Differential cardiorespiratory and sympathetic reflex responses to microinjection of
neuromedin U in rat rostral ventrolateral medulla

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Abbreviations

AUC, area under the curve; BP, blood pressure; CNS, central nervous system; HR, heart rate; I, inspiratory; LSNA, lumbar sympathetic nerve activity; MAP, mean arterial pressure; NMU, neuromedin U; NMU1, neuromedin U receptor 1; NMU2, neuromedin U receptor 2; NTS, nucleus tractus solitarius; PBS, phosphate buffered saline; PE, phenylephrine hydrochloride; PI, post-inspiratory; PNA, phrenic nerve activity; PNamp, phrenic nerve amplitude; PNf, phrenic nerve frequency; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla; SNP, sodium nitroprusside; SSNA, splanchnic sympathetic nerve activity; Tᵢ, inspiratory period; Tₑ, expiratory period.
Abstract

The rostral ventrolateral medulla (RVLM) regulates sympathetic vasomotor outflow and reflexes. Intracerebroventricular neuromedin U (NMU) increases sympathetic nerve activity (SNA), mean arterial pressure (MAP) and heart rate (HR), but the central nuclei that mediate these effects are unknown. In urethane-anaesthetized, vagotomized and artificially ventilated male Sprague-Dawley rats (n = 36) the effects of bilateral microinjection of NMU (50 nl, each side) into RVLM on cardiorespiratory variables, somatosympathetic, arterial baro- and chemo- reflexes were investigated. Microinjection of NMU into RVLM elicited a hypertension, tachycardia, and an increase in splanchnic SNA (SSNA) and lumbar SNA (LSNA) at lower doses (25 and 50 pmol). At higher dose (100 pmol), NMU caused a biphasic response, a brief hypertension and sympathoexcitation followed by a prolonged hypotension and sympathoinhibition. The peak excitatory and inhibitory response was found at 100 pmol NMU with an increase in MAP, HR, SSNA and LSNA of 36 mmHg, 20 bpm, 34% and 89%, respectively, and a decrease of 33 mmHg, 25 bpm, 42% and 52%, respectively, from baseline. NMU, in the RVLM, also increased phrenic nerve amplitude, expiratory period, and reduced inspiratory period. NMU (100 pmol) attenuated the somatosympathetic reflex, and the sympathoexcitatory and respiratory responses to hypoxia and hypercapnia. Following NMU injection in RVLM, the maximum gain of the SSNA baroreflex function curve was increased, but that of the LSNA was reduced. The present study provides functional evidence for a complex differential modulatory activity of NMU on the cardiovascular and reflex responses that are integrated in the RVLM.
Introduction

The rostral part of the rostral ventrolateral medulla (RVLM) is a key nucleus containing bulbospinal neurons that are inhibited by baroreceptor activation (presympathetic neurons), and provide a monosynaptic excitatory drive to preganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord (Oshima et al., 2006; Oshima et al., 2008). Overall sympathetic outflow is largely determined by the balance of excitatory and inhibitory inputs to the presympathetic neurons in the RVLM (Dampney, 1994; Pilowsky and Goodchild, 2002). The inputs that affect the activity of RVLM neurons may arise from other parts of the brain and spinal cord, or from afferent neurons in the periphery. At the present time, a complete understanding of the source of these different inputs, the neurotransmitters that they use, and their postsynaptic receptors is not known.

Neuromedin U (NMU), originally isolated from porcine spinal cord, is so named because of its potent contractile activity on uterine smooth muscle (Minamino et al., 1985). The N-terminal region of NMU varies greatly from species to species (mammals to amphibians), but the C-terminal sequence is highly conserved and is the biologically active part of the molecule. NMU acts equally at two G-protein coupled receptors (G_{q/11} and to a lesser extent G_{i/o}), NMU receptor 1 (NMU1) and NMU receptor 2 (NMU2) (Brighton et al., 2004; Gartlon et al., 2004). NMU1 and NMU2 are located predominantly, but not exclusively, in peripheral tissues and the central nervous system (CNS), respectively (Howard et al., 2000; Raddatz et al., 2000; Szekeres et al., 2000; Westfall et al., 2002). NMU injection into brain decreases food intake and body weight gain (Howard et al., 2000; Nakazato et al., 2000; Peier et al., 2009), increases release of oxytocin, arginine vasopressin and ACTH (Ozaki et al., 2002; Rokkaku et al., 2003), increases gross locomotor activity and body temperature (Howard et
al., 2000; Nakazato et al., 2000) and induces stress (Hanada et al., 2001; Zeng et al., 2006) and pain (Yu et al., 2003; Nakahara et al., 2004b; Zeng et al., 2006), suggesting differential physiological and pharmacological roles of NMU.

Neurons expressing NMU are present in the key cardiorespiratory areas of the brainstem and spinal cord including the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus, inferior olive and area postrema (Howard et al., 2000), raising the possibility that NMU may participate in the central regulation of cardiorespiratory function. NMU2 are expressed predominantly in paraventricular nucleus, the wall of 3rd ventricle, hippocampus, dorsal root ganglia and dorsal horn of spinal cord (Howard et al., 2000). Intracerebroventricular injection of NMU increases sympathetic nerve activity (SNA), blood pressure (BP), heart rate (HR) and plasma noradrenaline (Chu et al., 2002; Tanida et al., 2009). Microinjection of NMU into the NTS decreases BP and HR (Tsubota et al., 2003). We have previously demonstrated that NMU in the spinal cord shows differential effect on sympathetic outflow, respiration and sympathetic reflexes (Rahman et al., 2011). To date, no study has examined the effects of NMU in the RVLM on cardiorespiratory function and sympathetic reflexes.

The objective of the present study was 1) to determine the cardiovascular and respiratory effects elicited by microinjection of NMU into the RVLM of anaesthetized rats, and 2) to evaluate the effects of NMU on somatosympathetic, baro- and chemo- reflexes in the RVLM. Our principal findings are that bilateral microinjection of NMU in the RVLM increases mean arterial pressure (MAP), HR, splanchnic SNA (SSNA), lumbar SNA (LSNA) at lower doses, but shows biphasic effect at higher dose. NMU in the RVLM increases respiratory drive. NMU also attenuates somatosympathetic, and peripheral and central chemoreceptor reflexes.
SSNA exhibits an increase in maximum gain of baroreflex function curve with a reduced operating range of MAP following NMU injection, whereas LSNA shows an increase in operating range with reduced gain. These findings suggest a differential role of NMU in the modulation of central cardiorespiratory control.
Materials and Methods

Experiments in this study were approved by the Animal Ethics Committee of Macquarie University, Sydney, Australia and conducted in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NSW – Animal Research Act 1985).

General procedures

Experiments were carried out on adult male Sprague-Dawley rats (n = 36, 400-500 g). Rats were anaesthetized with an initial dose (1.2-1.4 g/kg, ip) of urethane. The depth of anaesthesia was assessed by monitoring changes in arterial pressure in response to pinching a hind paw every 60 min; supplemental doses of urethane (20-30 mg, iv) were given if baseline BP rose more than 10 mmHg. Body temperature was monitored and maintained at 37±0.5°C by placing rats on a feedback-controlled heating blanket for the duration of the experiment (Harvard Apparatus, Holliston, MA, USA).

The left carotid artery and right jugular vein were cannulated with polyethylene tubing (internal diameter = 0.58 mm; outer diameter = 0.96 mm) to enable monitoring of BP and regular injections of drugs and fluids. In 5 animals, both femoral veins were cannulated for the administration of sodium nitroprusside (SNP) or phenylephrine hydrochloride (PE). HR was derived from arterial BP. A tracheotomy was performed to allow artificial ventilation and end-tidal CO₂ monitoring. Nerve recordings were obtained from SSNA, LSNA and phrenic
nerve activity (PNA). Through a retroperitoneal approach, the left greater splanchnic and lumbar nerves were isolated and dissected; the left phrenic nerve was approached dorsally. The distal end of the nerves were tied with 5/0 silk thread and cut to permit recording of efferent nerve activity. In some experiments, the sciatic nerve was isolated, tied and cut. Once the nerves were isolated, they were covered with saline soaked cotton wool for the duration of the remainder of surgical preparation to prevent desiccation.

Rats were placed in a stereotaxic frame and the head tilted downwards at a 45° angle to the horizontal. All animals were paralyzed (pancuronium bromide; 0.8 mg iv initially, then 0.4 mg/h iv) and artificially ventilated with oxygen-enriched room air. A bilateral vagotomy was performed to ensure the phrenic nerve discharge was not synchronized to the cycle of mechanical ventilation. End-tidal CO₂ and pH were maintained at 4.0-4.5% and 7.35–7.45, respectively, by adjusting the rate and depth of ventilation after arterial blood gas analysis (pH 7.4 ± 0.02, PaCO₂ 40.5 ± 0.4). Animals were infused with 5% glucose in water (1.0– 2.0 ml/h) to ensure hydration. Nerve recordings were made with bipolar silver wire electrodes. The recording electrodes were immersed in a pool of liquid paraffin oil to prevent dehydration and for electrical insulation. After nerves were placed on the recording electrodes, rats were allowed to stabilize for 30-60 min. The neurograms were amplified (x10,000; CWE Inc., Ardmore, PA, USA), band pass filtered (0.1–2 kHz), sampled at 3 kHz (1401 plus, CED Ltd, Cambridge, UK) and recorded on computer using Spike2 software (v7, CED Ltd, Cambridge, UK).

The dorsal surface of the medulla was exposed by partial occipital craniotomy. The dura was cut and reflected laterally and the nerve tissue was kept moist by physiological saline until
the experiment protocol began. Brain microinjections and, functional identification of the RVLM, was performed as described previously (Rahman et al., 2012; Shahid et al., 2011b). Briefly, bilateral microinjection of test agents into the RVLM, at a fixed volume of 50 nl, was carried out stereotaxically and sequentially with single- or multi- barrel glass pipettes over a 10-s period. At the beginning of each experiment, RVLM was identified functionally on either side by an increase of >30 mmHg in MAP following microinjection of L-glutamic acid (5 nmol). The preliminary coordinates used to find the RVLM were 1.8 mm rostro-caudal, 1.8 mm medio-lateral, and 3.5 mm dorso-ventral to calamus scriptorius. Vehicle solutions (PBS) contained 2% rhodamine beads to aid in subsequent histological verification of the injection site. The brains were then removed from the skulls, fixed in 4% paraformaldehyde and sectioned at 50 µm to verify the microinjection sites. Only rats with microinjection sites within the boundaries of the RVLM were used for data analysis.

Activation of sympathetic reflexes

Reflexes were evoked as described previously (Rahman et al., 2011; Shahid et al., 2011a; Shahid et al., 2011b). The somatosympathetic reflex was activated by electrical stimulation (5-15 V, 50 sweeps, 0.2 ms pulses at 1 Hz) of the sciatic nerve. Sympathetic baroreflex function curves were generated by sequential intravenous injection of SNP (0.01 mg/kg) and PE (0.01 mg/kg) via two different venous lines. Peripheral and central chemoreceptors were activated by ventilating the animals with 100% N₂ for 12-14 s (brief hypoxia; BOC gas and gear, NSW, Australia) and 10% CO₂:90%O₂ for 1 min (hypercapnia; BOC gas and gear, NSW, Australia), respectively.

Experimental protocol
To investigate the effects of NMU on cardiovascular and respiratory responses in the RVLM, three doses of NMU (500 µM, 1 mM, 2 mM equivalent to 25, 50 and 100 pmol) were injected into the RVLM. PBS was microinjected as a volume and vehicle control 30 min after the completion of glutamate application. This time lag was adopted to ensure complete recovery from the glutamate-induced pressor response before bilateral microinjection into the RVLM of vehicle. Another gap of 25 min was given before injection of NMU. Each animal received only one treatment and vehicle to avoid the tachyphylaxis. The effect of NMU on basal MAP, HR, SSNA, LSNA or PNA was then evaluated for 60 min post-treatment.

The following sympathetic reflexes were evoked before and after bilateral microinjection of vehicle or NMU (100 pmol), respectively: (i) somatosympathetic reflex; (ii) baroreflex; (iii) peripheral chemoreceptor reflex; and (iv) central chemoreflex. One reflex was conducted in a single rat. Each reflex was evoked at both the peak excitatory and inhibitory phases of NMU response.

Data acquisition and analysis

For averaging purpose, neurograms were rectified and smoothed (SSNA and LSNA, 1 s time constant; PNA, 50 ms). Zero SNA value was obtained from the minimum background activity after death and was subtracted from SNA before analysis with off-line software (Spike 2 version 7). Baseline values were obtained by averaging 60 s of data 5 min prior to NMU or PBS injection and maximum changes were expressed as absolute (MAP, HR, phrenic nerve frequency (PNf), inspiratory period (T_i), and expiratory period (T_E)) or percentage (SSNA, LSNA, and phrenic nerve amplitude (PNamp)) changes from baseline values. Phrenic-triggered ensemble averages of SSNA and LSNA were generated from 60-s
portions of data before and after NMU injection to evaluate cardiorespiratory coupling. The area under the curve (AUC), less baseline, of SSNA/LSNA activity during the inspiratory (I) and post-inspiratory (PI) phases was determined. SSNA and LSNA were rectified and smoothed at 5 ms and 1 s time constants to analyze somatosympathetic reflex and baroreceptor reflex, respectively. To analyze reflexes, SNA was normalized between the activity of SNA before PBS injection (100%) and the SNA after death (0%). The SSNA/LSNA response to sciatic nerve stimulation was analyzed using peristimulus waveform averaging. The AUC of the sympathoexcitatory peaks was analyzed. The responses to hypoxia (100% N₂ inhalation for 12-14 s) and hypercapnia (10% CO₂ in 90% O₂ for 1 min) were quantified by comparing the average maximum SSNA/LSNA during hypoxia or hypercapnia with a baseline period during normal hyperoxic ventilation. To normalize the difference in baseline values, efficiency of the somatosympathetic and chemoreceptor reflexes were assessed by calculating percent change from the control. The percentage changes for cardiorespiratory coupling, somatosympathetic, peripheral chemo- and central chemo- reflexes were calculated according to Eq 1.

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\text{(Response to drug or vehicle or control/control response) } \times 100\% \quad \text{Eq 1}
\]

To analyze the data from the baroreflex function tests, mean MAP was divided into 1 s consecutive bins and the average SSNA/LSNA during each bin was determined; successive values were tabulated and graphed as XY plots, taking MAP as the abscissa and SSNA/LSNA as the ordinate. Each data set was then analysed to determine the sigmoidal curve of best fit (Kent et al., 1972), which is described by Eq 2.
where $y$ is SSNA or LSNA, $x$ is MAP, $A_1$ is the $y$ range ($y$ at the top plateau – $y$ at the bottom plateau), $A_2$ is the gain coefficient, $A_3$ is the value of $x$ at the midpoint (which is also the point of maximum gain), and $A_4$ is $y$ at the bottom plateau. The computed baroreflex function curves were differentiated to determine the gain of SNA of the baroreflex across the full range of MAP and the peak gain of each curve was determined. The range of SNA was calculated as the difference between the values at the upper and lower plateaus of the curve. The threshold and saturation values for MAP were defined (McDowall and Dampney, 2006) as the values of MAP at which $y$ was 5% (of the $y$ range) below and above the upper and lower plateaus, respectively.

Statistical analysis was conducted with GraphPad Prism (version 5.0) (GraphPad, La Jolla, CA, USA). Grouped data are expressed as mean ± SE. Paired t-test was used to analyze peak effects and reflexes. $P < 0.05$ was considered significant.

Drugs

NMU (MW = 2643; Cat. No: 2421; Lot No: 30955) was obtained from, Auspep (Australia). Urethane, L-glutamic acid, glucose, PE and SNP were purchased from Sigma-Aldrich (NSW, Australia), pancuronium bromide from AstraZeneca Pty Ltd (NSW, Australia), rhodamine microbeads from Molecular Probes (NSW, Australia) and PBS (10 mM in 0.9% NaCl) tablets from AMRESCO Inc. (Solon, OH, USA). NMU and rhodamine (2% v/v) were dissolved and further diluted in PBS (10 mM; pH 7.4). PBS, PE and SNP were prepared in de-ionised water. Urethane was dissolved in 0.9% NaCl and L-glutamic acid in PBS.
Results

Cardiorespiratory responses to microinjection of NMU into the RVLM

To determine the effect of NMU in the RVLM on the MAP, HR, SSNA and LSNA, NMU was microinjected bilaterally at three different doses (25, 50 and 100 pmol) into the functionally identified and histologically verified sites within the RVLM. In the anaesthetized rat, baseline MAP and HR were 99 ± 7 mmHg and 496 ± 5 bpm, (n = 16) respectively. Microinjection of NMU into the RVLM elicited a dose-dependent excitatory response on the cardiovascular parameters recorded (Figure 1A, B). At lower doses (25 and 50 pmol, n = 4 and 6), NMU increased MAP, HR, SSNA and LSNA (Figure 1B). Higher dose of NMU (100 pmol, n = 6) promoted a short hypertensive, tachycardiac and sympathoexcitatory response followed by a prolonged hypotensive, bradycardiac and sympathoinhibitory response (Figure 1A). The duration of the excitatory response to NMU was similar at lower and higher doses but the magnitude of the response was greater at higher doses. The maximum response was observed at 100 pmol injection of NMU with an increase in MAP, HR, SSNA and LSNA of 36 ± 2 mmHg (P<0.001), 20 ± 3 bpm (P<0.001), 34 ± 3 % (P<0.001) and 89 ± 6 %
(P<0.001), respectively; and a decrease of 33 ± 3 mmHg (P<0.001), 25 ± 4 bpm (P<0.01), 42 ± 5 % (P<0.001) and 52 ± 7 % (P<0.01), respectively, as compared to PBS (Figure 1B). The effects were almost immediate and peaked about 30 seconds after bilateral administration of the peptide (Figure 1A). The excitatory response lasted for 5-7 min when the inhibitory response started that returned to baseline in about 30 min (Figure 1A). The 100 pmol dose of NMU was used for the reflex studies. Microscopic examination of microinjection sites, marked with rhodamine microbeads, confirmed that all injections used in this study were within 500 µm of the caudal end of the facial nucleus (Figure 1C).

Microinjection of NMU (25, 50 and 100 pmol) into the RVLM elicited a dose-dependent increase in PNamp without any significant change in PNf (Figure 2A). The maximum amplitude of PNamp response was obtained with 100 pmol NMU with an increase of 28 ± 3 % (P<0.001) as compared to PBS (Figure 2B). NMU (100 pmol) caused a significant decrease in Ti (-0.13 ± 0.02 s vs -0.005 ± 0.002 s of PBS, n = 4, P<0.05) but significantly increased Te (0.37 ± 0.01 s vs -0.015 ± 0.02 s of PBS, n = 4, P<0.001; Figure 2A, B).

**Effect of NMU on SNA-respiration related rhythm in the RVLM**

Peri-phrenic averaging of the sympathetic nerve activity produces inspiratory (I) and post-inspiratory (PI) peaks in SSNA (Figure 3A). On the other hand, LSNA showed only one distinct respiratory-related peak during PI-period (Figure 3C), consistent with our previous report (Miyawaki et al., 2002). However, we measured the AUC of the area in LSNA corresponding to the I-period. At the excitatory phase of NMU (100 pmol), the AUC of I-peak of both SSNA and LSNA was increased by 42 ± 11 % (n = 4, P<0.05) and 37 ± 9 % (n = 4, P<0.05), respectively as compared to control whereas the AUC of PI-peak of both SSNA and LSNA were decreased significantly by 47 ± 7 % (n = 4, P<0.01) and 46 ± 7 % (n = 4,
During the inhibitory phase of NMU (100 pmol) response, the AUC of both the I- and PI- peaks of SSNA and LSNA were significantly reduced (SSNA: 42 ± 9 % and 59 ± 10 % respectively, n = 4, *P* <0.01; LSNA: 59 ± 14 % and 65 ± 14 % respectively, n = 4, *P* <0.05) as compared to control (100%) (Figure 3A-D).

**Effect of NMU injection into the RVLM on somatosympathetic reflex**

To investigate whether NMU injection into the RVLM altered the integration of somatosympathetic reflex as represented by two characteristic excitatory peaks in SSNA and LSNA, sciatic nerve was stimulated intermittently at baseline (control) and after PBS and NMU (100 pmol) injection (Figure 4A,C). The latencies of the peaks of SSNA (91 ± 2 and 181 ± 3 ms, respectively; n = 6) or LSNA (112 ± 3 and 201 ± 6 ms, respectively; n = 6) were not significantly altered by PBS (SSNA: 91 ± 1 and 181 ± 3 ms, respectively; LSNA: 110 ± 3 and 198 ± 7 ms, respectively; n = 6) or NMU (SSNA: 95 ± 3 and 183 ± 5 ms, respectively, at excitatory phase and 88 ± 2 and 175 ± 5 ms, respectively, at inhibitory phase; LSNA: 114 ± 3 and 196 ± 5 ms, respectively, at excitatory phase and 118 ± 9 and 200 ± 11 ms, respectively, at inhibitory phase; n = 6) injection. The AUC of both the excitatory peaks of both SSNA and LSNA were significantly reduced at excitatory (SSNA: 62 ± 5 %, *P* <0.001, and 52 ± 7 %, *P* <0.01, respectively, n = 6; LSNA: 60 ± 6 % and 54 ± 10 %, respectively, n = 6, *P* <0.01; as compared to control (100%)) and inhibitory phase (SSNA: 47 ± 8 %, *P* <0.01, and 43 ± 7 %, *P* <0.001, respectively, n = 6; LSNA: 54 ± 4 %, *P* <0.001, and 51 ± 12 %, *P* <0.05, respectively, n = 6; as compared to control (100%)) of NMU (100 pmol) following bilateral microinjection in the RVLM (Figure 4A-D).
Effect of NMU injection into the RVLM on baroreflex

Increase in systemic blood pressure following i.v. injection of PE activated arterial baroreceptors that elicited reflex sympathoinhibitory response in SSNA and LSNA (Figure 5A). The HR component of the baroreflex was not assessed because the rats were vagotomized. For SSNA, NMU (100 pmol) at its excitatory phase in the RVLM significantly increased the lower plateau, upper plateau, range of SSNA and maximum gain but reduced the operating range significantly as compared to control (n = 4, Figure 5B, Table 1) thereby increasing the barosensitivity. NMU (100 pmol) at its inhibitory phase, on the other hand, did not cause any significant alteration in any of the parameters of baroreflex function curve as compared to control (n = 4, Figure 5B, Table 1). For LSNA, at the excitatory phase of NMU (100 pmol) response in the RVLM, the threshold level and maximum gain of baroreflex function curve were significantly reduced whereas the upper plateau, saturation level and operating range were increased significantly as compared to control (n = 5, Figure 5C, Table 1). At the inhibitory phase, NMU increased the saturation level and operating range and decreased maximum gain without any significant change in other parameters (n = 5, Figure 5C, Table 1).

Effect of NMU injection into the RVLM on chemoreflex

Brief hypoxia (100% N₂ for 12-14 s) in rat activated peripheral chemoreceptors and resulted in sharp and brief increase in MAP, HR, SSNA, LSNA, PNamp and PNf (Figure 6A). NMU (100 pmol), at both excitatory and inhibitory phases, in the RVLM significantly reduced the effects of hypoxia on MAP (65 ± 8 and 67 ± 9 %, respectively, n = 5, P<0.05), HR (70 ± 9 and 79 ± 6 %, respectively, n = 5, P<0.05), SSNA (72 ± 2 %, P<0.001, and 77 ± 8 %, P<0.05, respectively, n = 5), LSNA (60 ± 10 and 54 ± 9 % respectively, n = 5, P<0.05) and
PNf (66 ± 5 %, \( P < 0.01 \), and 64 ± 9 %, \( P < 0.05 \), respectively, \( n = 5 \)) as compared to control (100%), without altering PNamp response (Figure 6A,B).

Hypercapnia (10% CO₂ for 1 min) in rat activated central chemoreceptors that elicited an increase in MAP, HR, SSNA, LSNA and PNamp (Figure 7A). NMU (100 pmol), at both excitatory and inhibitory phases, in the RVLM significantly reduced the effects of hypercapnia on MAP (33 ± 11 and 45 ± 6 %, respectively, \( n = 4, P < 0.01 \)), SSNA (64 ± 5 and 40 ± 5 %, respectively, \( n = 4, P < 0.01 \)), LSNA (63 ± 9 %, \( P < 0.05 \), and 49 ± 8 %, \( P < 0.01 \), respectively, \( n = 4 \)) and PNamp (60 ± 5, and 47 ± 9 %, respectively, \( P < 0.01, n = 4 \)) but increased tachycardiac response (146 ± 11 and 151 ± 14 %, respectively, \( n = 4, P < 0.05 \)), as compared to control (100%) (Figure 7A,B).

**Discussion**

The novel findings of the study are 1) NMU microinjection into the RVLM causes sympatthoexcitation, hypertension and tachycardia at lower doses, while at a higher dose, NMU produces biphasic cardiovascular response in the RVLM; 2) Respiratory modulation of SSNA and LSNA are differentially affected after microinjection of NMU; 3) Microinjection of NMU into the RVLM increases respiratory drive as represented by an increases in PNamp, \( T_e \) and a decrease in \( T_i \); 4) The excitatory peaks of both SSNA and LSNA evoked by sciatic nerve stimulation are attenuated by NMU injection into the RVLM; 5) Baroreflex sensitivity of SSNA is increased, but the barosensitivity of LSNA, is decreased following NMU injection; 6) NMU, at both excitatory and inhibitory phases, attenuates the pressor,
tachycardia, sympathoexcitatory and PNf responses to hypoxia in the RVLM; 7) The sympathoexcitatory, pressor and PNamp responses to hypercapnia are reduced while the tachycardia response is increased by NMU.

To the best of our knowledge, this is the first report demonstrating that NMU microinjected into the RVLM produces cardiorespiratory effects in anaesthetized rats. The functional role of the RVLM in cardiovascular responses is well established and it is recognized as the key site for the tonic and reflex control of sympathetic outflow (Pilowsky and Goodchild, 2002; Pilowsky et al., 2009). At lower doses (25 and 50 pmol), NMU, injected into the RVLM, produced only an excitatory cardiovascular response. But at a higher dose (100 pmol), NMU elicited a biphasic response on the cardiovascular variables consisting of a brief excitatory followed by a prolonged inhibitory phase. To date, two NMU receptors have been identified and NMU2 is suggested to be expressed predominantly in the CNS (Howard et al., 2000). But the presence of NMU1 or as yet unidentified/unknown receptors for NMU in the CNS cannot be excluded (Nakahara et al., 2004a; Zeng et al., 2006; Peier et al., 2009). The nature of NMU1 and NMU2, and their ability to increase or decrease neuronal excitability is controversial. Agonist binding to NMU1 and NMU2 is reported to activate both $G_{q/11}$ and $G_{q0}$ (Brighton et al., 2004; Brighton et al., 2008). By contrast, other studies suggest that 1) NMU1 and NMU2 couple only to $G_{q/11}$ (Raddatz et al., 2000; Shan et al., 2000) or 2) activation of NMU2 increases neuronal excitability suggesting an excitatory role for NMU2 (Qiu et al., 2003) or 3) NMU1 activation inhibits both T-type and L-type $Ca^{2+}$ channels, suggesting an inhibitory role of NMU1 (Zhang et al., 2010; Wang et al., 2011). Whether the biphasic response induced by the higher dose of NMU is a result of activation of two pharmacologically distinct NMU
receptors in the RVLM or due to NMU-induced release of endogenous signalling molecules, could not be addressed due to the lack of commercially available receptor antagonists.

Our study demonstrates that NMU injection into the RVLM dose-dependently increases respiratory drive as indicated by an increase in PNamp and TE, and a decrease in TI. The RVLM is a functionally heterogeneous region with cardiovascular and respiratory (Bötzinger and pre-Bötzinger) regions intermingled (Pilowsky et al., 1990; Kanjhan et al., 1995; Pilowsky et al., 2009). This close relationship, and synaptic input from respiratory neurons to RVLM neurons (Sun et al., 1997), along with close appositions between bulbospinal tyrosine hydroxylase immunoreactive neurons of the C1 cell group and boutons from identified Bötzinger neurons (Sun et al., 1994) suggest a possible site of action for the known interactions between the two systems and a possible explanation for the effects of NMU on respiration observed in this study.

RVLM presympathetic neurons receive a direct input from respiratory neurons (Sun et al., 1997) and their discharge is synchronous with different phases of PNA (Haselton and Guyenet, 1989; Miyawaki et al., 1995; Miyawaki et al., 1996; Pilowsky et al., 1996). It is also reported that at baseline, SSNA has two distinct respiratory related peaks whereas LSNA has only one peak, i.e., during PI-period (Miyawaki et al., 2002). The findings here are consistent with such earlier studies. Following NMU injection in the RVLM, I-peak of LSNA became obvious with a clear increase in the AUC. The AUC of I-peak of SSNA is potentiated in the excitatory phase of the NMU response in the RVLM, but in the inhibitory phase the I-peak of both SSNA and LSNA are reduced. On the other hand, the AUC of the PI-peak of both SSNA and LSNA is reduced at excitatory as well as inhibitory phase of NMU effect. A likely explanation for this finding is that NMU modulates RVLM neurons differentially,
receiving input from inspiratory and expiratory neurons of the respiratory column, either pre-synaptically or post-synaptically.

RVLM neurons receive information from myelinated and/or unmyelinated somatic afferent neurons, integrate the information and relay the efferent signal to different sympathetic beds with at least two identifiable excitatory peaks in SSNA and LSNA (Sato and Schmidt, 1973). An interesting finding of this study is that both the sympathoexcitatory peaks of SSNA and LSNA are attenuated by the higher dose of NMU at its excitatory, as well as its inhibitory phase. It is noteworthy that a significant inhibition of the somatosympathetic reflex was seen in both SSNA and LSNA where the ongoing activity was bidirectionally affected by NMU injection in the RVLM. Therefore, the selective inhibition of the somatosympathetic reflex cannot simply be related to the baseline shift following NMU injection. This inhibition at both excitatory and inhibitory phases of NMU response suggests an effect on the axon terminals or interneurons involved in the afferent limb of the somatosympathetic reflex, rather than a direct effect on RVLM neurons.

Short term change in BP is sensed by baroreceptors and the signal is transmitted to the barosensitive RVLM neurons via NTS and caudal ventrolateral medulla. Barosensitive RVLM neurons, projecting to the spinal cord, then provide the descending excitatory/inhibitory sympathetic input in response to a fall/rise in BP (Pilowsky and Goodchild, 2002; Pilowsky et al., 2009). Baroreflex function is known to exhibit regulation in different sympathetic outputs as represented by wide regional variability in gain, range, and maximum inhibition (Scislo et al., 1998). We found that, at the control period, the gain for SSNA is distributed over a greater range of MAP than that of LSNA consistent with
previous work (Scislo et al., 1998). Our study also reveals that the maximum gain for LSNA is higher than that of SSNA at baseline. Following the higher dose of NMU injection in the RVLM the baroreflex gain of SSNA is distributed over a narrower range of MAP with an increase in maximum gain at the excitatory phase. The opposite is observed for LSNA at both the excitatory and inhibitory phases of the NMU response. The results suggest that NMU differentially modulates different barosensitive subpopulation of RVLM neurons that in turn project to and modulate different sympathetic outflows.

RVLM neurons are activated, directly and indirectly, by hypoxia resulting in sympathoexcitatory, pressor and tachycardiac responses, along with an increase in PNA in vagotomized rats (Guyenet, 2000). The inhibition of all cardiovascular and respiratory responses to hypoxia at the higher dose of NMU injection suggests a pre- and/or post-synaptic role on cardiovascular and/or intermingled respiratory neurons in the RVLM.

Activation of central chemoreceptors influences presympathetic RVLM neurons and increases sympathetic outflow via their effects on central respiratory network or independent of it through retrotrapezoid nucleus (RTN) (Guyenet et al., 2010). To date, it remains unclear whether or not RTN neurons form monosynaptic, polysynaptic or both types of connection with RVLM neurons. The mechanisms by which NMU inhibit sympathoexcitatory and phrenic response to hypercapnia at both excitatory and inhibitory phases of its response is likely to be complicated, and the nature of the receptors involved in the responses are yet to be revealed.
Sympathetic outflow to the adrenal gland (SSNA) is regulated differently to the outflow targeting skeletal muscle (LSNA) by the presympathetic RVLM neurons as well as by the baroreceptors, chemoreceptors and somatic receptors (Dampney, 1994; Scislo et al., 1998; Pilowsky et al., 2009). Here we found that the excitatory response observed in LSNA following administration of NMU, is significantly greater than that seen in SSNA. Directionally, SSNA and LSNA show similar types of modulation following NMU administration in response to the activation of somatic, peripheral chemo- and central chemoreceptors, although the amplitudes of the responses differ. On the other hand, NMU causes directionally opposite responses in barosensitivity in LSNA compared with SSNA. The results suggest a differential role of NMU in the RVLM in modulating sympathetic outflow that controls different vascular beds.

In conclusion, exogenous NMU microinjected into the RVLM causes differential effects on sympathetic output to blood vessel and skeletal muscle in vivo, and on sympathetic baroreflex function. NMU in the RVLM attenuates the somatosympathetic, peripheral chemoreceptor and central chemoreceptor reflexes. The finding that NMU agonism in the RVLM exerts directionally opposite effects on baroreceptor function in the splanchnic compared with the lumbar sympathetic bed may have important implications for the development of drugs that control blood pressure, since the lumbar sympathetic bed is known to be of greater importance in determining total peripheral resistance.

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Authorship Contributions

Participated in research design: Rahman, Shahid and Pilowsky

Conducted experiments: Rahman and Shahid

Performed data analysis: Rahman, Shahid and Pilowsky

Wrote or contributed to the writing of the manuscript: Rahman, Shahid and Pilowsky
References


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* A.A.R and I.Z.S. contributed equally to this work

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Figure Legends

Figure 1. Effect of bilateral microinjection of neuromedin U (NMU) into the rostral ventrolateral medulla (RVLM). A: Representative recording of blood pressure (BP) (grey – pulsatile and black – mean), heart rate (HR), splanchnic sympathetic nerve activity (SSNA) and lumbar SNA (LSNA) (grey – raw and black - rectified and integrated) [arbitrary unit (a.u.)], before and after injection of glutamate (Glu), phosphate buffered saline (PBS) or NMU (100 pmol). B: Grouped data of maximum cardiovascular effects following PBS or NMU (25, 50 and 100 pmol). Peak effects are shown as absolute (mean arterial pressure (MAP), HR) or percentage (SSNA, LSNA) change from respective basal values. C: Microinjection sites in the RVLM and a section showing an injection site stained following an injection of rhodamine microbeads. NA: nucleus ambiguus; sp5: spinal trigeminal tract; IOL: inferior olive; Py: pyramidal tract; LHS: left hand side; RHS: right hand side. Data are expressed as mean ± SE. Number of animals are shown in parentheses. *** P < 0.001, ** P < 0.01, * P < 0.05 compared with PBS. bpm, beats per minute.

Figure 2. Effect of bilateral microinjection of neuromedin U (NMU) in the rostral ventrolateral medulla on phrenic nerve activity (PNA). A: Representative trace of data from a recording of rectified PNA [arbitrary unit (a.u.)], phrenic nerve frequency (PNf), phrenic nerve amplitude (PNamp), inspiratory period (T₁) and expiratory period (T₂) before and after injection of PBS or NMU (100 pmol). B: Comparison of peak respiratory effects produced by PBS or NMU (25, 50 and 100 pmol). Peak effects are shown as absolute or percentage change from respective basal values. Values are expressed as mean ± SE. Number of animals are shown in parentheses. *** P < 0.001, ** P < 0.01, * P < 0.05 compared with PBS. bpm, bursts per minute.
Figure 3. Effect of neuromedin U (NMU) on phrenic nerve discharge-related rhythmicity of splanchnic sympathetic nerve activity (SSNA) and lumbar SNA (LSNA) in the rostral ventrolateral medulla. A, C: Phrenic-triggered average of SSNA and LSNA before and after bilateral NMU(100 pmol, each side) injection. B, D: Grouped data illustrating the effects of NMU (100 pmol, n=4) on inspiratory (I) and post-inspiratory (PI) peaks of SSNA and LSNA. Values are expressed as mean ± SE. Number of animals are shown in parentheses. ** P < 0.01, * P < 0.05 compared with control.

Figure 4. Effect of bilateral microinjection of neuromedin U (NMU) on somatosympathetic reflex. A, C: Grouped effect of sciatic nerve-evoked stimulation of splanchnic sympathetic nerve activity (SSNA) and lumbar SNA (LSNA) at control period and after injection of phosphate buffered saline (PBS) and NMU. Data are mean (black) ± SE (grey). Arrows indicate the time of stimulation. B, D: Grouped data illustrating the effects of PBS or NMU (100 pmol, n=5) on the AUC of 1st and 2nd sympathoexcitatory peaks. *** P < 0.001, ** P < 0.01, * P < 0.05 compared with control.

Figure 5. Effect of bilateral microinjection of neuromedin U (NMU) in the rostral ventrolateral medulla on the arterial baroreflex evoked by intravenous injection of sodium nitroprusside (SNP) and phenylephrine hydrochloride (PE). A: Representative experimental recording of the effect of changes in blood pressure (BP) on splanchnic sympathetic nerve activity (SSNA) and lumbar SNA (LSNA) due to SNP or PE before (control) or after NMU injection. B: Average splanchnic sympathetic baroreflex function curves generated for data before (control) or after NMU (100 pmol, n=4) injection. Trace at right represents baroreflex
gain for SSNA (error bars are omitted for clarity - see Table 1). C: Average lumbar sympathetic baroreflex function curves generated for data before (control) or after NMU (100 pmol, n=5) injection. Trace at right represents baroreflex gain for LSNA (error bars are omitted for clarity - see Table 1).

**Figure 6.** Effect of bilateral microinjection of neuromedin U (NMU) in rostral ventrolateral medulla on the cardiovascular and respiratory responses to hypoxia with 100% N₂ for 12-14s. A: Experimental recording of hypoxic episodes at control period and after phosphate buffered saline (PBS) or NMU injection. B: Grouped data of peak effects on cardiovascular (mean arterial pressure (MAP), heart rate (HR), splanchnic sympathetic nerve activity (SSNA) and lumbar SNA (LSNA)) and respiratory function (phrenic nerve amplitude (PNamp) and phrenic nerve frequency (PNf)) after injection of PBS and NMU (100 pmol, n=5) in response to brief hypoxia. Values are expressed as mean ± SE. *** P < 0.001, ** P < 0.01, * P < 0.05 compared with control. bpm, beats per minute for HR or bursts per minute for PNf.

**Figure 7.** Effect of bilateral microinjection of neuromedin U (NMU) in rostral ventrolateral medulla on the cardiovascular and respiratory responses to hypercapnia with 10% CO₂ in oxygen for 1 min. A: Experimental recording of hypercapnic episodes at control period and after PBS or NMU injection. B: Grouped data of peak effects on cardiovascular (mean arterial pressure (MAP), heart rate (HR), splanchnic sympathetic nerve activity (SSNA) and lumbar SNA (LSNA)) and respiratory function (phrenic nerve amplitude (PNamp) and phrenic nerve frequency (PNf)) after injection of PBS and NMU (100 pmol, n=4) in response to hypercapnia. Values are expressed as mean ± SE. ** P < 0.01, * P < 0.05 compared with control. bpm, beats per minute for HR or bursts per minute for PNf.
Table 1. Parameters describing baroreflex control of SSNA and LSNA after bilateral microinjection of NMU (100 pmol) in the RVLM

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<tr>
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<th>Lower Plateau (%</th>
<th>Upper Plateau (%</th>
<th>Mid Point (mmHg)</th>
<th>Max. Gain (%/mmHg)</th>
<th>Range of SNA (%)</th>
<th>Threshold Level (mmHg)</th>
<th>Saturation Level (mmHg)</th>
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<td><strong>SSNA</strong></td>
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<td>Control</td>
<td>14.6 ± 1.7</td>
<td>108.3 ± 4.8</td>
<td>127.3 ± 2.4</td>
<td>-1.4 ± 0.07</td>
<td>93.7 ± 5.0</td>
<td>76.4 ± 3.3</td>
<td>178.3 ± 4.1</td>
<td>101.9 ± 5.7</td>
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<td>NMU (excitatory</td>
<td>28.8 ± 2.9</td>
<td>149.9 ± 14.5</td>
<td>138.4 ± 9.4</td>
<td>-2.9 ± 0.2</td>
<td>121.1 ± 12.6</td>
<td>108.0 ± 9.3</td>
<td>168.9 ± 9.7</td>
<td>60.9 ± 3.1</td>
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<td>phase)</td>
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<td>NMU (inhibitory</td>
<td>19.9 ± 2.8</td>
<td>117.4 ± 11.4</td>
<td>136.6 ± 10.8</td>
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<td>Control</td>
<td>16.9 ± 8.1</td>
<td>97.4 ± 10.1</td>
<td>139.7 ± 12.2</td>
<td>-2.3 ± 0.5</td>
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<td>NMU</td>
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<td>-1.7 ± 0.4</td>
<td>138.9 ± 10.5</td>
<td>73.7 ± 16</td>
<td>198.9 ± 12.9</td>
<td>125.2 ± 14.3</td>
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Values are mean ± SE (n =4-5). Maximum (Max.) gain is the slope of the sigmoid curve of best fit at the MAP corresponding to steepest part of the curve. ns, non-significant; ** P < 0.01, * P < 0.05 compared with control.

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<td>NMU</td>
<td>19.5 ± 9.1</td>
<td>113.4 ± 27.3</td>
<td>138.5 ± 9.6</td>
<td>-1.2 ± 0.5</td>
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