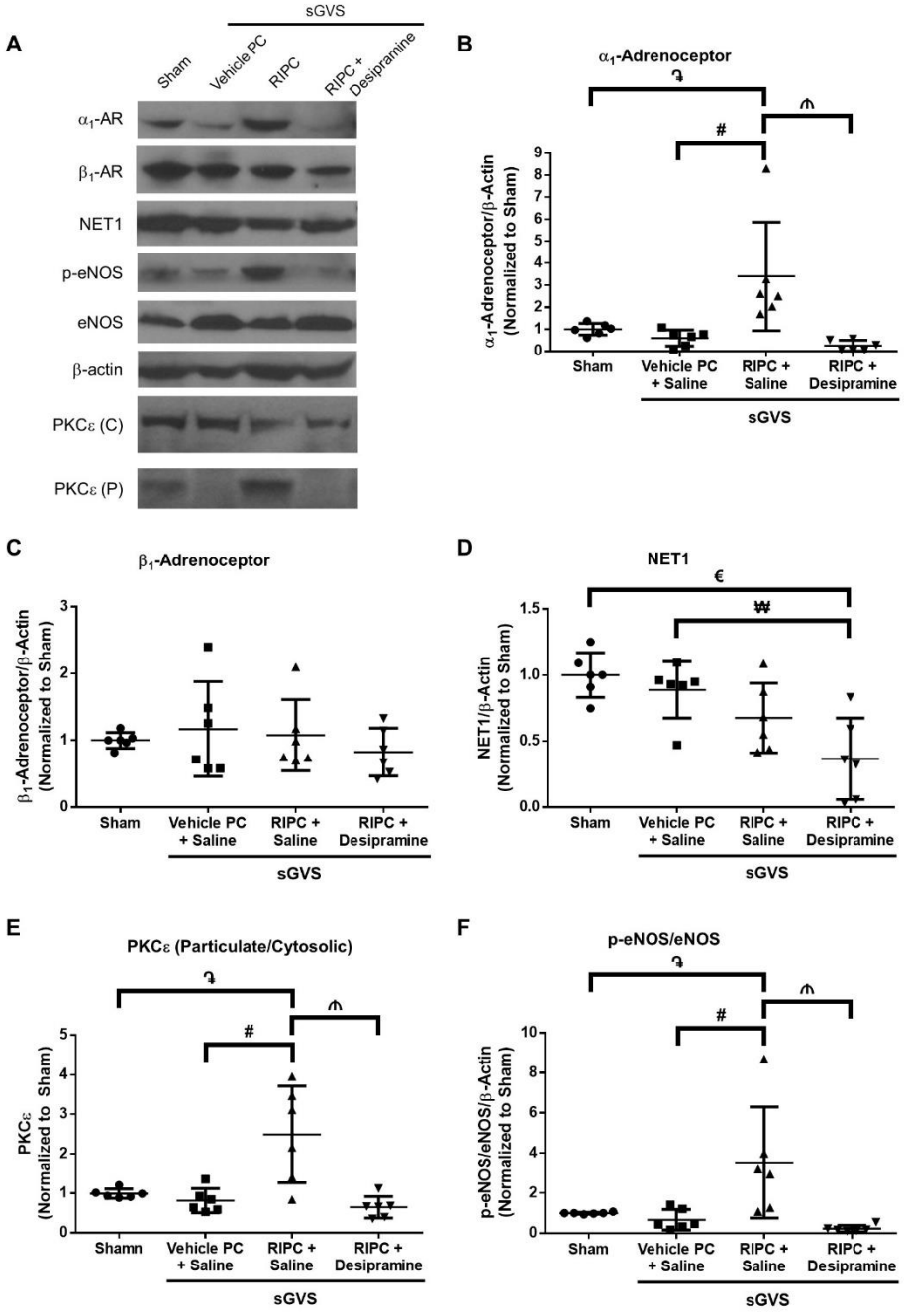
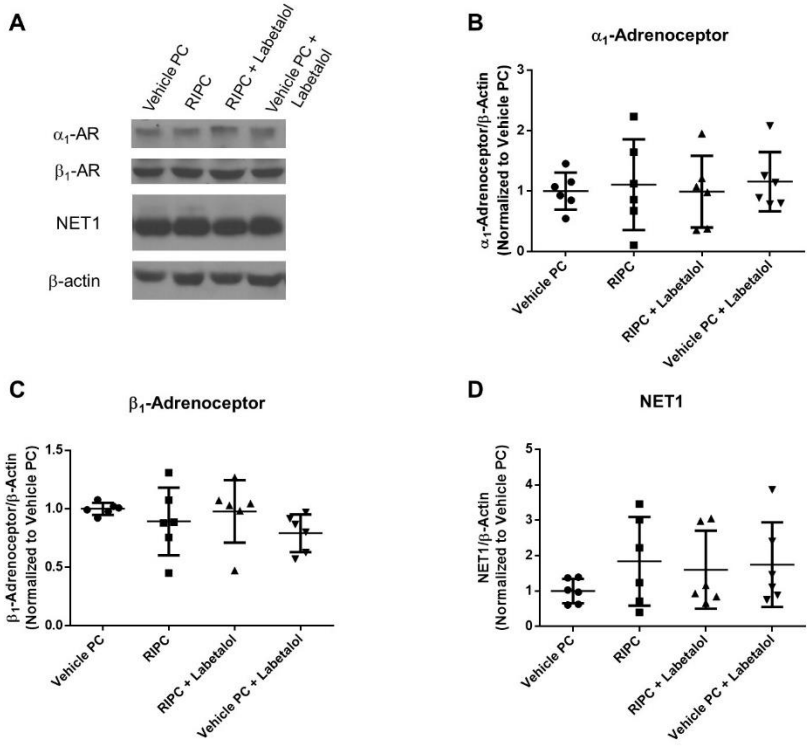


Figure S11. Heart Expressions of α_1- and β_1-Adrenoceptors, and NET1 after Preconditioning. **A.** Representative Western blots. **B-D.** Quantification of the Western blot films for α_1-adrenoceptor, β_1-adrenoceptor, and NET1. No statistical significance is observed between any group pairings for any of the proteins. n=6/group. Dots indicate individual values. Mean and SD are plotted. One-way ANOVA with Tukey post-hoc.



Supplemental References

1. Jardine DL. Vasovagal syncope: New physiologic insights. *Cardiol Clin*. 2013;31:75-87
2. Zhao L, Nowak TS, Jr. Cbf changes associated with focal ischemic preconditioning in the spontaneously hypertensive rat. *J Cereb Blood Flow Metabol*. 2006;26:1128-1140
3. Ostrowski RP, Schulte RW, Nie Y, Ling T, Lee T, Manaenko A, Gridley DS, Zhang JH. Acute splenic irradiation reduces brain injury in the rat focal ischemic stroke model. *Transl Stroke Res*. 2012;3:473-481
4. Boarini DJ, Kassell NF, Coester HC, Butler M, Sokoll MD. Comparison of systemic and cerebrovascular effects of isoflurane and halothane. *Neurosurgery*. 1984;15:400-409
5. Kuroda Y, Murakami M, Tsuruta J, Murakawa T, Sakabe T. Preservation of the ratio of cerebral blood flow/metabolic rate for oxygen during prolonged anesthesia with isoflurane, sevoflurane, and halothane in humans. *Anesthesiology*. 1996;84
6. Ornstein E, Young WL, Fleischer LH, Ostapkovich N. Desflurane and isoflurane have similar effects on cerebral blood flow in patients with intracranial mass lesions. *Anesthesiology*. 1993;79:498-502
7. Turner DM, Kassell NF, Sasaki T, Comair YG, Boarini DJ, Beck DO. Time-dependent changes in cerebral and cardiovascular parameters in isoflurane-nitrous oxide-anesthetized dogs. *Neurosurgery*. 1984;14:135-141
8. Gürdal H, Can A, Uğur M. The role of nitric oxide synthase in reduced vascocontractile responsiveness induced by prolonged a_1-adrenergic receptor stimulation in rat thoracic aorta. *Br J Pharmacol*. 2005;145:203-210