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TONICALLY ACTIVE cAMP DEPENDENT SIGNALLING IN THE VENTROLATERAL MEDULLA REGULATES SYMPATHETIC AND CARDIAC VAGAL OUTFLOWS

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RUNNING TITLE: cAMP signalling in the ventrolateral medulla

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List of non-standard abbreviations

AP - arterial pressure

cAMP - cyclic adenosine monophosphate

EPAC - exchange protein activated by cAMP

GPCR - G-protein coupled receptors

H2 receptor - histamine 2 receptor

HCN - hyperpolarization activated cyclic nucleotide–gated

HR - heart rate

MC3/4 receptor - melanocortin 3/4 receptor

PACAP - pituitary adenylate cyclase-activating peptide

PKA - protein kinase A

RVLM - rostral ventrolateral medulla

sSNA - splanchnic sympathetic nerve activity

ABSTRACT

The ventrolateral medulla contains presympathetic and vagal preganglionic neurons that control vasomotor and cardiac vagal tone respectively. G protein coupled receptors influence the activity of these neurons. Gαs activates adenylyl cyclases which drive cAMP-dependent targets: protein kinase A (PKA), the exchange protein activated by cAMP (EPAC) and hyperpolarization activated cyclic nucleotide –gated (HCN) channels. The aim was to determine the cardiovascular effects of activating and inhibiting these targets at presympathetic and cardiac vagal preganglionic neurons. Urethane-anaesthetized rats were instrumented to measure splanchnic sympathetic nerve activity (sSNA), arterial pressure (AP), heart rate (HR) as well as baroreceptor and somatosympathetic reflex function or, were spinally transected and instrumented to measure HR, AP and cardiac baroreflex function. All drugs were injected bilaterally. In the rostral ventrolateral medulla Sp-cAMPs and 8-Br-cAMP, which activate PKA, as well as 8-pCPT, which activates EPAC, increased sSNA, AP and HR. Sp-cAMPs also facilitated the reflexes tested reflexes. Sp-cAMPs also increased cardiac vagal drive and facilitated cardiac baroreflex sensitivity. Blockade of PKA, using Rp-cAMPs or H-89 in RVLM increased sSNA, AP and HR and increased HR when cardiac vagal preganglionic neurons were targeted. BFA, which inhibits EPAC, and ZD7288, which inhibits HCN channels, alone each had no effect. Cumulative, sequential blockade of all three inhibitors resulted in sympathoinhibition. The major findings indicate that Gαs linked receptors in the ventral medulla can be recruited to drive both sympathetic and parasympathetic outflows and that tonically active PKA-dependent signaling contributes to the maintenance of both sympathetic vasomotor and cardiac vagal tone.

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INTRODUCTION

Key centres for the autonomic control of vasomotor tone and heart rate are located in the ventrolateral medulla oblongata.

Presympathetic neurons of the rostral ventrolateral medulla (RVLM) regulate the activity of sympathetic preganglionic neurons of the spinal cord, predominantly those controlling vasomotor tone (Dampney, 1994; Pilowsky & Goodchild, 2002; Guyenet, 2006). Cardiac vagal preganglionic neurons are localised primarily in the nucleus ambiguus and innervate cardiac ganglia to control heart rate (Wang *et al.*, 2001). The tonic activity of both these neuronal populations in the ventrolateral medulla is now accepted to be dependent on synaptic drive resulting from the sum of excitatory and inhibitory input (Wang *et al.*, 2001; Lipski *et al.*, 2002; Guyenet, 2006). Blockade of ionotropic glutamate receptors in both regions however fails to decrease sympathetic vasomotor tone or increase HR respectively despite the fact that blockade of GABA-A receptors in these regions has clear directionally opposite responses (Dampney *et al.*, 2003; Hildreth & Goodchild, 2010). Inputs arising from multiple brain sites are encoded by a plethora of not only ionotropic but also G-protein coupled receptors (GPCR) present in the region (Lovick, 1985; Dampney, 1994; Bowman *et al.*, 2013). Those GPCR's that can be recruited to drive or tonically modulate these two neuronal populations have not been clearly identified.

The multitude of GPCRs are linked to heterotrimeric G-proteins whose α subunits signal via three major intracellular proteins: adenylyl cyclase, phospholipase C-β and Rho (Brown & Sihra, 2008). Despite this convergence, the expression and functions of G-protein related signalling molecules in controlling cardiovascular autonomic functions mediated by the ventrolateral medulla are poorly understood. We have previously demonstrated that mRNA for all Gα proteins are expressed in the ventrolateral medulla, with Gαs most abundant (Parker *et al.*, 2012). Gαs mRNA is present in all adrenergic C1 neurons, an important cardiovascular subpopulation within the region. Gαs proteins couple to adenylyl cyclases which catalyse the conversion of ATP to cAMP. cAMP in turn can activate three downstream targets: cAMP dependent protein kinase (PKA), exchange proteins activated by cAMP (EPAC) and hyperpolarization activated cyclic nucleotide gated (HCN) channels (Beavo & Brunton, 2002; Bos, 2003; Holz *et al.*, 2006). Cardiovascular autonomic functions regulated by cAMP in ventrolateral medulla are the focus of this study.

The objective is to determine whether Gαs linked receptors can drive and/or tonically modulate outputs from the ventral medulla. Specifically the aims are to determine: the effects of (1) activating or (2) inhibiting cAMP-dependent effectors on splanchnic sympathetic outflow, blood pressure,

heart rate, and baroreceptor and somatosympathetic reflex function mediated by RVLM presympathetic and cardiac vagal pathways originating in the ventrolateral medulla.

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MATERIALS AND METHODS

All experiments were approved by the Macquarie University Animal Ethics Committee (Protocol Number 2009-019) and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgical preparation

A total of 47 male Sprague-Dawley rats (350 - 450 g) were used. Rats were anaesthetized with urethane (1.2 – 1.3 g / kg; i.p.) and depth of anaesthesia was assessed every 30 – 40 min by monitoring withdrawal, respiratory, or blood pressure responses to firm pinch of the hind paw. Additional doses of urethane (20 - 30 mg, i.v.) were given as required. Core temperature was maintained between 36.5 °C and 37.0 °C with a feedback-controlled heating blanket (Harvard Apparatus, Holliston, MA).

Both femoral veins and the right femoral artery were cannulated for the administration of drugs and fluids and for the measurement of arterial blood pressure, respectively. Heart rate was derived from R-wave of the electrocardiogram (ECG) obtained from leads attached to both forepaws and one hind limb. A tracheotomy was performed to permit artificial ventilation. Rats were secured in a stereotaxic frame.

Procedures specific for assessment of RVLM vasomotor function.

Rats were vagotomised. The left greater splanchnic nerve was dissected via a retroperitoneal approach and cut at the distal end to permit recording of efferent nerve activity. The sciatic nerve was isolated, cut at the distal end and stimulated to drive the somatosympathetic reflex response. Rats were paralyzed with pancuronium bromide (0.4 mg given as 0.2 ml bolus i.v, then an infusion of 20% pancuronium in 0.5% glucose in saline at 1.5 ml/hr), and artificially ventilated with oxygen-enriched room air. End-tidal CO_2 was monitored and blood gases measured regularly; ventilation was adjusted to maintain P_aCO_2 and pH within a physiological range (P_aCO_2 40 ± 3 mmHg; pH 7.35 - 7.45). The dorsal medullary surface was exposed by occipital craniotomy and nerves were mounted on bipolar silver wire electrodes and covered in paraffin oil.

The RVLM was mapped on both sides by pneumatic microinjection of glutamate, as previously described (Burke *et al.*, 2008). Pressor sites were located 1.8 – 2.2 mm rostral and 1.6 – 2.0 mm lateral to the calamus scriptorius and between 3.3 – 3.8 mm ventral to the brainstem surface. A site was considered to be within the RVLM if a 50 nl microinjection of 100 mM glutamate caused a rise in blood pressure ≥ 35 mmHg.

Procedures specific for assessment of cardiac vagal function.

Rats were spinally transected between cervical segments 7-8 and cardioinhibitory regions of both sides of the brain were identified by glutamate microinjection (50 nl, 100 mM) as described previously (Hildreth & Goodchild, 2010). Sites in and around the nucleus ambiguus from which bradycardic responses greater than 50 bpm were selected for injection of drugs.

Experimental protocols

In the RVLM, in initial studies, the cumulative dose response evoked by drugs was performed in order to determine effective doses to be used. Bilateral injections of 50 nl per side were made with increasing doses of each drug. Only one drug and vehicle was used in each animal.

Each drug was then assessed using the same protocol. Following a control period of recording bilateral microinjections of 50 nl per side of the test drug (or vehicle) were made into the selected sites. Supramaximal somatosympathetic (2-15 V sciatic nerve stimulation, 50 x 0.1 ms pulses at 1 Hz) and baroreceptor reflexes (sequential injection of SNP (10 μg in 0.4 ml saline) and PE (10 μg in 0.4 ml saline) via two different femoral venous cannulae), as previously described (Burke *et al.*, 2008) were activated before, and every 5-10 min after drug injection for up to one hour. Only one drug was tested in each animal except for one study in which the three inhibitors were sequentially injected. Parameters measured were sSNA, AP, HR, sympathetic baroreflex and somatosympathetic reflex function. Cardiac baroreflex function was not measured in these animals.

For experiments investigating cardiac vagal pathways, two 100 nl injections of the vehicle followed by the test drug were made, on each side approximately 600 μm apart, to effectively target cardiac vagal preganglionic neurons (as described previously (Hildreth & Goodchild, 2010)). Injection of PE (10 μg/kg) permitted calculation of heart rate baroreflex sensitivity (BRS) before and after vehicle and drug injection and effects on HR were also monitored.

At the conclusion of recordings injection sites were marked with 50-100 nl of ink/dye and the animal was euthanized (0.8 ml of 3 M KCl, i.v.). Brainstems were removed, drop-fixed in 4 % formaldehyde overnight and cryo-preserved until histological processing. 100 μm coronal sections were cut on a vibratome and injection sites verified.

Data acquisition and analysis

Neurograms were amplified (gain: 10 000, CWE Inc, Ardmore, PA, USA), band- pass filtered (0.1 - 2 kHz), sampled at 3 kHz (1401 plus, CED Ltd, Cambridge, UK) and rectified and smoothed with a 2 sec time constant (Spike 2, CED Ltd). For RVLM microinjections, bilateral injections of

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phosphate buffered saline (PBS) preceded all drug microinjections. Peak changes in mean arterial pressure (MAP), heart rate (HR) and splanchnic sympathetic nerve activity (sSNA) were measured with respect to control data measured over 120 s 5 min prior to drug/PBS injection. For time course analysis 120 s blocks of data were averaged every 10 min. sSNA activity was normalized with respect to background noise post-mortem (0%) and baseline activity prior to vehicle injection (100%). sSNA response to sciatic nerve stimulation was analyzed using peri-stimulus waveform averaging (McMullan *et al.*, 2008); baroreceptor function curves were generated as previously described (Burke *et al.*, 2008). Analyses for baroreceptor and somatosympathetic reflex function were conducted 5 - 20 min post target drug injection. For CVPN microinjection, peak changes in HR and BRS were calculated as described previously (Hildreth & Goodchild, 2010).

Analysis was conducted using GraphPad Prism (v 5.0). All values are expressed as mean ± standard error. One-way ANOVA or paired or unpaired Student's t-test was used to analyze drug effects on baseline and reflex parameters. $p < 0.05$ was considered significant.

Drugs

The following drugs were used in this study; L-glutamate disodium salt, Sp-cAMPs (Sp-Diastereomer of adenosine 3', 5'-cyclic monophosphorothioate), 8-Br-cAMP (8-Bromoadenosine 3',5'-cyclic monophosphate, Rp-cAMPs (Rp-Diastereomer of adenosine 3', 5'-cyclic monophosphorothioate), H-89 (*N*-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride), 8-pCPT (8-pCPT-2'-O-Me-cAMP), BFA (Brefeldin A), ZD-7288, phenylephrine (PE) and sodium nitroprusside (SNP) and all were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pancuronium bromide was obtained from AstraZeneca Pty Ltd (NSW, Australia). Drugs were dissolved in phosphate buffered saline PBS (10 mM; pH 7.4) except for BFA which was first solubilized in ethanol before dilution in PBS. Urethane, PE and SNP were prepared in 0.9 % NaCl.

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RESULTS

Activating cAMP dependent pathways in the RVLM

Cell permeable drugs that activate downstream effectors PKA, EPAC and HCN channels were microinjected into the RVLM in order to determine the effects of cAMP stimulation on cardiovascular tonic and reflex function.

cAMP analogues in the RVLM: effects on baseline parameters

Bilateral cumulative microinjection of two cAMP analogues Sp-cAMPs (0.5, 1.5 and 5 nmol, n=4) and 8-Br-cAMP (1 and 10 nmol, n=4) increased sSNA (Sp-cAMPs: $F_{(3, 11)} = 5.9$, $p = 0.011$; Br-cAMP: $F_{(2, 6)} = 60.8$, $p = 0.0001$) and MAP (Sp-cAMPs: $F_{(3, 11)} = 8.4$, $p = 0.0035$; Br-cAMP: $F_{(2, 6)} = 20.7$, $p = 0.002$) in a dose-dependent manner (Fig 1). Effects were rapid and prolonged. 5 nmol Sp-cAMPs was selected for detailed investigation.

Microinjection of Sp-cAMPs (5 nmol, n = 6) evoked increases in sSNA, HR and MAP that were maximal at 10-20 minutes whereas vehicle had little effect (Fig 2A-D). sSNA and HR remained elevated for the remainder of the experiment (>1 hour), whereas MAP recovered within 40 minutes (Fig 2A-D). Sp-cAMPs (5 nmol, n = 6) evoked significant peak increases in sSNA, MAP and HR compared to control (PBS n=6) ($p < 0.01$ for all parameters) (Fig 2E).

Sp-cAMP in the RVLM: effect on sympathetic reflexes

The effect of Sp-cAMPS in the RVLM was tested on reflexes that are dependent upon the GABAergic (baroreflex (Schreihofer & Guyenet, 2003)) or glutamatergic (somatosympathetic reflex (Kiely & Gordon, 1993)) synapses within the RVLM.

Microinjection of Sp-cAMPs significantly increased the upper plateau and maximum gain of the sympathetic baroreflex function curve compared to control (PBS) (Fig 3A & Table 1). The data were acquired from experiments represented in Fig 2. Importantly cardiac baroreflex changes are not reported following drug injection into the RVLM (however see Fig 8).

Intermittent stimulation of the sciatic nerve resulted in a characteristic two-phase excitatory response in sSNA. The total AUC (22.8 ± 4.9 vs 13.6 ±3.1 au, Sp-cAMPs vs PBS p = 0.0083) but not the amplitude of the two peaks (89.6 ± 21.2 % (peak 1) and 87.7 ± 29.8 % (peak 2) vs 120 ± 19.6 % and 103.6 ± 28.8 % sSNA; Sp-cAMPs vs PBS n.s.) was significantly affected by Sp-cAMPs compared to PBS (Fig 3B). The effect of Sp-cAMPs was to increase the width of the 2nd peak particularly at more delayed latencies.

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8-pCPT in RVLM: effects on baseline parameters

Bilateral microinjection of 8-pCPT (5 nmol in 50nl, n=6), a cAMP analogue which selectively activates EPAC (Vliem *et al*., 2008), into the RVLM increased sSNA, AP and HR (Fig 4A). The grouped time-course data are shown in Fig 4B-D and the peak responses compared to bilateral PBS injection are shown in Fig 4E. 8-pCPT significantly increased sSNA (p<0.01). Increases in MAP and HR were not statistically significantly different.

8-pCPT in RVLM: effects on sympathetic reflexes

8-pCPT significantly increased the upper plateau and the maximum gain of the sympathetic baroreflex (Fig 3C & Table 1). In contrast, 8-pCPT evoked no significant effect on somatosympathetic reflex parameters (Fig 3D): total AUC (8.7 ± 2.6 vs 6.3 ± 1.4 au, 8-pCPT vs PBS n.s.) and peak heights (66.9± 21.9 % (peak 1) and 39.23 ± 23.9 (peak 2) vs 64.1 ± 9.7 % and 44.3 ± 18.1 sSNA; 8-pCPT vs PBS, n.s.).

Blocking cAMP dependent pathways in the RVLM

In order to determine whether the downstream effectors of cAMP, PKA, EPAC and HCN channels are tonically activated in the RVLM their effects were individually blocked using cell permeable, selective pharmacological agents.

Inhibition of PKA in RVLM: effects on baseline parameters

Bilateral microinjection of the PKA inhibitor, Rp-cAMPs (5 nmol in 100 nl, n = 5) into the RVLM evoked increases in sSNA, biphasic changes in AP and small increases in HR (Fig 5A). The grouped time-course data are shown in Fig 5B-D and peak changes shown in Fig 5E. Rp-cAMPs compared to PBS evoked significant increases in sSNA (p <. 0.001), MAP p < 0.01) and HR (p < 0.05).

Similar effects were observed following bilateral microinjection of another inhibitor of PKA, H-89 (1 and 10 nmol in 50 nl, n = 3) although these recordings lasted only 30 min. There was a significant effect of H-89 on sSNA ($F_{(2, 9)}$ =4.5, p=0.04) and MAP ($F_{(2, 9)}$ =16.1, p=0.001) following one-way ANOVA. H-89 evoked peak increases in sSNA of 17 ± 5% and 35% ± 10% and MAP of 15 ± 7 mmHg and 32 ± 5 mmHg (1 and 10 nmol, respectively).

Inhibition of PKA in RVLM: effects on sympathetic reflexes

Rp-cAMPs evoked no significant effects on baroreceptor (Figure 3E & Table 1) or somatosympathetic (Fig 3F) reflex parameters. Total AUC (14.1 ± 3.4 vs 9.1 ± 1.2 au, Rp-cAMPs

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vs PBS n.s.) and peak heights (87.3 ± 15.4 % (peak 1) and 95.4 ± 30.2 % (peak 2) vs 100.0 ± 46.4 % and 57 ± 22.2 % sSNA; Rp-cAMPs vs PBS, n.s.) were not altered.

Inhibition of EPAC with BFA in the RVLM blocks the sympathoexcitation evoked by 8-pCPT but alone has no effect

Bilateral microinjection of BFA (100 pmol in 100 nl, n = 4), an inhibitor of EPAC, into the RVLM had no significant effect on sSNA, MAP or HR (Fig 6) or on somatosympathetic or the baroreceptor reflex function (data not shown). However, when injections of 8-pCPT (5 nmol in 50 nl) were preceded 5 min earlier by bilateral injection of BFA (n = 3) no effect was seen over 60 min (Fig 6) indicating that BFA blocked the effects of 8-pCPT alone (data taken from Fig 4).

Blocking HCN channels in RVLM with ZD-2788 evokes no effects

Microinjection of ZD-7288, a specific antagonist of HCN channels (300 pmol in 100 nl, n=3) bilaterally into the RVLM did not alter sSNA, MAP or HR (data not shown) as reported previously (Miyawaki *et al.*, 2003).

Combined cAMP effector blockade in the RVLM evokes sympathoinhibition

In order to determine in the RVLM the combined effect of blocking three downstream effector proteins (PKA, EPAC and HCN channels) each was inhibited in succession (n = 5, Fig 7). Blockade of PKA was followed by inhibition of EPAC and then blockade of HCN channels at 10 min intervals. Figure 7A shows a representative example of sequential blockade of cAMP effectors and the grouped time course effects are shown in Fig 7B-D. The summed effect of blockade evoked a fall in all parameters with a peak decrease in sSNA of -30.8 ± 7.6% ($p < 0.05$) but non-significant decreases in MAP (-15 ± 6 mmHg, $p = 0.09$) and HR (-20 ± 7 bpm, $p = 0.08$).

Effects of activating or inhibiting cAMP-dependent pathways at cardiac vagal preganglionic neurons

In order to determine whether cAMP-dependent effects could be evoked in other functional pathways originating in the ventral medulla responses from cardiac vagal preganglionic neurons were evaluated.

Sp-cAMPs at cardiac vagal preganglionic neurons decreases HR and facilitates the cardiac baroreflex

Figure 8A shows the time course and effects evoked by bilateral microinjection of Sp-cAMPs (2 injections per side of 10 nmol in 100 nl, n = 5) at cardiac vagal preganglionic neurons. A decrease

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in HR was evoked and the haemodynamic and cardiac effects of modifying baroreceptor reflex function (PE) is seen. Sp-cAMPs decreased resting heart rate (307 ± 10 pre vs 273 ± 6 bpm post, $p < 0.01$) (Fig 8C) and increased BRS (0.46 ± 0.09 pre vs 0.71 ± 0.12 post bpm/mmHg, $p < 0.01$) (Fig 8C). Injection of vehicle at these sites evoked no significant effect on heart rate or BRS.

Rp-cAMPs at cardiac vagal preganglionic neurons decreases HR but has no effect of the cardiac baroreflex

Figure 8B shows the time course and effects evoked by subsequent bilateral microinjection of Rp-cAMPs (2 injections per side of 10 nmol in 100 nl, n = 4) at cardiac vagal preganglionic neurons. A decrease in HR was evoked and the haemodynamic and cardiac effects of modifying cardiac baroreceptor reflex function are seen. Rp-cAMPs injection decreased resting heart rate 299 ± 8 pre vs 240 ± 5 post bpm, $p < 0.05$) (Fig 8D) but did not significantly alter BRS (0.65 ± 0.12 pre vs. 0.66 ± 0.13 post bpm/mmHg, $p = 0.98$) (Fig 8D).

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DISCUSSION

This is the first study to comprehensively investigate the role that cAMP-dependent signalling pathways play in regulating neural activities in the ventrolateral medulla. The major findings are that: 1) Activation of PKA in the RVLM is sympathoexcitatory and enhances the sympathetic baroreflex and somatosympathetic reflex whilst, at cardiac vagal preganglionic neurons, evokes bradycardic and augments the cardiac baroreflex; 2) Activation of EPAC in the RVLM, using 8-pCPT, also evokes sympathoexcitation, which is blocked by BFA; 3) Blockade of PKA within the RVLM and at cardiac vagal preganglionic neurons is also sympathoexcitatory and cardioinhibitory respectively but did not alter sympathetic reflex function; 4) Blockade of EPAC or HCN channels in the RVLM has no effect; however, 5) In the RVLM the summed effect of sequential and cumulative blockade of PKA, EPAC and HCN channels is sympathoinhibitory.

Our results indicate that cAMP-dependent pathways, which are likely naturally stimulated by GPCRs linked via Gαs proteins, can be recruited to activate both sympathetic and parasympathetic outflows in the ventrolateral medulla and contribute to basal levels of sympathetic vasomotor and cardiac vagal tone. Blocking PKA alone has a net excitatory effect both in the RVLM and at cardiac vagal preganglionic neurons. One simple explanation may be that inhibitory inputs to both groups of neurons are tonically driven by cAMP-dependent signalling and blockade of PKA causes disinhibition. As sympathetic baroreflex function is unaffected by PKA blockade, such active inhibitory inputs to the RVLM are unlikely to be of baroreceptor origin. However as sympathoinhibition results following blockade of all cAMP-dependent signalling this suggests excitatory as well as inhibitory substrates within the RVLM may be tonically influenced by cAMP dependent signalling.

Methodological considerations

All drugs used in this study to alter cAMP-dependent signalling were cell permeable and largely resistant to phosphodiesterases (Schaap *et al.*, 1993; Dostmann, 1995). Both Sp-cAMPs and 8-Br-cAMP are analogues of cAMP and effectively activate all downstream effectors. Both are potent activators of PKA and EPAC (Christensen *et al.*, 2003). Both activators evoked significant dose-related sympathoexcitation and pressor or vagally mediated bradycardic effects in the ventral medulla. The doses of Sp-cAMPs used were similar to those used in other brain regions (Paine *et al.*, 2009). 8-pCPT selectively activates EPAC without effect at PKA (Christensen *et al.*, 2003; Brown *et al.*, 2014) although it may have some non-specific/EPAC-independent effects, at least as identified in platelets (Herfindal *et al.*, 2013). Nevertheless, the effects of 8-pCPT were similar to

those evoked by Sp-cAMPs and blocked by prior treatment with BFA, which alone had no effect as described elsewhere (Zhong & Zucker, 2005). ZD7288 is a commonly used selective blocker of HCN channels (Harris & Constanti, 1995) although some effect on sodium channels has been suggested (Wu *et al.*, 2012).

Rp-cAMPs, which inhibits PKA, has little effect at EPAC (Christensen *et al.*, 2003; Brown *et al.*, 2014) and H-89, which also inhibits PKA evoked similar dose-dependent effects although H-89 actions could also be via other kinases (Lochner & Moolman, 2006). The pressor effect evoked by H-89 in the RVLM confirm that previously noted (Xu & Krukoff, 2006). ZD 7288, which blocks HCN channels, although ineffective alone in the RVLM, as described previously (Miyawaki *et al.*, 2003), contributed to inhibitory effects when preceded by other drugs. Nevertheless, as in most pharmacological studies of this type, it is possible that the effects evoked by the drugs used may not be due to the substrates targeted.

Heart-rate, sympathetic and blood pressure responses evoked by drug injection in vagotomized spinal-cord intact animals are interpreted as sympathetically mediated, albeit modified by competing baroreflex pathways. The splanchnic nerve innervates functionally diverse targets including the gut vasculature, gastrointestinal muscles and the adrenal gland, and cannot therefore be interpreted as a purely vasomotor output. Conversely, data from spinally transected animals are interpreted as consequences of direct drug effects on cardiac vagal motor circuits, as described previously (Hildreth & Goodchild, 2010), as all sympathetic outputs were disrupted also providing conditions of maximal baroreflex unloading.

Drug interaction with medullary interneurons presynaptic to sympathetic/parasympathetic outputs are likely. We have previously shown select effects on respiratory function within subregions of the ventrolateral medulla (Burke *et al.*, 2013) and it is possible that changes in respiratory-sympathetic coupling contribute to effects seen here.

Sites of cAMP activation in the ventral medulla

Activation of cAMP-dependent pathways in the RVLM evoked sympathoexcitation and a pressor effect and at cardiac vagal preganglionic neurons bradycardia. This is in keeping with our finding that the Gαs subunit mRNA is abundant in the RVLM (Parker *et al.*, 2012) and consistent with a post-synaptic site of action, as suggested previously in neonatal RVLM brain slice preparations, in which 8-Br-cAMP and the adenylyl cyclase activator forskolin, increased the firing rate of RVLM 'pacemaker' neurons in the presence of tetrodotoxin (Sun & Guyenet, 1990). Activation of Gαs-linked receptors in the RVLM using PACAP evokes sympathoexcitation and pressor responses

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although reflex functions were unaffected (Farnham *et al.*, 2012). On the other hand cardiac vagal nerve activity is increased by systemic adenosine (da Silva *et al.*, 2012) or by activation of β-adrenergic receptors, specifically β1 which reduces GABAergic and glycinergic (as well as glutamatergic) conductances at cardiac vagal preganglionic neurons (Bateman *et al.*, 2012). Recently, β1 and β2 receptors have been identified on putative presympathetic RVLM neurons and their selective activation evoked depolarization and hyperpolarization, respectively (Oshima *et al.*, 2014). These data suggest that cAMP-dependent signalling can be elicited by catecholamine release in the ventrolateral medulla.

Injections of PKA and EPAC activators enhanced both sympathetic and cardiac baroreflex functions. This could be explained by the activation of cAMP in presympathetic neurons and/or in inhibitory inputs and, in cardiac vagal preganglionic neurons and/or in excitatory inputs respectively. The effect, at least of PKA, on the somatosympathetic reflex (mediated by glutamatergic synapses in the RVLM (Kiely & Gordon, 1993) could indicate modulation of glutamatergic inputs or postsynaptic effects particularly as facilitation appeared more prominent at slowly conducting possibly catecholaminergic cells in the region.

Tonically active PKA dependent signalling in the ventral medulla

Blockade of PKA, using both Rp-cAMPs and H-89, evoked sympathoexcitation and vagally mediated bradycardia indicating tonically-active PKA-dependent signalling in the ventrolateral medulla. Although a pressor effect initially accompanied the sympathoexcitation elicited by both agents, at later time points a depressor response which was not accompanied by splanchnic sympathoinhibition was evoked by Rp-cAMPs. This biphasic effect may indicate that splanchnic sympathetic drive is counteracted by other effectors such as inhibition of excitatory input supplying other sympathetic vasomotor outflows. Nevertheless the effects on MAP suggest that vasomotor pathways are affected as well as both sympathetic and parasympathetic pathways controlling heart rate. As the tonic activity of RVLM neurons supplying vasomotor tone is dependent upon the balance of tonic excitatory and inhibitory input we speculate that the early net excitatory action of Rp-cAMPs could be mediated by effects at inhibitory inputs however, as the sympathetic baroreflex (mediated by inhibitory presynaptic input) was unaffected, actions at other functional inhibitory inputs would be indicated. GABA-A receptor blockade at both the RVLM and cardiac vagal preganglionic neurons indicate significant levels of tonic inhibitory input to neurons controlling vasomotor (Schreihofer & Guyenet, 2003) and cardiac function (Hildreth & Goodchild, 2010). Furthermore there is some precedent for PKA dependent disinhibition as modulation of glycinergic release occurs in spinal cord (Katsurabayashi *et al.*, 2004). It is possible that blocking PKA may

redistribute the active pool of cAMP to other effectors however, at least in the RVLM, blocking either EPAC or HCN channels alone had little effect. There is little evidence supporting the idea of tonically active peptides in the RVLM (Burke *et al.*, 2008; Pilowsky *et al.*, 2008; Farnham *et al.*, 2012). Nevertheless the findings here suggest that a neurotransmitter acting via Gαs linked receptor/s is active in the ventrolateral medulla. One possibility is a catecholamine acting at β receptors where, at least in the neonatal RVLM, β2 receptor blockade depolarised neurons (Oshima *et al.*, 2014). However, an alternative explanation could be that such a receptor is constitutively active (Milligan, 2003; Costa & Cotecchia, 2005) and candidates that are Gαs linked in the RVLM include the H2 and MC3/4 receptors (Granata & Reis, 1987; Kawabe *et al.*, 2006). When activated in the RVLM only the H2 receptor causes sympathoinhibition, most likely via excitation of an inhibitory input (Granata & Reis, 1987).

Although blockade of PKA in RVLM evoked sympathoexcitation, blockade of other cAMP effectors each had no effect. Nevertheless combined blockade resulted in sympathoinhibition suggesting actions at both inhibitory and excitatory synaptic sites. It should be noted that the cAMP effectors are restricted to spatially separated microdomains within cell bodies and terminals in the ventral medulla (Karpen & Rich, 2004; Calebiro & Maiellaro, 2014) so sequential blockade may have disturbed the balance within intracellular compartments.

Conclusions

Our data show that cAMP-dependent pathways, signaling via PKA and EPAC, can be recruited in the ventrolateral medulla to evoke excitation in sympathetic circuitry controlling the heart, vasculature and baroreflex as well as excitation of the cardiac vagus and circuitry controlling the cardiac baroreflex. Importantly, the results indicate that PKA-dependent pathways are tonically active in a region controlling the basal level of sympathetic and cardiac vagal tones. These effects are in contrast to the effects of blocking excitatory ionotropic receptors in the RVLM or at cardiac vagal preganglionic neurons which do not alter the level of sympathetic activity or HR respectively (Dampney *et al.*, 2003; Hildreth & Goodchild, 2010). Thus GPCRs utilizing Gαs proteins in the ventrolateral medulla contribute to setting the level of sympathetic tone including sympathetic vasomotor as well as cardiac vagal tone.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Goodchild, Hildreth Tallapragada
Conducted experiments: Tallapragada, Hildreth, Raley, Burke
Performed data analysis: Tallapragada, Hildreth, Burke, Hassan
Wrote or contributed to the writing of the manuscript: Goodchild, Tallapragada, Hildreth, Hassan, Burke, McMullan

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FOOTNOTES*

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FIGURE LEGENDS

Figure 1. The change in sympathetic nerve activity (SNA) and mean arterial pressure (MAP) evoked by increasing doses of analogues of cAMP microinjected bilaterally and cumulatively in the RVLM. **A)** shows the change in sSNA elicited by vehicle (PBS), Sp-cAMPs (0.5, 1.5, 5 nmol, n=4) and 8-Br-cAMP (1 and 10 nmol) (n=3). **B)** shows the change in MAP evoked by the same agents (n=4 for both). Data are reported as mean ± SEM, *p<0.05, **p<0.01, ****p<0.0001

Figure 2. Time-course and peak responses in sSNA, MAP and HR evoked by bilateral microinjection of the cAMP analogue Sp-cAMPs (5 nmol/side) or vehicle (PBS) in the RVLM. **A)** shows a representative example of the responses evoked by Sp-cAMPs. Stimulation of the sciatic nerve to evoke the somatosympathetic reflex (SSR) and injection of vasoactive agents phenylephrine (PE) and sodium nitroprusside (SNP) are indicated. These stimuli are applied multiple times but are indicated only once for clarity. Sp-cAMPs elicited an increase in all parameters measured. **B-D)** show the time course of effects on sSNA, MAP and HR, respectively. The effects of PE and SNP on heart rate may be due to Starling's law following rapid i.v. injection in a vagotomised rat or the SNP induced bradycardia may be caused by pressure induced reduction in coronary perfusion pressure. HR baroreflex changes were not analysed in this part of the study (however, see Fig 8). **E)** shows the peak response in sSNA, MAP and HR evoked by Sp-cAMPs compared to vehicle (PBS) (n=6). Data are reported as mean ± SEM, **p<0.01, ****p<0.0001.

Figure 3. Effects of Sp-cAMPs (A,B), 8-pCPT (C,D) or RP-cAMPs (E,F) bilaterally injected into the RVLM on baroreflex (A,C,E) or somatosympathetic reflex function (B,D,F). Effects are shown before (black) and 15-20 min after (grey) drug injection. For the somatosympathetic reflex prior to drug injection (before) two characteristic peaks in sSNA were evoked (black). The averaged response + SEM are shown. The increase in sSNA evoked by all drugs is evident.

Figure 4. Time-course and peak responses in sSNA, MAP and HR evoked by bilateral microinjection of a cAMP analogue that selectively activates EPAC, 8-pCPT (5 nmol/side), in the RVLM. **A)** shows a representative example of the responses evoked. Stimulation of the sciatic nerve to evoke the somatosympathetic reflex (SSR) and injection of vasoactive agents phenylephrine (PE) and sodium nitroprusside (SNP) are indicated. 8-pCPT elicited an increase in all parameters measured. **B-D)** show the time course of effects on sSNA, MAP and HR, respectively. See legend for Fig 1 for additional details. **E)** shows the peak response evoked in each parameter measured compared to the effect evoked by vehicle (PBS) (n=6). Data are reported as mean ± SEM **p<0.01, ****p<0.0001.

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Figure 5. Time-course and peak responses in sSNA, MAP and HR evoked by bilateral microinjection of the PKA inhibitor Rp-cAMPs (5 nmol/side) in the RVLM. **A)** shows a representative example of the responses evoked. Stimulation of the sciatic nerve to evoke the somatosympathetic reflex (SSR) and injection of vasoactive agents phenylephrine (PE) and sodium nitroprusside (SNP) are indicated. Rp-cAMPs elicited an increase in all parameters measured. **B-D)** show the time course of effects on sSNA, MAP and HR, respectively. See legend for Fig 1 for additional details. **E)** shows the peak response evoked in each parameter measured compared to the effect evoked by vehicle (PBS) (n=6). Data are reported as mean ± SEM, *p<0.05 **p<0.01, ***p<0.001.

Figure 6. Time-course effects on sSNA (A), MAP (B) and HR (C) of 8-pCPT (data taken from Fig 4, black square), an inhibitor of EPAC, BFA (open circle) n=4) and when 8-pCPT microinjection was preceded by BFA (open triangle, n=3). BFA blocked the effect of 8-pCPT. Data are reported as mean ± SEM.

Figure 7. Time-course responses in sSNA, MAP and HR evoked by bilateral microinjection in the RVLM of Rp-cAMPs, followed by BFA, followed by ZD7288 (300 pmol/side) at 10 min intervals. **A)** shows a representative example of the responses evoked. **B-D)** show the time course of effects on sSNA, MAP and HR, respectively. Data are reported as mean ± SEM.

Figure 8. Effects on HR and cardiac baroreflex sensitivity (BRS) of dual bilateral microinjections of Sp-cAMPs (each injection 10 nmol) and Rp-cAMPs (each injection 10 nmol) in the ventral medulla targeting cardiac vagal preganglionic in spinally transected animals. (A) shows a representative example of the effects of Sp-cAMPs on HR and also the effect of the vasoactive agent phenylephrine (PE) used to determine BRS. (B) shows a representative example of the effects of Rp-cAMPs on HR. (C) shows the grouped data of the effects of SP-cAMPs on resting HR and BRS and (D) shows the grouped data of the effects of Rp-cAMPs on resting HR and BRS. Data are reported as mean ± SEM, *p<0.05 **p<0.01.

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TABLES

Drug		Lower Plateau (%)	Upper Plateau (%)	Mid Point (mmHg)	Range of sSNA (%)	Max. Gain
Sp-cAMPs	before	9.9±5.2	94.6±7.2	125.1±4.1	84.7±11.1	-1.5±0.0.1
	15-20min	13.9±18.6	164.3±10.8	147.8±4.8	150.5±28.2	-2.4±0.3
	P value	ns	**0.0043**	**0.0003**	ns	**0.039**
8-pCPT	before	18.3±10.8	96.8±1.0	130.5±8.9	78.5±10.9	-1.6±0.3
	15-20min	27.3±6.9	141.1±2.1	147.3±7.8	113.8±6.9	-2.2±0.4
	P value	ns	**0.0001**	ns	**0.0098**	**0.04**
Rp-cAMPs	before	24.3±7.7	97.2±0.9	122.0±7.0	73.0±7.9	-1.2±0.3
	15-20min	34.8±7.1	119.8±3.0	130.9±8.5	85.0±8.3	-1.4±0.2
	P value	ns	**0.0005**	ns	ns	ns

Table 1. Baroreflex control of sSNA after drug microinjection into the RVLM

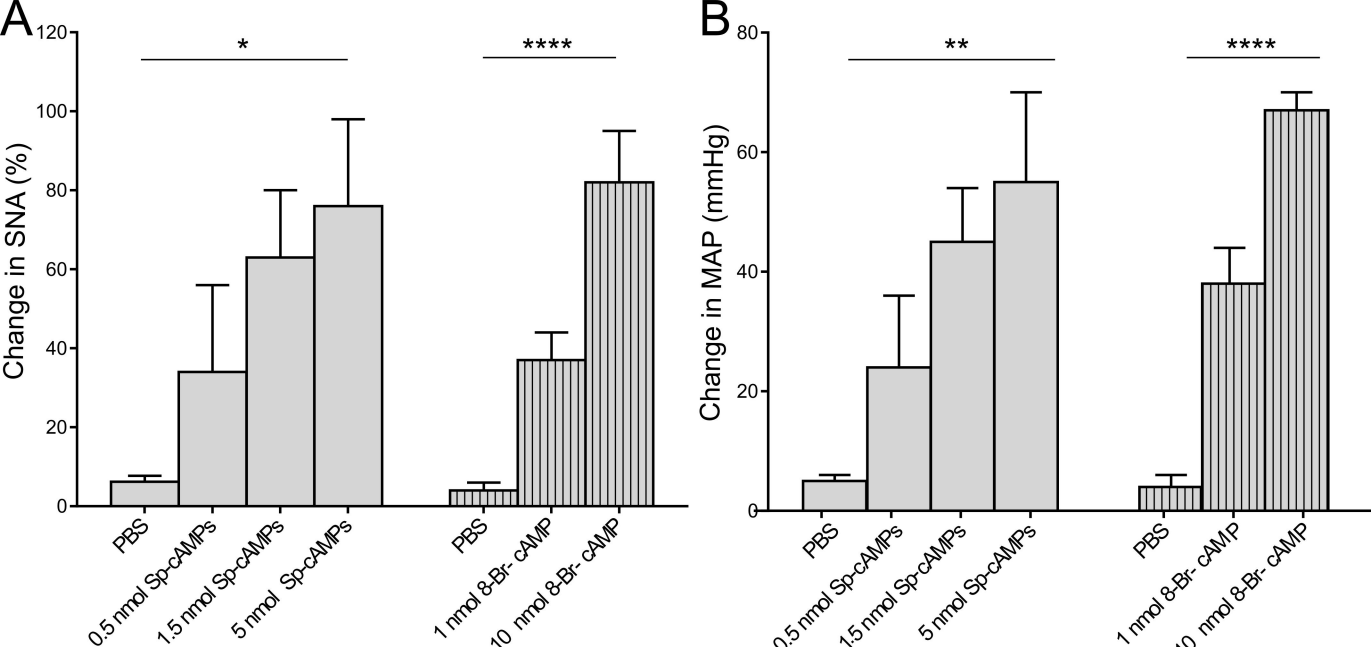
A
Change in SNA (%)
120
100
80
60
40
20
0
*
****
PBS
0.5 nmol Sp-cAMPs
1.5 nmol Sp-cAMPs
5 nmol Sp-cAMPs
PBS
1 nmol 8-Br- cAMP
10 nmol 8-Br- cAMP
B
Change in MAP (mmHg)
80
60
40
20
0
**
****
PBS
0.5 nmol Sp-cAMPs
1.5 nmol Sp-cAMPs
5 nmol Sp-cAMPs
PBS
1 nmol 8-Br- cAMP
10 nmol 8-Br- cAMP

Figure 1

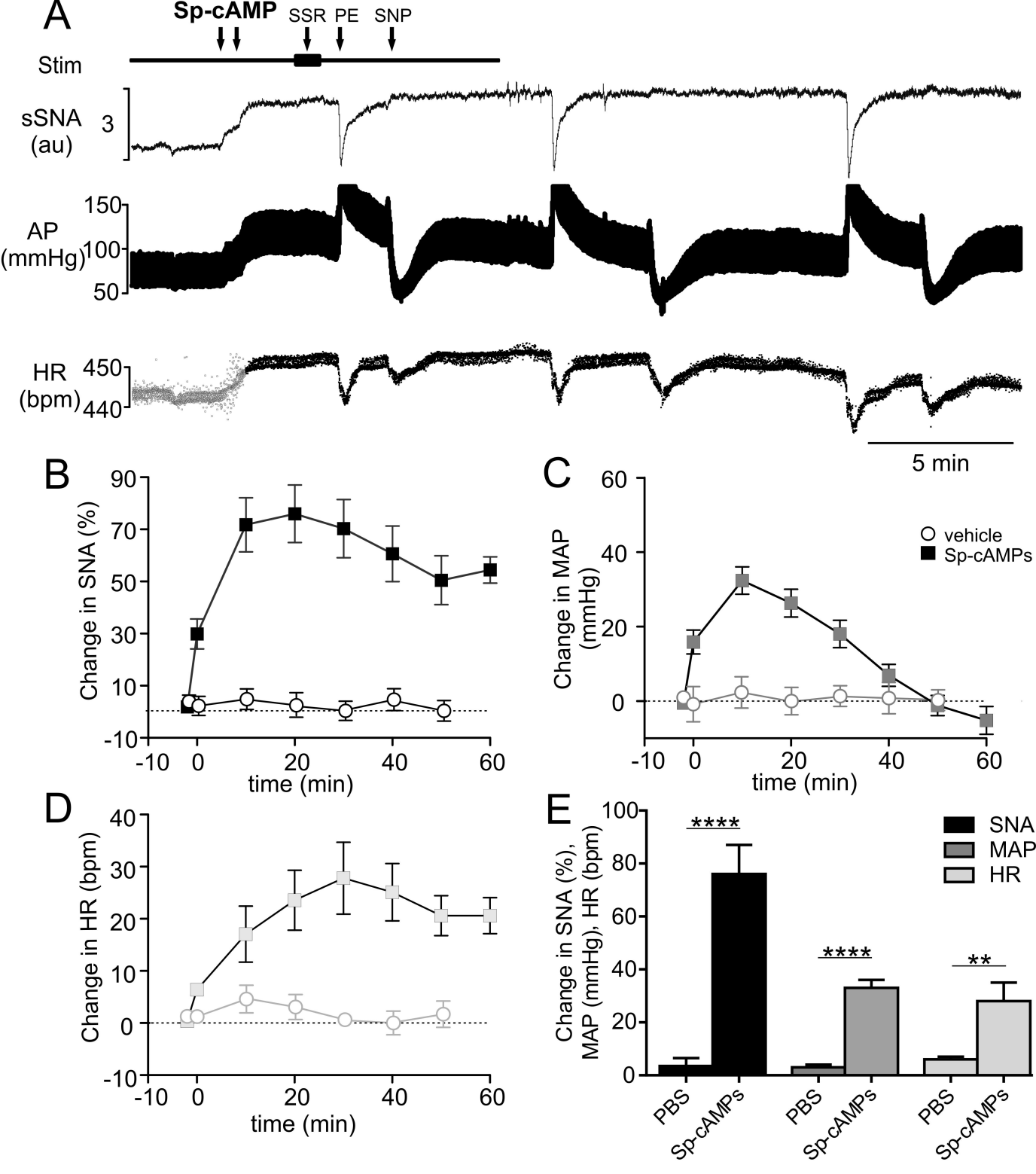
A
Sp-cAMP
SSR
PE
SNP
Stim
sSNA (au)
3
AP (mmHg)
150
100
50
HR (bpm)
450
440
5 min
B
Change in SNA (%)
time (min)
C
Change in MAP (mmHg)
vehicle
Sp-cAMPs
time (min)
D
Change in HR (bpm)
time (min)
E
Change in SNA (%), MAP (mmHg), HR (bpm)
SNA
MAP
HR
****
****
**
PBS
Sp-cAMPs
PBS
Sp-cAMPs
PBS
Sp-cAMPs

Figure 2I

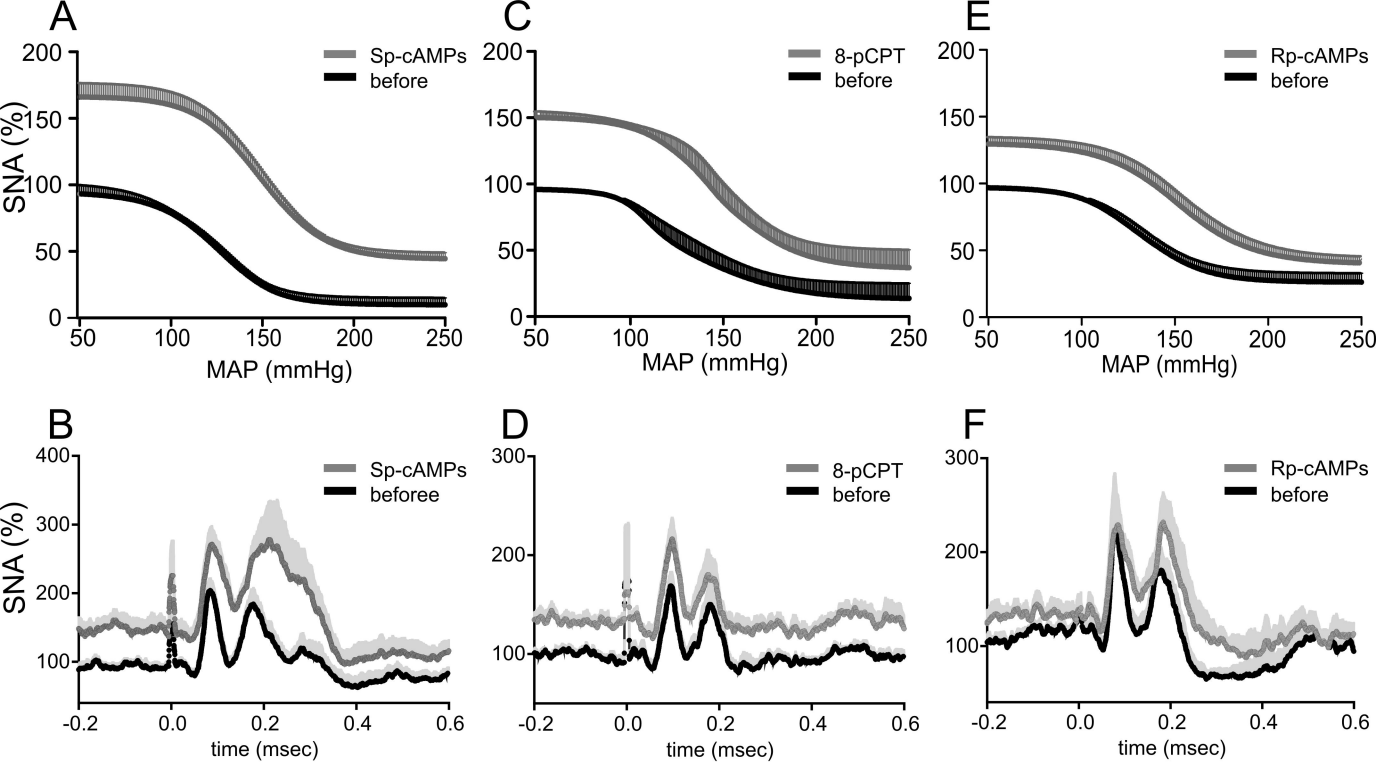
A
C
E
B
D
F
SNA (%)
MAP (mmHg)
time (msec)
Sp-cAMPs
before
beforee
8-pCPT
Rp-cAMPs
200
150
100
50
0
250
400
300
-0.2
0.0
0.2
0.4
0.6

Figure 3

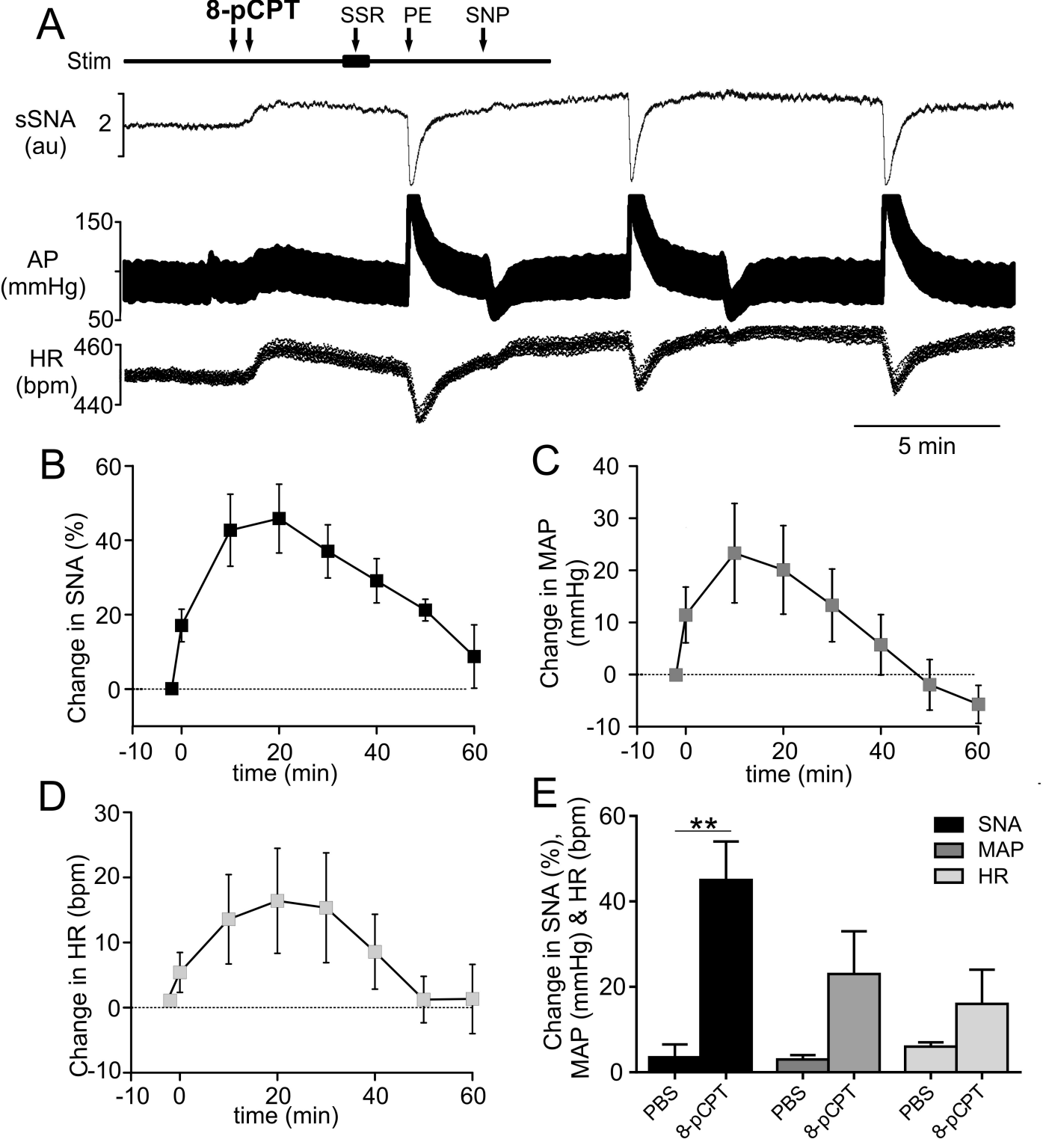
A
8-pCPT
SSR
PE
SNP
Stim
sSNA (au)
2
AP (mmHg)
150
50
HR (bpm)
460
440
5 min
B
Change in SNA (%)
time (min)
C
Change in MAP (mmHg)
time (min)
D
Change in HR (bpm)
time (min)
E
Change in SNA (%), MAP (mmHg) & HR (bpm)
**
SNA
MAP
HR
PBS
8-pCPT
PBS
8-pCPT
PBS
8-pCPT

Figure 4

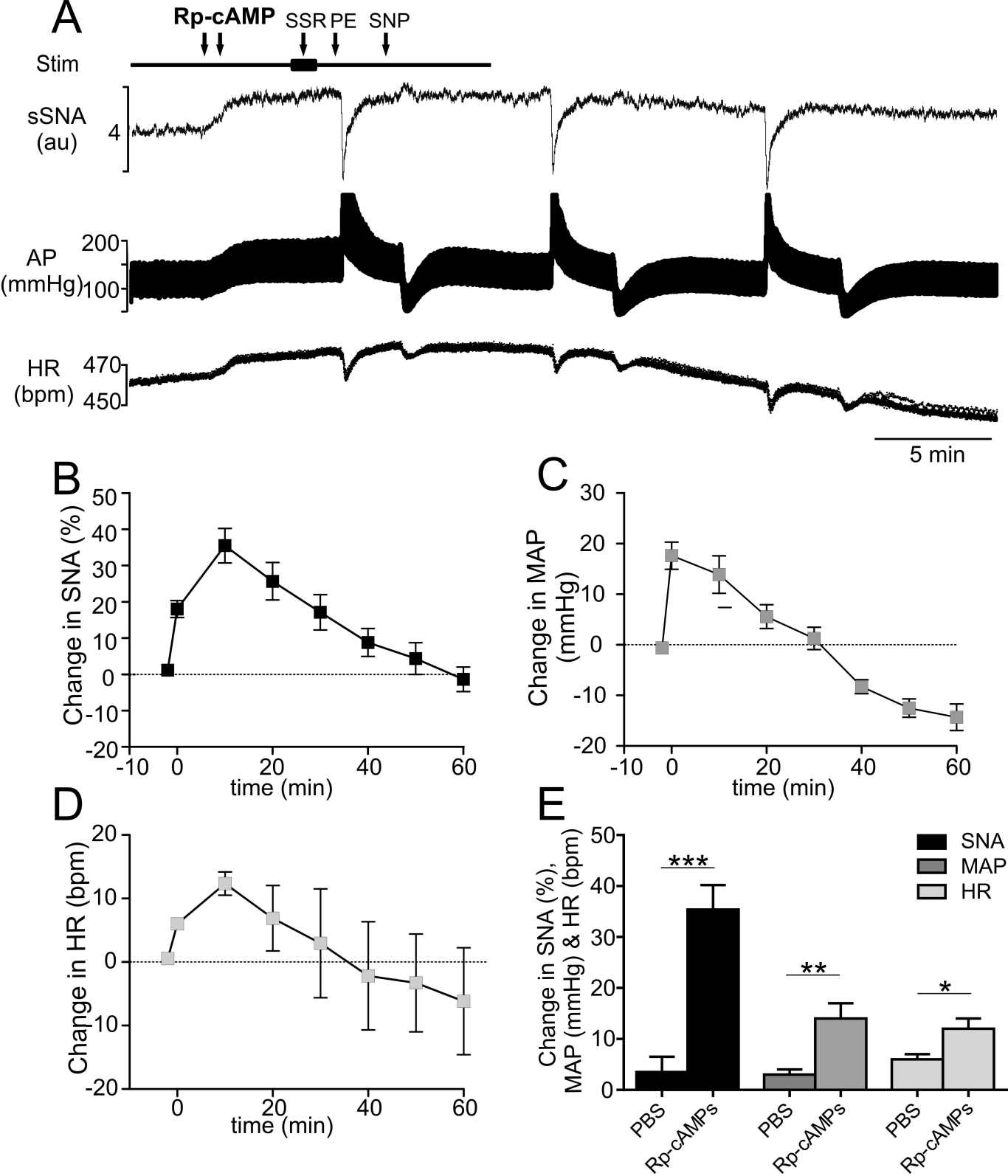
A
Rp-cAMP
SSR
PE
SNP
Stim
sSNA (au)
4
AP (mmHg)
200
100
HR (bpm)
470
450
5 min
B
Change in SNA (%)
time (min)
C
Change in MAP (mmHg)
time (min)
D
Change in HR (bpm)
time (min)
E
Change in SNA (%), MAP (mmHg) & HR (bpm)
SNA
MAP
HR
***
**
*
PBS
Rp-cAMPs

Figure 5

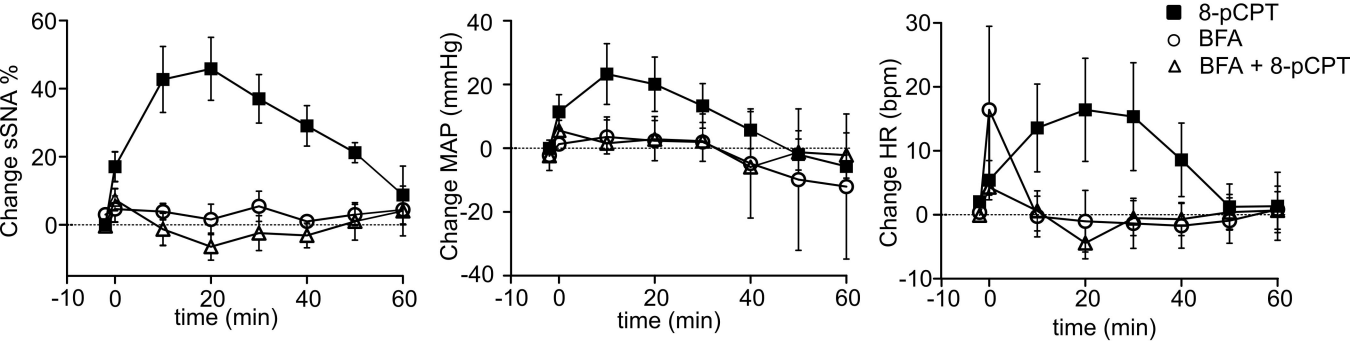
Change sSNA %
60
40
20
0
-10
0
20
40
60
time (min)
Change MAP (mmHg)
40
20
0
-20
-40
-10
0
20
40
60
time (min)
Change HR (bpm)
30
20
10
0
-10
-10
0
20
40
60
time (min)
8-pCPT
BFA
BFA + 8-pCPT

Figure 6

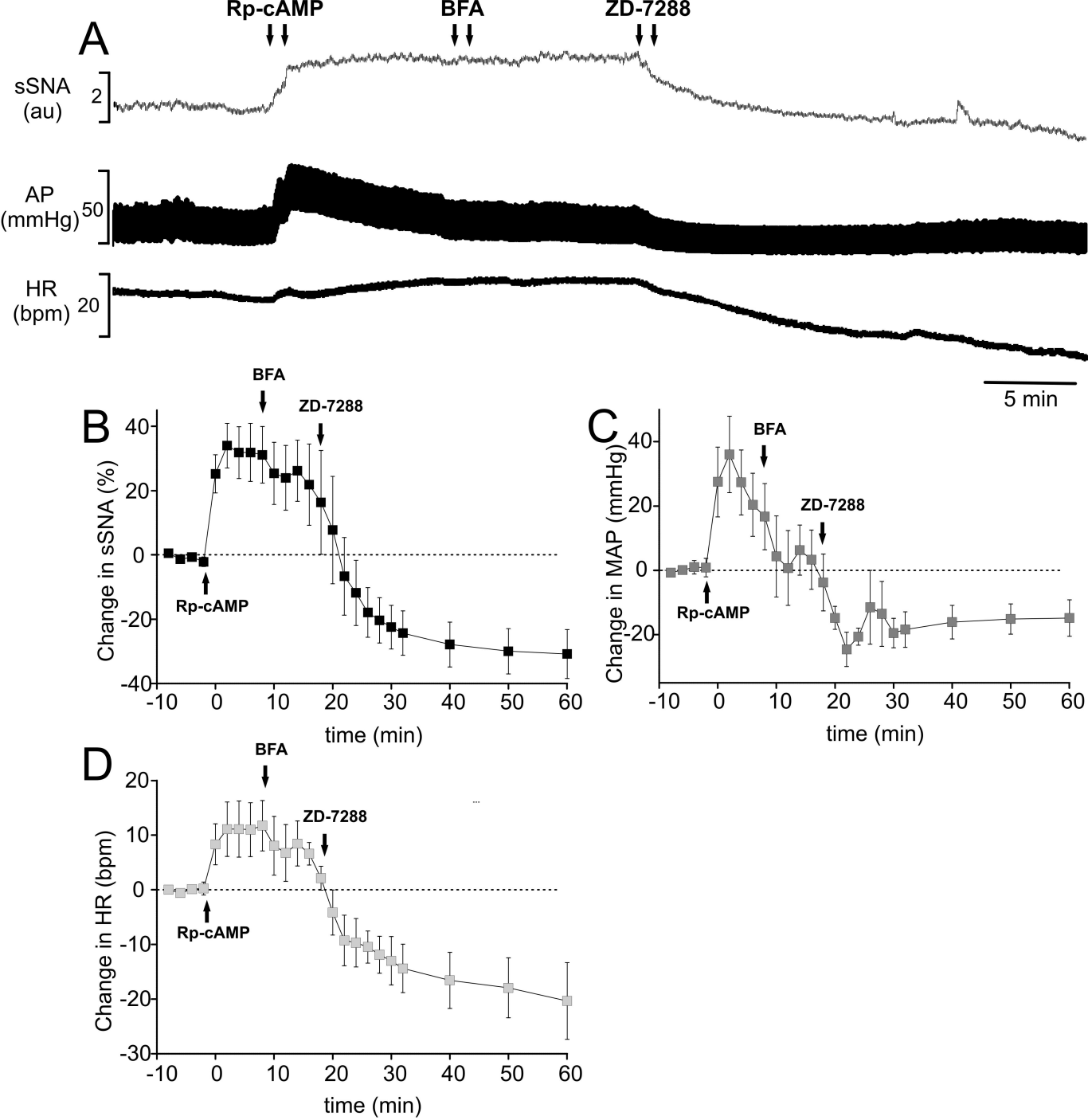
A
Rp-cAMP
BFA
ZD-7288
sSNA (au)
2
AP (mmHg)
50
HR (bpm)
20
5 min
B
BFA
ZD-7288
Rp-cAMP
Change in sSNA (%)
time (min)
C
BFA
ZD-7288
Rp-cAMP
Change in MAP (mmHg)
time (min)
D
BFA
ZD-7288
Rp-cAMP
Change in HR (bpm)
time (min)

Figure 7

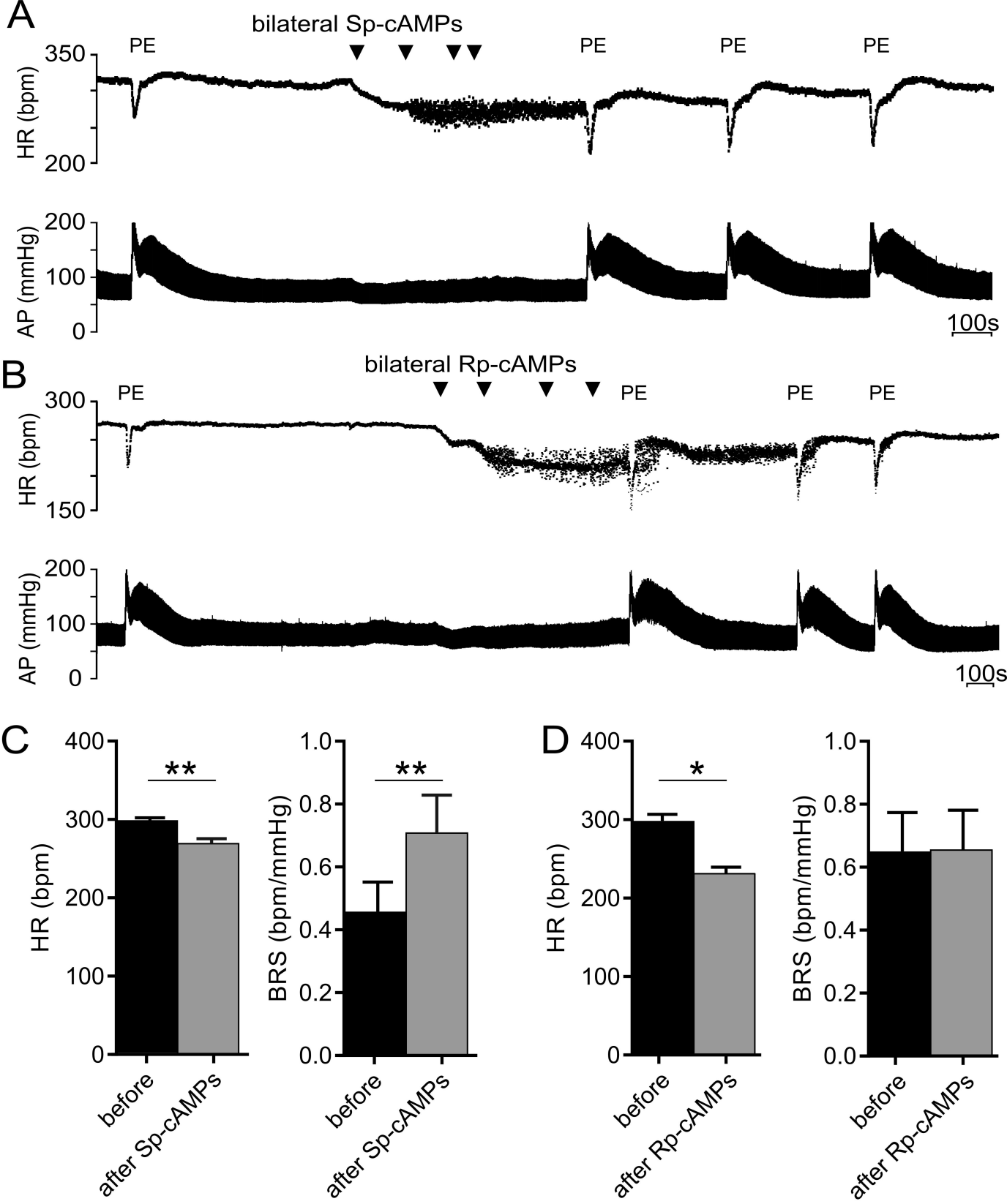
A
bilateral Sp-cAMPs
PE
PE
PE
PE
350
200
HR (bpm)
200
100
0
AP (mmHg)
100s
B
bilateral Rp-cAMPs
PE
PE
PE
PE
300
150
HR (bpm)
200
100
0
AP (mmHg)
100s
C
400
300
200
100
0
HR (bpm)
**
before
after Sp-cAMPs
1.0
0.8
0.6
0.4
0.2
0.0
BRS (bpm/mmHg)
**
before
after Sp-cAMPs
D
400
300
200
100
0
HR (bpm)
*
before
after Rp-cAMPs
1.0
0.8
0.6
0.4
0.2
0.0
BRS (bpm/mmHg)
before
after Rp-cAMPs

Figure 8