Central Command Regulation of Circulatory Function Mediated by Descending Pontine Cholinergic Inputs to Sympathoexcitatory Rostral Ventrolateral Medulla Neurons

James R. Padley, Natasha N. Kumar, Qun Li, Thomas B.V. Nguyen, Paul M. Pilowsky, Ann K. Goodchild

Abstract—Central command is a feedforward neural mechanism that evokes parallel modifications of motor and cardiovascular function during arousal and exercise. The neural circuitry involved has not been elucidated. We have identified a cholinergic neural circuit that, when activated, mimics effects on tonic and reflex control of circulation similar to those evoked at the onset of and during exercise. Central muscarinic cholinergic receptor (mACHR) activation increased splanchnic sympathetic nerve activity (SNA) as well as the range and gain of the sympathetic baroreflex via activation of mACHR in the rostral ventrolateral medulla (RVLM) in anesthetized artificially ventilated Sprague–Dawley rats. RVLM mACHR activation also attenuated and inhibited the peripheral chemoreflex and somatosympathetic reflex, respectively. Cholinergic terminals made close appositions with a subpopulation of sympathoexcitatory RVLM neurons containing either preproenkephalin mRNA or tyrosine hydroxylase immunoreactivity. M2 and M3 receptor mRNA was present postsynaptically in only non–tyrosine hydroxylase neurons. Cholinergic inputs to the RVLM arise only from the pedunculopontine tegmental nucleus. Chemical activation of this region produced increases in muscle activity, SNA, and blood pressure and enhanced the SNA baroreflex; the latter effect was attenuated by mACHR blockade. These findings indicate a novel role for cholinergic input from the pedunculopontine tegmental nucleus to the RVLM in central cardiovascular command. This pathway is likely to be important during exercise where a centrally evoked facilitation of baroreflex control of the circulation is required to maintain blood flow to active muscle. (Circ Res. 2007;100:284-291.)

Key Words: baroreflex ■ exercise ■ chemoreflex ■ somatosympathetic

A distinct pattern of tonic and reflex cardiovascular adjustments is mediated by central command to ensure appropriate muscle and organ perfusion during different arousal or behavioral states, such as sleep and exercise.1–3 Limited evidence implicates some regions within the pons and hypothalamus that could provide descending input to cardiovascular control sites4–6; however, the neural circuitry and neurotransmitters involved are yet to be elucidated.

Activation of the central cholinergic system has a profound effect on cardiovascular and other autonomic functions.7–18 Systemic or central administration of acetylcholinesterase inhibitors or muscarinic agonists increases blood pressure,7–11 lowers body temperature,12 and alters respiration.13,14 Pressor responses can be evoked via activation of muscarinic receptors (mACHR) within several cardiovascular nuclei, including the posterior hypothalamus,7 nucleus of the solitary tract,15 and rostral ventrolateral medulla (RVLM).10,11 Effects of central mACHR activation on cardiovascular reflexes are well understood.8,16,17 Sympathoexcitatory and hypertensive effects of intravenously administered physostigmine are largely mediated by excitation of RVLM neurons.10,11,18 The RVLM generates basal sympathetic vasomotor activity and is a critical synaptic relay in cardiovascular reflexes.19,20 Descending cholinergic projections to the RVLM arise from neurons in the pedunculopontine tegmental nucleus (PPT),21 although local medullary neurons may also be a source of cholinergic input.22 The function of this input into the RVLM is unknown. A dense cholinergic terminal field is present within the RVLM,11,22,23 although supportive anatomical evidence that cholinergic terminals provide input to C1 or non-C1 spinally projecting neurons is lacking. Activation of the inhibitory M2 mACHR subtype in the RVLM is thought to mediate pressor responses,10,11 but its cellular location or that of other mACHR subtypes within the RVLM is unknown.

We hypothesized that cholinergic input to the RVLM from the PPT is involved in central command–mediated effects on cardiovascular function. Previous studies have shown that the
PPT is involved in initiation of movement and modulation of muscle tone during locomotion, exercise, and arousal. Additionally, the PPT connects albeit indirectly with both motor and sympathetic outflows.

We aimed, firstly, to determine the role of the RVLM in the autonomic responses and effects on reflex control of the circulation evoked by central mAChR activation. Secondly, we identified the mAChR subtypes involved by examining gene expression within phenotypically identified RVLM neurons and determined the exact sources of cholinergic input to the RVLM. Finally, we determined the tonic and reflex cardiovascular effects generated by chemical stimulation of the PPT.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. Studies were approved by the Animal Care and Ethics Committee of Royal North Shore Hospital/University of Technology, Sydney. Following experimentation, rats were euthanized while under surgical anesthesia by KCl (3 mol/L, 1 mL IV).

Male Sprague–Dawley rats (n=17) were anesthetized (urethane, 1.2 g/kg, 10% IP; Sigma-Aldrich) and maintained at surgical depth throughout experiments. Rats were intubated and arterial (femoral or carotid) and intravenous catheters inserted for arterial pressure (AP) measurement or drug administration. Splanchnic sympathioexcitatory activity (SNA) and phrenic nerve activity (PNA) were recorded using bipolar electrodes and in four rats tail blood flow (TBF) was also recorded using a laser Doppler flow probe (Oxford Optronics, Oxford, UK). In 2 rats electromyographic (EMG) activity from the left biceps femoris was also recorded. All signals were acquired online using Spike 2 software (CED Ltd, Cambridge, UK) as described previously. Microinjections were made into the RVLM as described previously in paralyzed (pancuronium, 0.8 mg), nonvagotomized animals (n=7), or vagotomized animals before and after paralysis (n=7, P<0.05) (Figure 1D). The increase in LF oscillations evoked by OXO (0.3±0.1 versus 36.5±15.1 mm Hg², P<0.05; 4.6±1.4 versus 47.5±17.9 SNA%/mL, P<0.05). Respiratory-related oscillations of SNA also tended to be increased (P=0.051). SCOP injected bilaterally into the RVLM had no effect on baseline parameters but prevented the increase in LF oscillations evoked by OXO (0.3±0.1 versus 4.0±0.1 mm Hg², 3.3±1.0 versus 7.3±2.5 SNA%/mL, n=7, P<0.05) (Figure II in the online data supplement).

**Protocols**

Animals were pretreated with mATR (2 mg/kg), and OXO was injected intravenously (0.2 mg/kg); SCOP was then injected bilaterally into the RVLM (9 nmol per side). Reflexes were tested before and after OXO in the absence or presence of SCOP. Injections of DLH or bicuculline were made into the PPT and effects of SNMP injection and TN stimulation were tested before and after SCOP intravenous injection (2 mg/kg).

Close appositions between vAChT immunoreactivity (vAChT-IR) and CTB/TH-IR or non–TH-IR neurons in the RVLM were examined at ×100 magnification; serial cell counts of vAChT-IR appositions or M2 receptor/CTB/TH were made from every fourth section. For detailed data and statistical analysis, see the online data supplement.

**Results**

**RVLM mAChR Mediate Sympathoexcitatory but Not Other Autonomic Effects Evoked by Central mAChR Activation**

Central mAChR activation (OXO) significantly increased AP, mean and postinspiratory-related discharge of SNA, heart rate (HR) and TBF and reduced PNA amplitude (Figure 1A, 1B, and 1D). Bilateral injection of SCOP into the RVLM (Figure 1C) significantly attenuated the increase in AP (n=8, P<0.01), SNA (n=8, P<0.01), HR (n=8, P<0.05) and post-inspiratory activation of SNA (n=4, P<0.05) but had no effect on changes in TBF (n=4; P=NS) or PNA amplitude (n=4; P=NS) evoked by OXO (Figure 1D).

Spectral analysis of systolic AP (SAP) and SNA revealed an increase in low frequency (LF, 0.1–0.4 Hz) oscillations following OXO (0.3±0.1 versus 36.5±15.1 mm Hg², P<0.05; 4.6±1.4 versus 47.5±17.9 SNA%/mL, P<0.05).

**Activation of RVLM mAChR Facilitates the Sympathetic Baroreflex and Inhibits the Somatosympathetic and Chemoreflexes**

OXO significantly enhanced the reflex sympatoexcitatory and inhibitory responses evoked by equipotent doses of SNAP and PE (Figure 2). This effect was reproducible following repeat injection of OXO (Figure 2A). OXO significantly increased the maximum plateau (146±4 versus 321±12%, P<0.01) and maximum gain of the SNA baroreflex (4.4±0.5 versus 8.2±0.6%/mm Hg, P<0.05) (Figure 2B and 2C). The operating point (resting MAP) also shifted closer to the point of maximum gain (Figure 2C).

Figure 3 shows the effects of OXO on cardiovascular reflexes before and after blockade of mAChR bilaterally in
the RVLM. OXO increased the magnitude of SNA inhibition evoked by aortic nerve stimulation (166±13% control, n=6, P<0.01) or excitation following SNP administration (4±1 versus 17±2% SNA/50 mmHg, n=4, P<0.01). In contrast, OXO inhibited both excitatory peaks of SNA evoked by TN stimulation (early peak 37±3% control, P<0.01, late peak 41±5% control, n=9, P<0.01). Sympathoexcitatory and pressor responses to brief hypoxia were attenuated and inhibited, respectively (53±6% control, P<0.01; +33±2 mm Hg versus -17±5 mm Hg, n=7, P<0.001).

Bilateral injection of SCOP into the RVLM reversed effects seen in A and B. Data shown are mean±SEM; bpu indicates blood perfusion units; au, arbitrary units.

Figure 1. The role of RVLM mAChR in mediating autonomic effects of central mAChR activation in urethane-anesthetized rats. A, Following pretreatment with mATR, OXO (0.2 mg/kg IV) evokes an increase in SNA, AP, and TBF but a reduction in PNA amplitude. Following identification of RVLM pressor sites (L-glutamic acid [glut]), SCOP (9 nmol per site) is injected into the RVLM bilaterally substantially reducing the pressor, sympathetic, and HR effects but not the TBF or PNA response to OXO. Increases in HR are caused by sympathetic activation. B, Average SNA (bold line) and PNA (thin line) waveforms showing increase in postinspiratory (P-I)-related discharge of SNA following OXO and blockade of this effect by SCOP injection in the RVLM. C, Injection sites in the RVLM (open circles) (only unilateral sites shown). D, Group data from 8 animals illustrating reflex was only partially restored (66±4% control, P<0.01; late peak P<0.01). Grouped data are illustrated in supplemental Figure I.

Cholinergic Terminals Closely Appose Sympathoexcitatory RVLM Neurons

vAChT-IR terminals were found throughout the VLM and in cell bodies within the facial and ambiguous motor nuclei, consistent with previous reports.33,34 A choline acetyltransferase–positive cell group previously identified in the ventromedial medulla22 was not present using vAChT labeling (supplemental Figure IV). vAChT-IR terminals were closely apposed to CTB-labeled spinally projecting cells in the RVLM; 32.6±7.4% (379/1118 cells, n=3) of all CTB-IR neurons and 31.1±5.6% (66/206 cells, n=3) of TH-positive CTB-IR cells (Figure 4A and 4B). vAChT-IR varicosities also formed perisomatic appositions with NeuN-positive non-TH RVLM neurons that expressed PPE, as well as other PPE-negative NeuN-positive cells (Figure 4C).

M2 and M3 Receptor mRNA Is Expressed in Spinally Projecting Non-TH Neurons in the RVLM

All mAChR subtypes were expressed in an RVLM tissue punch (supplemental Figure III). We analyzed the cellular distribution of M2 receptor expression in the RVLM and found that no spinally projecting TH neurons contained M2 receptor mRNA (0/310, n=5) (Figure 5A). In contrast, 23±4% of spinally projecting non-TH RVLM neurons did express M2 receptor mRNA (78/367, n=5) (Figure 5C). M3 receptor mRNA was also expressed in some TH-IR/non-CTB-IR and some CTB-IR/non-TH neurons (Figure 5B and 5D).

Direct Cholinergic Projections to the RVLM From the PPT

To determine the source of cholinergic input to the RVLM, discrete injections of CTB were made unilaterally into the RVLM (Figure 6A) and cholinergic neurons were identified by vAChT-IR. CTB-IR neurons were found in regions previously described, including the parabrachial nucleus, the paraventricular nucleus of the hypothalamus (Figure 6B), central nucleus of the amygdala, and the cortex.21,35 vAChT-IR neurons were also found in regions previously described33,34 (Figure 6C and 6D). Neurons that were double-labeled for CTB and vAChT had a restricted distribution and were confined within the PPT (Figure 6C and 6D).

Chemical Stimulation of the PPT Increases Muscle Activity and SNA and Facilitates the Sympathetic Baroreflex via mAChR Activation

Bilateral injection of bicuculline into the PPT evoked increases in AP and EMG activity (Figure 7A). EMG activity but not the increase in AP was abolished by subsequent neuromuscular (NM) blockade (Figure 7A). Injection of DLH into the PPT produced an increase in SNA and AP and increased the magnitude of SNA excitation produced by injection of SNP (Figure 7B). These effects could be evoked...
throughout the rostrocaudal extent of the PPT (≈7 to 9 mm caudal to Bregma). Smaller increases in SNA were evoked at sites dorsal or ventral but facilitation of baroreflex-evoked SNA responses was restricted to the PPT (6.5 to 7.5 mm ventral) (Figure 7B). Transient alterations in PNA frequency were observed following stimulation of the PPT and surrounding brain areas, whereas the somatosympathetic reflex was unaffected. Prior blockade of central mAChR receptors with SCOP intravenous injection prevented the facilitation of baroreflex-evoked SNA responses (n=3, P<0.05) but did not abolish sympathoexcitation produced by DLH injection into the PPT (Figure 7C).

Discussion

The novel findings of this study are (1) mAChR activation in the RVLM facilitates the sympathetic baroreflex and attenuates and inhibits the sympathetic chemoreflex and somatosympathetic reflex, respectively; (2) identified sympathoexcitatory neurons in the RVLM receive cholinergic input and differentially express M2 and M3 receptor subtypes; (3) chemical stimulation of the PPT, which provides the only direct cholinergic input to the RVLM, evokes a similar pattern of tonic and baroreflex SNA responses to that seen following RVLM mAChR activation. Neurons in the PPT control muscle tone during...
locomotion, exercise, and arousal. Our findings indicate that tonic and reflex cardiovascular adjustments are also evoked from the PPT via direct cholinergic projections to the RVLM. These data support our hypothesis that cholinergic input to the RVLM is involved in central command.

RVLM mAChRs mediate the increase in SNA and HR evoked by centrally acting OXO, in agreement with previous studies. Postinspiratory-related discharge of SNA was also enhanced, indicating a direct effect on respiratory-related inputs to the RVLM that presumably contributes to the mean increase in SNA evoked by OXO. This effect was elicited independently of the OXO-evoked depression of phrenic amplitude, which is mediated by sites other than the RVLM. The OXO-evoked increase in TBF, presumably contributing to the well-described hypothermic effect of this drug, is also mediated via other central sites. These may include the preoptic area of the hypothalamus as it receives cholinergic input and carbachol microinjections here evoke hypothermia.

We showed, for the first time, that the reflex responses of SNA to baroreceptor loading or unloading, demonstrated following vasoactive drug administration and by direct stimulation of baroreceptor afferents, were markedly enhanced by OXO and were mediated by RVLM mAChRs. Furthermore, baroreflex-related LF oscillations of both SAP and SNA were enhanced.

Our findings show that RVLM mAChR activation resets the SNA baroreflex to higher pressures and increases its range and gain. Earlier studies showed that the pressor effect of bilateral carotid occlusion was greater after central or systemic administration of physostigmine. Caputi et al demonstrated an upward shift in baroreflex HR responses without changes in range or gain following intracerebroventricular injection of physostigmine. A limitation of the present study was that pretreatment with mATR to block peripheral mAChR precluded analysis of vagally mediated HR responses. Our findings indicate that RVLM mAChR activation facilitates sympathetic vasomotor responses to baroreflex activation, whereas cholinergic effects at brain sites important in reflex vagal control, including the nucleus ambiguus, may evoke resetting of the HR baroreflex without changing its gain.

OXO evoked differential effects on cardiovascular reflexes via RVLM mAChR activation. Baroreflex SNA responses

Figure 4. Neurons in the RVLM are closely apposed by vAChT-IR varicosities. A, RVLM neurons retrogradely labeled from the thoracic intermediolateral cell column (CTB-IR, brown reaction product) are surrounded by vAChT-IR varicosities (black reaction product) that make close appositions with their cell bodies (arrowheads). Note the neurons in the more dorsal nucleus ambiguus that are immunoreactive for vAChT. B, CTB-IR cells or CTB-IR/TH-positive cells are closely apposed by vAChT-IR (arrowhead or *, respectively). C, Neurons in the RVLM identified by the neuron-specific marker NeuN that express PPE mRNA (arrows) are closely apposed by vAChT-IR, as are PPE-negative neurons. Scale bars: 400 μm (A, left); 50 μm (A, right); 25 μm (B and C). pyr indicates pyramidal tract. IOL indicates inferior olivary nucleus; NAc, nucleus ambiguus pars compacta.

Figure 5. Cellular distribution of M2 (A and C) or M3 receptor mRNA (B and D) and TH-IR neurons in the RVLM. M2 receptor mRNA is colocalized in RVLM neurons that project to the thoracic intermediolateral cell column (CTB-IR) (C), but none of these contain TH-IR. M3 receptor mRNA is expressed in some CTB-IR neurons (arrows) or in TH-IR cells lacking CTB-IR (arrowheads) (boxed area in B shown in D). Scale bars: 250 μm (A and B); 100 μm (C and D).
mediated by direct inhibition or disinhibition of RVLM neurons were enhanced. Somatosympathetic and chemoreflex SNA responses mediated by direct excitation of RVLM neurons were inhibited and attenuated, respectively. The clear inhibition of the somatosympathetic reflex suggests that these effects were not indirectly caused by raised sympathetic activity. To our knowledge, a study in anesthetized cats also showed that a somatosympathetic reflex evoked by intercostal nerve stimulation was inhibited by OXO.41 As single RVLM neurons receive largely convergent input from baroreceptors, peripheral chemoreceptors, somatic afferents, and central respiratory neurons, 3 mechanisms are possible to explain our data: OXO activates inhibitory presynaptic mAChRs located on reflex inputs to RVLM neurons, postsynaptic excitatory mAChRs on RVLM neurons, or a combination of both. Pre- and postsynaptic effects of carbachol on RVLM neurons have been demonstrated in vitro.18 Phenotypically identified sympathoexcitatory (CTB + TH) and putative sympathoexcitatory (CTB + non-TH, or PPE +) neurons in the RVLM were closely apposed by vAChT-IR varicosities. This is the first anatomical evidence indicating that cholinergic terminals may synapse with sympathoexcitatory RVLM neurons. Milner et al23 showed that choline acetyltransferase IR terminals formed abundant synaptic contacts in the ventral medulla but these were rarely seen with TH-containing neurons. In the study by Milner et al, however, only caudal sections of the RVLM were examined (0.5 to 2.0 mm caudal to the facial nucleus); these contain few spinally projecting neurons.29 Furthermore, compared with choline acetyltransferase, immunoreactivity to vAChT as used here gives better cholinergic terminal labeling.33,34 Our results showed, for the first time, that the M2 receptor was not expressed in TH neurons but was expressed in a subpopulation of spinally projecting non-TH neurons. M2 receptor-preferring antagonists prevent pressor effects of RVLM mAChR activation.11 The ligands used, however, do not display high affinity for any 1 particular subtype.46 If M2 receptors do mediate OXO-evoked sympathoexcitatory responses, then they are most likely located presynaptically in the RVLM or this effect is mediated by non-TH spinally projecting neurons. Huangfu et al have shown in neonatal RVLM that both C1 and non-C1 cells depolarized in response to mAChR activation.18 Because vAChT-IR terminals opposed both classes of RVLM neurons, we sought evidence for expression of other receptor subtypes. A subpopulation of spinally projecting non-TH RVLM neurons also contain M3 receptor mRNA. We have further demonstrated that mRNA for all 5 mAChR subtypes was present in the RVLM, confirming earlier studies in WKY and SHR rats.47 Our results suggest that different or multiple mAChR subtypes may be expressed by sympathoexcitatory RVLM neurons.

In agreement with Yasui et al,21 we found that the projection from the PPT to the RVLM is cholinergic. In addition, we showed that the PPT is the only cholinergic cell group that provides input to the RVLM. Local inputs from choline acetyltransferase–positive neurons in the ventromedial medulla22 are not functionally cholinergic, as we found that these cells did not contain vAChT. We demonstrated for the first time that chemical stimulation of the PPT facilitates baroreflex-evoked excitation of SNA, mimicking effects of RVLM mAChR activation. Blockade of mAChR with SCOP prevented this effect but did not completely abolish sympathoexcitation generated by PPT activation. Electrical stimulation of the PPT increases AP, HR, and renal SNA (with a lesser increase in lumbar SNA) in decerebrate animals.48,49 Sympathoexcitatory responses are also evoked from surrounding brain areas including the cuneiform nucleus.50 At present, we cannot explain the lack of effect of stimulating the PPT on other reflex responses that are modified by activation of RVLM mAChR.

Disinhibition of the PPT increased EMG activity, consistent with studies that reported increases in muscle activity following electrical or chemical stimulation of the PPT in anesthetized or decerebrate animals.24,48,49 Single cholinergic neurons in the PPT have dual connections with the motor cortex and stellate ganglion, as revealed by polysynaptic viral
tracing.\textsuperscript{5} The PPT may therefore be a key nodal point where changes in motor signals can be coordinated with descending modulation of sympathetic function. The simplest explanation of our data is that stimulation of the PPT evokes muscular activity and releases acetylcholine activating RVLM mAChR pre- and/or postsynaptically located on sympathoexcitatory neurons, causing an increase in AP and SNA as well as increasing the range and gain of the sympathetic baroreflex.

**Functional Implications**

The involvement of the PPT in initiating and modulating movement related to arousal and locomotion, including exercise, is well recognized.\textsuperscript{4,24,25} The present findings indicate that the cholinergic projection to the RVLM may be activated in parallel to elicit tonic and reflex cardiovascular adjustments that are appropriate to different behaviors. The pattern of effects bears a striking similarity to those evoked by central command during exercise.\textsuperscript{2,3,5,1}

Exercise is accompanied by a resetting of baroreflex control of SNA and HR to higher AP.\textsuperscript{2,51–53} This is thought to be crucial to AP elevation at exercise onset and AP stabilization during exercise and can oppose other reflex influences on circulation, including nociceptive and peripheral chemoreflexes.\textsuperscript{3} In addition to an increase in AP and SNA, the increase in the range and gain of the sympathetic baroreflex as seen here strongly resembles that evoked during treadmill exercise in conscious rats.\textsuperscript{52} Studies showing complete sympathetic baroreflex function curves during exercise in humans are sparse, although some studies have demonstrated large increases in linear baroreflex gain of muscle SNA during static exercise\textsuperscript{53} or no change during moderate intensity arm cycling.\textsuperscript{54} In contrast, exercise appears to reset the cardiac component of the baroreflex to a higher AP without changing its gain,\textsuperscript{51} also resembling effects on the HR baroreflex evoked by central administration of physostigmine.\textsuperscript{16} Recent evidence indicates that the cardiac baroreflex is transiently inhibited at exercise onset, which may facilitate immediate vagal withdrawal.\textsuperscript{55}

In conclusion, our data indicate that the cholinergic projection from the PPT to the RVLM is an integral component of the central command pathway that regulates circulatory function during exercise and possibly other arousal or behavioral states.

**Acknowledgments**

We thank Simon McMullan and Peter Burke for helpful discussions.

**Sources of Funding**

Supported by the National Health and Medical Research Council of Australia (211023, 211196), Garnett Passe and Rodney Williams Memorial Foundation, North Shore Heart Research Foundation (6-05/06), and Northern Sydney Central Coast Area Health (2006:03). J.R.P. and N.N.K. receive Australian Postgraduate awards.

**Disclosures**

None.

**References**


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_Circ Res._ 2007;100:284-291; originally published online January 4, 2007; doi: 10.1161/01.RES.0000257370.63694.73

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Expanded Materials and Methods

Animals

Studies were approved by the Animal Care and Ethics Committee of Royal North Shore Hospital / University of Technology Sydney. Following experimentation rats were killed whilst under deep surgical anaesthesia by injection of potassium chloride (KCl 3M, 1mL iv).

Anaesthesia, surgical procedures and recording

Male Sprague Dawley (SD) rats (n=17) were anaesthetised with urethane (ethyl carbamate, Sigma-Aldrich, 1.2 g/kg, 10% ip). The withdrawal response to hindpaw pinch, testing of corneal reflexes and pattern of phrenic nerve discharge were used to monitor the depth of anaesthesia and level of neuromuscular blockade. Additional anaesthetic (urethane, 30mg) was administered when required. Rats were intubated and arterial (femoral or carotid) and intravenous catheters inserted for blood pressure (AP) measurement or drug administration. The left splanchnic sympathetic and phrenic nerves were isolated for recording \(^1\) and in four rats tail blood flow (TBF) was recorded via a laser Doppler flow probe (\(\lambda = 780 \pm 10\) nm, power = 0.5 – 1.0 mW,
Oxford Optronics, UK). All signals were amplified and recorded differentially (Bioamplifier, CWE Inc., USA) and acquired at 1000 Hz online (Spike 2, Cambridge Electronic Design, CED Ltd., UK) \(^1\)-\(^3\).

Microinjections into the RVLM were made following exposure of the dorsal surface and the RVLM was identified as the site where injection of glutamate evoked a sharp transient increase in AP (>50 mmHg) and splanchnic nerve activity (SNA) (>200 %) \(^1\). Microinjections into the pons were made by placing rats in a flat-skull position and exposing the parietal cortex bilaterally. In these rats both vagi were cut and in two animals EMG from the left biceps femoris was recorded. Rats were paralysed with pancuronium dibromide (0.8mg + 0.4mg/hr, 2mg/ml) and ventilated with 100% O\(_2\) mixed with room air adjusted to maintain end-tidal CO\(_2\) between 4 – 5 % of expired gases (Capstar-100, CWE Inc., USA).

Activation of cardiovascular reflexes

Reflexes were evoked as described previously \(^2\)-\(^4\). Briefly, changes in AP were evoked by sequential iv injection of sodium nitroprusside (SNP) and phenylephrine (PE) (10 \(\mu\)g/kg). Submaximal baroreflex inhibition of SNA was evoked by intermittent electrical stimulation of the AN (0.5 Hz x 100, twin pulses separated by 2.5 ms, pulse width 1 ms, 5-10 V). The somatosympathetic reflex was evoked by stimulation of the TN (0.5 Hz x 100, twin pulses separated by 2.5 ms, pulse width 1 ms, 15-20 V). Carotid chemoreceptor activation was achieved by substitution of the inhaled gas mixture for 100% N\(_2\) for 15s.

Drugs used
The following drugs were used for iv injection; all were dissolved in saline (0.9% NaCl, pH 7.4): atropine methylnitrate (mATR, a peripheral mAChR blocker, Sigma, 5mg/ml), oxotremorine sesquifumarate salt (OXO, a broad spectrum mAChR agonist, Sigma, 0.5 mg/ml), (-) scopolamine hydrobromide (SCOP, a broad spectrum mAChR antagonist, 5 mg/ml), SNP (Faulding) and PE (ICN Biomedicals Inc.). All drugs were administered in a volume of 0.4 ml/kg.

The following drugs were used for microinjection; all were dissolved in phosphate buffered saline (PBS, 0.01M, pH 7.4): l-glutamic acid (glut, monosodium salt, Sigma, 100mM (5nmol/50nl)); (-) scopolamine hydrobromide, a broad spectrum mAChR antagonist (SCOP, Sigma, 60mM (3nmol/50nl)); DL-homocysteic acid (DLH, MP Biomedicals USA, 100mM); (-)-bicuculline methiodide (Sigma, 4 mM); injection site markers, either colloidal gold (Sigma, 20nM) or methylene blue (4%) 5, 6.

Experimental protocol

All animals were pretreated with mATR to block peripheral mAChR (2 mg/kg). In nine rats, all reflexes were activated before and after central mAChR activation with iv injection of OXO (0.2 mg/kg). SCOP was injected bilaterally into the RVLM to determine if this reversed effects of OXO on SNA, AP and reflexes. Repeat doses of OXO were administered and reflexes were again tested. In four rats reflexes were not tested during experiments to examine effects of OXO on SNA, AP, phrenic nerve activity (PNA), TBF and spectral parameters before and after SCOP injection into the RVLM. In four additional rats, DLH (8 nmol) or bicuculline (0.2 nmol) was injected into the pons at 6.5 – 9.5 mm caudal and 2 mm lateral to Bregma at varying depths (5.5 – 9 mm ventral) 7 to examine site-specific effects on AP, SNA and SNA baroreflex responses. In some animals effects on the SSR were also examined. Effects
on EMG were examined in two rats prior to neuromuscular blockade and nerve recording. In three animals sites within the PPT where stimulation evoked increases in SNA and SNA baroreflex responses were tested following SCOP (2 mg/kg) administered intravenously to block central mAChR.

Data Analysis

All data were analysed offline using Spike 2 software (CED Ltd, UK). As previously described $^2,^3$, the magnitude of respiratory modulation of SNA was quantified by peri-phrenic averaging and SNA responses to intermittent AN or TN stimulation were quantified by peri-stimulus averaging. Maximal changes in SNA and AP following brief hypoxia were expressed as a percent of baseline SNA or AP (mmHg) taken over 10 s prior to the stimulus. Baroreflex function curves describing the SNA-MAP relationship and their first derivatives for calculation of gain were calculated as described previously $^8$. Spectral parameters were quantified at low (0.25-0.75 Hz) and phrenic nerve frequencies (1-2.5 Hz) by fast Fourier transformation (size 256) of systolic AP and SNA time series uniformly resampled at 10 Hz.

Retrograde labeling from the spinal cord or the RVLM

In 10 rats anaesthetised with sodium pentobarbital (60 mg/kg ip) and administered carprofen (2.5 mg ip), injections of the retrograde tracing agent cholera toxin β subunit (CTB, 1 %, 200 nl) were made bilaterally into the intermediolateral cell column (IML) at T1/T2 as described previously $^9,^{10}$. In three additional rats, AP was recorded from the superficial femoral artery and CTB (20 nl) was injected into the RVLM identified functionally as a site where glut evoked a sharp rise in AP (>50 mmHg). Following recovery (36 hrs RVLM group, 72-96 hrs IML group), rats were
deeply anaesthetised with pentobarbital (70 mg/kg) and transcardially perfused with tissue culture medium (ph 7.4, Dulbecco’s Modified Eagle’s Medium, Sigma) and 4 % formaldehyde (0.1 M in phosphate buffer (PB)) (RVLM-injected group and 7 IML-injected rats) or 0.9% saline and freshly prepared 4 % paraformaldehyde (0.1 M in PB) (3 IML-injected rats).

Light and fluorescence immunohistochemistry, in situ hybridisation and PCR
Following overnight fixation, serial transverse sections (50 µm) of the entire brain or medulla and spinal cord were cut using a vibrating microtome (Leica VT1000S). For light or fluorescence immunohistochemistry, sections were washed serially in 50% ethanol (30 min) and Tris-phosphate-buffered saline (TPBS; Tris-HCl 0.01 M, sodium phosphate buffer 0.01 M, 0.9% NaCl, pH 7.4) (3 x 30 min). For fluorescence microscopy, sections were incubated with three primary antibodies (see below) diluted in 5% normal horse serum (NHS) prior to overnight incubation with fluorophore-conjugated secondary anti-sera diluted 1:500 in 2% NHS. For light microscopy, sections were reacted with species-specific primary and secondary antibodies, which were detected sequentially using enhanced diaminobenzidine reactions (nickel and imidazole) as described previously. RVLM and IML injection sites were visualised by immunohistochemistry to CTB.

For immunohistochemical detection of mRNA as well as protein, floating brain sections were processed with a combined method of in situ hybridisation (ISH) and fluorescence immunocytochemistry. Sections were firstly hybridized with preproenkephalin (PPE), M2 or M3 receptor antisense riboprobe, washed in descending concentrations of salt, then reacted with primary antibodies against
digoxigenin (DIG, alkaline phosphatase conjugated) and other proteins (see below) for 48 hrs at 4°C. The proteins were then detected by fluorophore-conjugated secondary antibodies (1:500, Jackson) and DIG-labelled in situ neurons by a histochemical reaction using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate salts.

Primary antibodies used were as follows. Spinally projecting neurons were visualised using goat anti-CTB (light 1:50 000, fluorescence 1:1000, List). Cholinergic perikarya and terminals were visualised using rabbit anti-vAChT (light 1:500, fluorescence 1:800, Chemicon, USA). Catecholaminergic neurons were visualised using mouse anti-TH (fluorescence 1:2000, Sigma, Australia). For visualisation of enkephalinergic neurons in the RVLM sections were reacted with primary antibodies against DIG (alkaline phosphatase conjugated sheep anti-DIG (1:1000, Roche, Germany), mouse anti-neuron-specific nuclear protein (NeuN, 1:2000, Chemicon, USA) and rabbit anti-vAChT (1:800). For visualisation of M2 or M3 receptor mRNA within the RVLM sections were reacted with primary antibodies against DIG, mouse anti-TH (1:2000) and rabbit anti-CTB (1:5000, Virostat, USA).

Secondary antibody mixtures varied and were as follows. All secondary antisera (Jackson Immunoresearch) were diluted 1:500 in TPBS-M containing 2% NHS. For visualisation of spinally projecting and catecholaminergic neurons in the RVLM, sections were incubated overnight in a mixture of Fluorescein Isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (for vAChT), 7-Amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated donkey anti-mouse IgG (for TH) and Texas Red (TR)-conjugated donkey anti-goat IgG (for CTB). For visualisation of enkephalinergic
neurons and cholinergic terminals in the RVLM, sections were incubated overnight in Cy3-conjugated donkey anti-mouse IgG (for NeuN), FITC-conjugated donkey anti-rabbit (for vAChT) and DIG-labelled in situ neurons (for PPE mRNA) were detected as described in detail previously. For visualisation of M2 or M3 receptor mRNA colocalised with catecholaminergic and spinally projecting neurons in the RVLM, sections were incubated for 24 hrs at 4°C with Cy3-conjugated donkey anti-mouse IgG (for TH) and FITC-conjugated donkey anti-rabbit IgG (for vAChT). DIG-labelled neurons (for M2 or M3 mRNA) were detected as described previously. For visualisation of cholinergic neurons projecting to the RVLM, sections were incubated with FITC-conjugated donkey anti-rabbit IgG (for vAChT) and TR-conjugated donkey anti-goat IgG (for CTB).

*Synthesis of DIG-labelled riboprobes for M2, M3 receptor and PPE*

DNA fragments for PPE was firstly amplified by PCR from rat brain cDNA using forward and reverse primers with SP6 and T7 promoters attached at the 5’ end, respectively. Homology analysis using nucleotide BLAST searches of the National Centre for Biotechnology Information (NCBI, USA) were carried out to ensure that the RNA probe sequence was specific to the mRNA of interest only. Antisense sequences, riboprobe length and GeneBank accession source numbers for M2 receptor, M3 receptor and PPE mRNA are shown in Table 1.
Supplemental Table 1. Sp6- and T7-tagged primer sequences for DIG-labelled riboprobes

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<tr>
<th>Gene</th>
<th>Primer</th>
<th>GenBank</th>
<th>Nucleotide sequence (5'--&gt;3')*</th>
<th>Size (bp)</th>
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*Upper case denotes Sp6 and T7 tag sequences and lower case denotes primer sequence

The antisense and sense riboprobes were then transcribed *in vitro* using DIG-11-UTP (Roche Applied Sciences) and Ampriscibe T7 or Sp6 transcription Kit (Epicenter, USA).

All sections were mounted onto slides with Prolong anti-fade (Molecular Probes, Invitrogen, Australia) or Vectorshield Hard Set (Vector laboratories, USA) and viewed using a fluorescent microscope (Leica DML or Zeiss imager Z.1, Germany). Images were acquired using a SPOT2 digital camera (Diagnostic Instruments).
Fluorescence images were merged using SPOT2 and contrast adjusted using Zeiss Imaging software.

**PCR detection of M1-M5 receptor mRNA in the RVLM**

SD (n=3) were anesthetised and perfused as previously described. The brainstem was cut into 350 μm sections in freezing conditions using a vibratome (Leica VT1000S). The RVLM (located ventral to the nucleus ambiguus in the two sections spanning approximately 700 μm immediately caudal to the facial nucleus) and the cerebellum (posterior lobe) were immediately dissected into RNAlater cryoprotectant reagent (Ambion Inc, USA) and stored at -70 °C.

Total RNA was isolated and reverse transcribed to cDNA as described previously. PCR (Corbett Rotorgene 3000) was performed in a reaction solution (20 μl) composed of 1 μl of cDNA, 100 μM each primer (see Table 2), 2 mM MgCl₂, 0.25 mM dNTPs, 10X immunobuffer, 0.125 U Immolase hot-start DNA polymerase (Bioline, Australia) and SYBR Green I fluorescent stain (1:40 000 dilution, Molecular Probes, USA). Samples of the amplified M1-M5 receptor gene products were electrophoresed in an ethidium bromide-stained 2% agarose gel. Gene products were recovered enzymatically using QiaQuick gel extraction kit (Qiagen, Australia) and the DNA was sequenced using the ABI Prism 3730 platform (SUPAMAC, Australia) to confirm specificity to the GenBank sequence.
### Supplemental Table 2. Primer sequences for RT-PCR detection of M1-M5 receptor mRNA

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Detection of close appositions and cell counts

Light microscopic sections were viewed at high magnification (x 100) to identify close appositions between vAChT-IR terminals (black reaction product) and CTB-labelled neurons in the RVLM (brown reaction product). CTB- and TH-immunoreactive (IR) cells were counted in every 4\textsuperscript{th} section in the RVLM from the caudal pole of the facial nucleus (VII) (Bregma -11.80 to -12.40 mm) \textsuperscript{9} to determine the proportion of cells that are closely apposed by vAChT-IR varicosities or express M2 receptor mRNA. Expression of M3 receptor mRNA and close appositions from vAChT-IR was also examined in NeuN-IR / PPE-positive cells.

Statistical analysis

All data are presented as mean ± standard error of the mean. A paired Students’ t-test was used to calculate effects of treatment versus control or following SCOP and \( P<0.05 \) was considered significant.
Supplemental Figure Legends

Figure S1  *MAChR activation within the RVLM enhances the baroreflex and inhibits the somatosympathetic and peripheral chemoreflex.* Grouped data from nine animals showing OXO-evoked effects on cardiorespiratory reflex function. OXO enhances SNA baroreflex responses, inhibits both early and late peaks of the somatosympathetic reflex and attenuates sympathoexcitatory and pressor effects of the peripheral chemoreflex. Most reflexes return to control level following SCOP injection bilaterally into the RVLM, although the early peak of the somatosympathetic reflex remains attenuated and chemoreflex activation evokes no change in AP. Prior injection of SCOP into the RVLM blocks all effects on cardiorespiratory reflexes evoked by OXO. Data are mean ± SEM, *P<0.05, ** P<0.01, ns = non significant.

Figure S2  *MAChR activation within the RVLM enhances low frequency (0.4 Hz) and respiratory-related oscillations of SNA.* Power spectra of SNA and SAP (uniformly resampled at 10 Hz) are illustrated showing that the amplitude of the LF peak is increased following OXO. Grouped data from seven animals are shown on right illustrating the significant increase in LF power of SNA and SAP following OXO and blockade of this effect by prior injection of SCOP bilaterally into the RVLM. OXO also tended to increase HF oscillations of SNA but not SAP. Data are mean ± SEM, *P<0.05, ** P<0.01, ns = non significant.

Figure S3  *M1-M5 receptor subtypes are expressed in the RVLM.* 2% Ethidium bromide stained agarose gel with high intensity amplicons confirming the presence of M1-M5 receptor mRNA in a tissue punch taken from the RVLM in an SD rat.
**Figure S4**  *Comparison of ChAT and vAChT immunoreactivity in the ventral medulla.* Coronal sections of the medulla at the same level of the RVLM are shown at low (left panel) and high power (right panel) from different SD rats that were perfused transcardially. 50 µm brainstem sections were reacted for immunoreactivity against ChAT (sheep, 1:500, Chemicon, USA) or vAChT (rabbit 1:500, Chemicon, USA) and detected using nickel-intensified diaminobenzidine reactions. ChAT labelling in the medulla identifies large motoneurons of the compact and loose formation of the nucleus ambiguus that can be seen to send fine ChAT containing fibres ventrally into the RVLM. Smaller ChAT-positive perikarya are clearly visible in the medial ventral medulla. In comparison, vAChT labelling identifies ambiguous motoneurons and cholinergic terminal fields in the RVLM but labelling in the ventromedial region is clearly absent. Scale bars = 200 µm. *Abbreviations:* NAc, nucleus ambiguus pars compacta; pyr, pyramidal tract; SpV, spinal trigeminal tract.
Supplemental References


Figure S1.
Figure S2.
Figure S3.
Figure S4.