Chapter 5

Effects of RVLM Neurokinin-1 Receptor Activation and Blockade

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5.1 Abstract

The effects of activation and blockade of the neurokinin-1 receptor in the rostral ventrolateral medulla (RVLM) on arterial blood pressure, splanchnic sympathetic nerve activity (sSNA), phrenic nerve activity, the somato-sympathetic reflex, baroreflex and chemoreflex were studied in urethane anaesthetized, and artificially ventilated Sprague-Dawley rats.

Bilateral microinjection of either the stable substance P analogue ($pGlu^5$, $MePhe^8$, Sar^9)-SP(5-11) (DiMe-SP) or the highly selective neurokinin-1 receptor agonist [Sar^9, $Met(O_2)^{11}$]-substance P into the RVLM resulted in an increase in arterial blood pressure, sSNA, and heart rate and an abolition of phrenic nerve activity. The effects of [Sar^9, $Met(O_2)^{11}$]-substance P were blocked by the selective non-peptide neurokinin-1 receptor antagonist WIN 51708. Neurokinin-1 receptor activation also dramatically attenuated the somato-sympathetic reflex elicited by tibial nerve stimulation whilst leaving the baroreflex and chemoreflex unaffected. This effect was again blocked by WIN 51708. Neurokinin-1 receptor antagonism in the RVLM with WIN 51708 significantly attenuated the sympathoexcitatory response to hypoxia, but had no effect on baseline respiratory function. These findings demonstrate that substance P and the neurokinin-1 receptor play a significant role in the cardiorespiratory reflexes integrated within the RVLM.

The results presented in this chapter were published in 2005 in the *American Journal of Physiology* (see appendix)-

Makeham JM, Goodchild AK, and Pilowsky PM. 2005. NK1 receptor activation in rat rostral ventrolateral medulla selectively attenuates somato-sympathetic reflex while antagonism attenuates sympathetic chemoreflex. American Journal of Physiology 288:R1707-R1715.

5.2 Introduction

The sympathoexcitatory cells of the rostral ventrolateral medulla (RVLM) project to the sympathetic preganglionic neurons of the spinal cord that are essential for the maintenance of resting sympathetic tone (Brown and Guyenet, 1985; Lipski *et al.*, 1995b; Sun 1995; Verberne *et al.*, 1999). The RVLM is also essential for the integration of cardiovascular reflexes such as the baroreflex, chemoreflex, somato-sympathetic reflex as well as respiratory modulation of the sympathetic outflow (Pilowsky and Goodchild, 2002). Given the importance of these reflexes in cardiovascular regulation, the modulation of the sympathoexcitatory RVLM neurons is of considerable interest. Recently our laboratory demonstrated that stimulation of delta opioid receptors in the RVLM inhibits respiratory related discharge of lumbar sympathetic nerve activity whilst having little effect on splanchnic sympathetic nerve activity, potently attenuates the somato-sympathetic reflex, but has no effect on the baroreflex or the chemoreflex. Stimulation of RVLM mu-opioid receptors, however, inhibits the baroreflex whilst leaving the somato-sympathetic reflex and chemoreflex unaffected (Miyawaki *et al.*, 2002a). Similarly, activation of 5-HT_{1A} receptors in the RVLM results in a potent, selective inhibition of the somato-sympathetic reflex but not the baroreflex or chemoreflex (Miyawaki *et al.*, 2001).

The undecapeptide tachykinin substance P, and its receptor, the neurokinin-1 receptor, have been implicated in the central regulation of the cardiovascular system. Microinjection of substance P into the NTS modulates the baroreflex (Cowan *et al.*, 2000; Seagard *et al.*, 2000). The RVLM contains substance P immunoreactive terminals and the neurokinin-1 receptor (Helke *et al.*, 1984; Nakaya *et al.*, 1994). The neurokinin-1 receptor is present on some C1 adrenergic neurons of the RVLM (see

chapter 4) (Makeham *et al.*, 2001). Furthermore, substance P immunoreactive terminals are known to contact C1 neurons (Milner *et al.*, 1988). Microinjection of a stable substance P analogue into the RVLM causes powerful pressor responses *in vivo* (Urbanski *et al.*, 1989), and both substance P and the selective neurokinin-1 receptor agonist [Sar^9, $Met(O_2)^{11}$]-substance P excite neonatal bulbospinal C1 neurons recorded using patch electrodes *in vitro* (Li and Guyenet, 1997).

This study examined the role of substance P and the neurokinin-1 receptor in the cardiovascular functions and reflexes mediated by the RVLM. The effects of neurokinin-1 receptor agonist and antagonist compounds on the baroreceptor, chemoreceptor, somato-sympathetic reflex and splanchnic sympathetic nerve activity (sSNA) were examined.

5.3 Materials and methods

5.3.1 General procedures

The general surgical procedures have been described in chapter 2. Briefly, male Sprague-Dawley rats (300-500g) were anaesthetized as described in section 2.1.3. The right carotid artery and jugular vein were cannulated as described in section 2.2.1. The phrenic nerve, aortic depressor nerve (ADN), splanchnic sympathetic nerve, and tibial nerve were dissected and prepared for recording or electrical stimulation as described in section 2.2.3. The animal was placed in the stereotaxic frame, ventilated, and the dorsal medulla exposed (see section 2.2.4.1).

5.3.2 Nerve recording

Bipolar silver wire electrodes were used to record sSNA and phrenic nerve activity as described in section 2.2.5. The zero level of sSNA was determined using supramaximal stimulation of the aortic depressor nerve (0.2 ms stimulation, 50 Hz for 5 seconds).

5.3.3 Activation of cardiovascular reflexes

The sympathetic baroreflex, somato-sympathetic reflex, and chemoreflex were activated and analyzed as described in sections 2.2.5 and 2.2.6

5.3.4 Microinjections

The stable substance P analogue ($pGlu^5$, $MePhe^8$, Sar^9)-SP(5-11) (DiMe-SP, 600pmol in 50nl; Sigma), the highly selective NK1 receptor agonist [Sar^9, $Met(O_2)^{11}$]-substance P ([Sar^9, $Met(O_2)^{11}$]-SP, 600pmol in 50nl; Sigma) and the non-peptide selective neurokinin-1 receptor antagonist Win 51708 (5nmol in 100nl; Sigma) were prepared for microinjection as described in section 2.2.8.

In the first series of experiments multibarrel micropipettes were prepared with either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP in one barrel and albumin-colloidal gold (Sigma) in a second barrel. In the second series of experiments, triple barrel micropipettes were used, containing DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP, Win 51708 and colloidal gold in the third barrel. The volume of injections was determined by direct observation of the movement of the fluid meniscus in the micropipette. In all experiments the micropipettes were placed bilaterally into the RVLM.

5.3.5 Experimental procedures

The pressor region of the RVLM was identified physiologically by microinjection of L-glutamate (50mM, 50nl) in a single barrel micropipette. When a site was identified where L-glutamate microinjection elicited a pressor response of >30mmHg, the pipette was removed and multibarrel micropipettes containing drugs were placed stereotaxically in the same sites. Reflexes were tested in the following order; *1)* baroreceptor activation by tetanic ADN stimulation; *2)* baroreceptor activation by intermittent ADN stimulation; *3)* activation of somatic afferent nerve fibres by intermittent electrical stimulation of the tibial nerve; and *4)* chemoreceptor activation by a brief period of hypoxia. Drugs were then microinjected into the RVLM bilaterally and the activation of the reflexes (*steps 1-4* above) was repeated. Reflex activation was repeated every 15-20 mins until the return of responses to pre-injection levels. Due to significant desensitisation following microinjection of neurokinin-1 receptor agonists into the RVLM, each animal received only one bilateral microinjection of either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP.

5.3.6 Histological procedure

At the end of the experiment the brainstem was removed and processed to visualize the microinjection sites as described in section 2.2.9.

5.3.7 Data analysis

As described in section 2.2.6, data were analysed during experiments and post acquisition using a CED 1401 data capture system and Spike 2 software (version 4.0,

Cambridge, U.K.). The average value over a 20 second period was used to evaluate sSNA and arterial blood pressure. Phrenic nerve frequency and phrenic nerve amplitude were determined using a phrenic nerve triggered waveform average over a 100 second period. The sSNA responses to intermittent ADN stimulation and tibial nerve stimulation were analysed using peristimulus waveform averaging. The amplitude of the sSNA from –200 to 0 ms prior to stimulation was taken as the baseline. The maximum response to stimulation was then expressed as a percentage change from the baseline. The response to hypoxia was quantified by comparing the average sSNA for 10 seconds following the onset of excitation of phrenic nerve discharge caused by the inhaled 100% N_2 as a percentage change from a control period of 10 seconds average sSNA prior to 100% N_2 inhalation.

Data are expressed as means and standard error of means (SEM). Statistical significance was assessed by paired *t*-tests to compare effects before and after injection of a drug. To assess the effect of treatment with DiMe-SP, WIN 51708 and [Sar^9, $Met(O_2)^{11}$]-SP, a one-way ANOVA followed by multiple t-tests with Bonferroni's correction (if ANOVA significant) was conducted. All statistical analysis was performed using GraphPad software.

5.4 Results

Microinjection sites were located between 0 and 500μm caudal to the caudal pole of the facial nucleus, 1.9 and 2.1 mm lateral from the midline, and ventral to the nucleus ambiguus. A typical injection site is seen in Fig. 5.1.

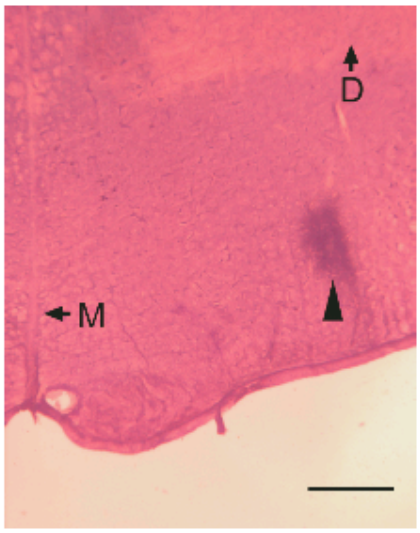

Figure 5.1. Microinjection site in the RVLM. The arrowhead identifies the microinjection site marked with silver-intensified gold particles. Injections into the RVLM were located caudal to the caudal pole of the facial nucleus and ventral to the compact formation of the nucleus ambiguus. D, dorsal; M, midline. Scale bar = 500µm.

5.4.1 Arterial blood pressure, sSNA and HR

Microinjection of DiMe-SP (600pmol, 50nl) bilaterally into the RVLM resulted in a rise in arterial blood pressure (ABP) of 22 ± 3 mm Hg from 96 ± 6 to 119 ± 5 mm Hg (n=8, P<0.001, Fig. 5.4*A*) Similarly, bilateral microinjection of [Sar^9, $Met(O_2)^{11}$]-SP in the RVLM resulted in a rise in ABP of 44 ± 4 mmHg from 115 ± 8 to 156 ± 11 mmHg (n=6, P<0.001, Figs. 5.2, 5.4*A*). This rise was maximal at 2-5 minutes and lasted 30-40 min

Splanchnic SNA (sSNA) and HR were also significantly increased following the microinjection of DiMe-SP and [Sar^9, $Met(O_2)^{11}$]-SP. DiMe-SP increased sSNA to 180 ± 7% baseline (n=7, P<0.001, Fig. 5.5*A*) and [Sar^9, $Met(O_2)^{11}$]-SP increased sSNA to 204 ± 29% baseline (n=6, P<0.001, Figs. 5.2, 5.5*A*). DiMe-SP increased HR 24±7 bpm from 384 ± 9 to 408 ± 9 (n=7, P<0.05, Fig. 5.4*B*), whereas [Sar^9, $Met(O_2)^{11}$]-SP increased HR 13 ± 3 from 428 ± 13 to 441 ± 15 (n=6, P<0.01, Fig. 5.4*B*).

Bilateral microinjection of the selective neurokinin-1 receptor antagonist WIN 51708 did not significantly change mean arterial pressure (118 ± 7 vs.124 ± 9, n=10, NS) or HR (436 ± 9 vs. 442 ± 10, n=10, NS). There was a small but significant increase in sSNA to 121 ± 6% baseline (n=10, P<0.01). Five to 10 minutes following pre-treatment with WIN 51708, either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP was injected into the same site from another barrel of the triple-barrel micropipette.

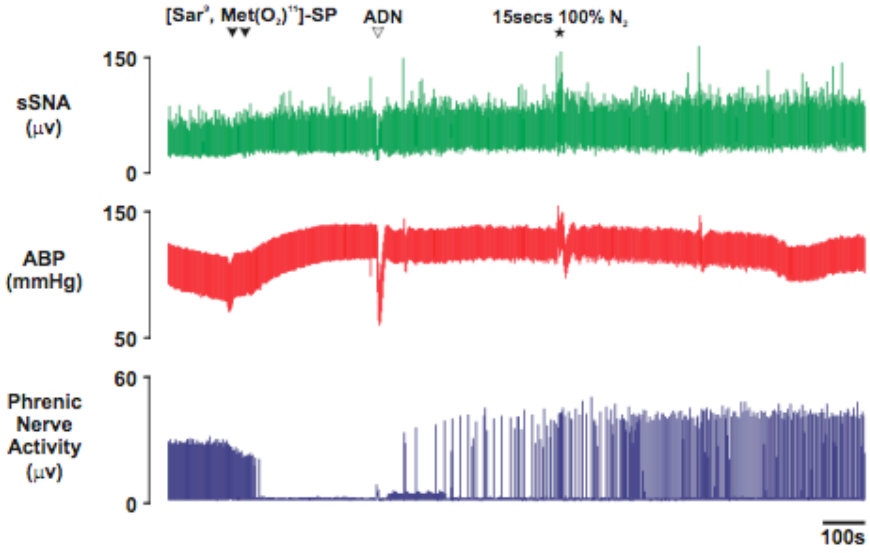

Figure 5.2. Bilateral microinjection of [Sar⁹, Met(O₂)¹¹]-SP into the RVLM (closed arrows) elicited a sympathoexcitatory response and a rise in arterial blood pressure. There was also complete inhibition of phrenic nerve activity, returning in approximately 400s. Open arrows indicate tetanic stimulation of the aortic depressor nerve (ADN). Closed star indicates ventilation with 100% N_2 for 15 seconds. During the sympathoexcitatory period, multiple reflexes were tested, including the baroreflex, somato-sympathetic reflex and chemoreflex. sSNA, splanchnic sympathetic nerve activity; ABP, arterial blood pressure.

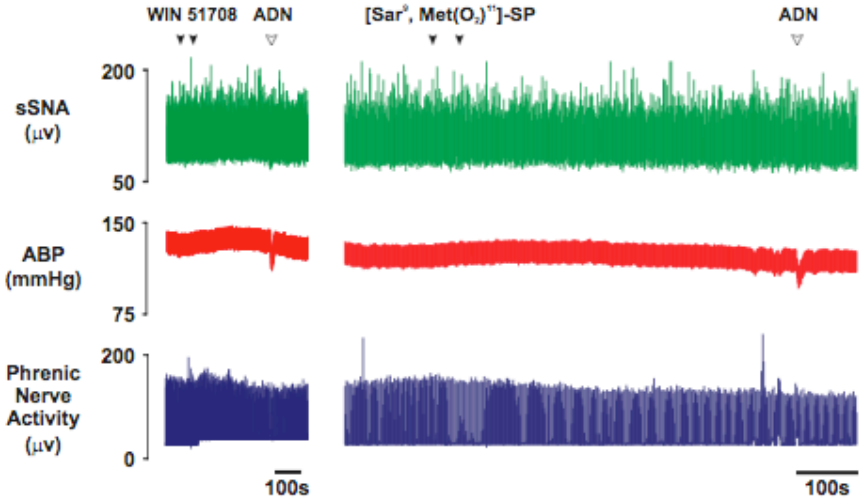

Figure 5.3. Bilateral microinjection of [Sar^{9}, Met(O$_2$)^{11}]-SP after pre-treatment with WIN 51708 in the RVLM. WIN 51708 had no significant effect on sympathetic or phrenic nerve activity, but did cause a small but significant rise in arterial blood pressure (*left*). Following pre-treatment with WIN 51708, [Sar^{9}, Met(O$_2$)^{11}]-SP had no significant effect on arterial blood pressure, sympathetic nerve activity or phrenic nerve activity (*right*). The open arrows indicate intermittent stimulation of the aortic depressor nerve (ADN).

Pre-treatment with WIN 51708 failed to fully block the rise in ABP following DiMe-SP, still significantly different from baseline with an increase of 11 ± 3mm Hg from 107 ± 5 to 119 ± 5mmHg (n=5, P<0.05, Fig 5.4*A*). However, as shown in Figures 5.3 and 5.4, pre-treatment with WIN 51708 blocked the rise in ABP following [Sar^9, $Met(O_2)^{11}$]-SP (139 ± 7 vs. 144 ± 11mmHg, n=5, NS). Pre-treatment with WIN 51708 blocked the HR response to DiMe-SP (from 454 ± 9 to 458 ± 9bpm, n=5, NS, Fig. 5.4*B*) and to [Sar^9, $Met(O_2)^{11}$]-SP (from 438 ± 18 to 433 ± 17 bpm, n=5, NS, Fig 5.4*B*). Pre-treatment with WIN 51708 blocked the sSNA response to DiMe-SP (114 ± 6% baseline, n=5, NS, Fig 5.5*A*) and to [Sar^9, $Met(O_2)^{11}$]-SP (112 ± 11% baseline, n=5, NS, Figs 5.3 and 5.5*A*).

5.4.2 Phrenic nerve activity

Phrenic nerve frequency was significantly reduced following bilateral DiMe-SP microinjection to 39 ± 7% baseline (n=8, P<0.001, Fig. 5.5*B*). Phrenic nerve activity was consistently abolished following [Sar^9, $Met(O_2)^{11}$]-SP microinjection. This inhibition lasted 2-5 mins (Fig. 5.2). There was no significant decrease in phrenic nerve amplitude following DiMe-SP (102 ± 5% baseline, n=8) or upon return of phrenic nerve activity after [Sar^9, $Met(O_2)^{11}$]-SP (104 ± 9% baseline, n=6). Bilateral microinjection of WIN 51708 did not significantly alter phrenic nerve frequency (30 ± 2 vs. 31 ± 3 bursts / min, n=10, NS) or phrenic nerve amplitude (102 ± 6% baseline, n=10, NS). Pre-treatment with WIN 51708 failed to block the phrenic nerve frequency response to DiMe-SP, reduced to 51 ± 11% baseline (34 ± 4 vs. 19 ± 6 bursts / min, n=5, P<0.05, Fig. 5.5*B*). Pre-treatment with WIN 51708 did block the phrenic nerve frequency response to [Sar^9, $Met(O_2)^{11}$]-SP (32 ± 5 vs. 22 ± 9 bursts / min, n=5, NS, Fig 5.5*B*).

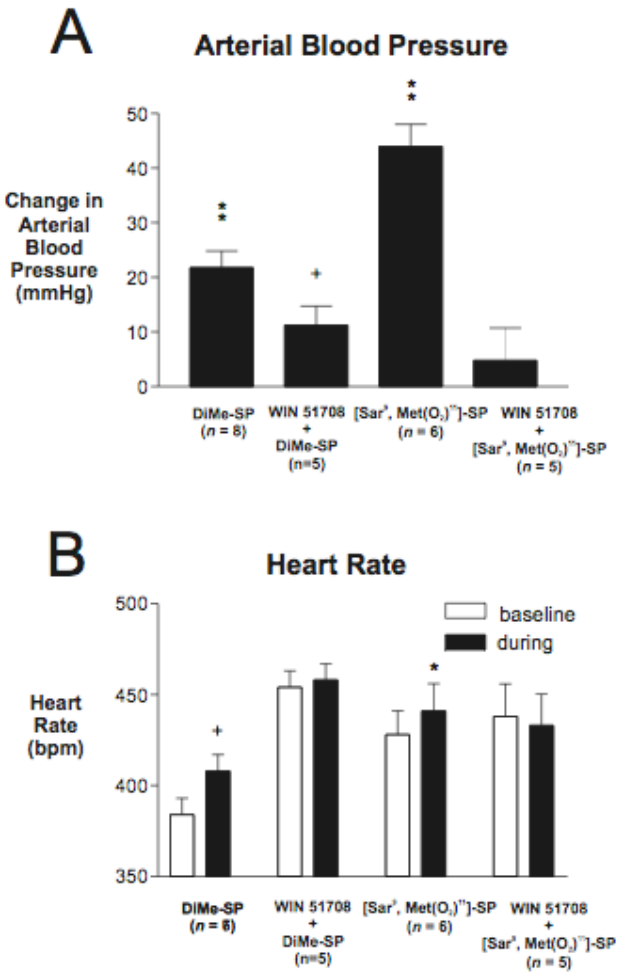

Figure 5.4. ***A,B***: Group data for the effects of DiMe-SP, [Sar^9, $Met(O_2)^{11}$]-SP and WIN 51708 within the RVLM on Arterial Blood Pressure (ABP) and Heart Rate (HR). Note the increase in ABP and HR following neurokinin-1 receptor activation with either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP. The highly selective neurokinin-1 receptor antagonist, WIN 51708, blocked the effects of [Sar^9, $Met(O_2)^{11}$]-SP, but only partially attenuated the effects of DiMe-SP on ABP. + $P<0.05$ vs. baseline. *$P<0.01$ vs. baseline. **$P<0.001$ vs. baseline.

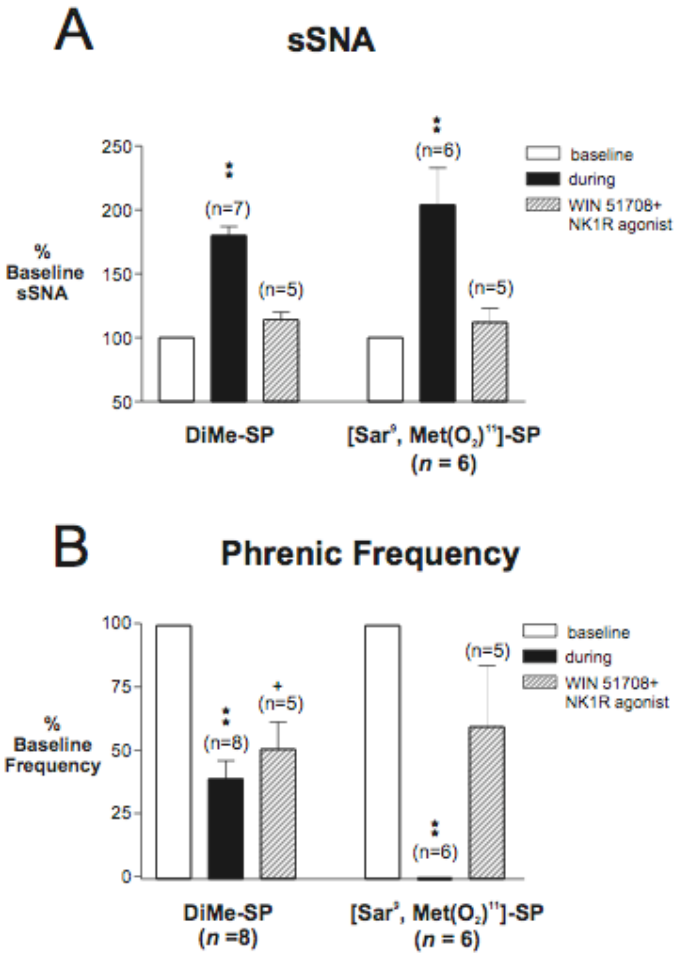

Figure 5.5. ***A,B*** Group data for the effects of DiMe-SP, [Sar^{9}, Met(O$_{2}$)^{11}]-SP and WIN 51708 within the RVLM on splanchnic Sympathetic Nerve Activity (sSNA) and Phrenic Frequency. Note the increase in sSNA and the decrease in Phrenic Frequency following neurokinin-1 receptor activation with either DiMe-SP or [Sar^{9}, Met(O$_{2}$)^{11}]-SP. The highly selective neurokinin-1 receptor antagonist, WIN 51708, blocked the effects of [Sar^{9}, Met(O$_{2}$)^{11}]-SP, but only partially attenuated the effects of DiMe-SP on Phrenic Frequency. (+) P<0.05 vs. baseline. **P<0.001 vs. baseline.

5.4.3 Somato-sympathetic reflex

The average SNA response to intermittent stimulation of the tibial nerve was tested both before and after microinjection of drugs. Intermittent tibial nerve stimulation resulted in a characteristic 2-peaked response in the sSNA with the latencies of 115 ± 2 ms and 211 ± 4 ms (n= 7, Fig. 5.6*A*). Bilateral microinjection of DiMe-SP in the RVLM significantly attenuated the first and second peaks to 27 ± 10% and 1 ± 8% baseline respectively (n=6, P<0.001). Bilateral microinjection of [Sar^9, $Met(O_2)^{11}$]-SP significantly attenuated the first peak to 15 ± 3% baseline (n=5, P<0.05, Fig. 5.6*A,C*). The second peak of the somato-sympathetic reflex was often absent during baseline stimulation in these experiments. On the 2 occasions it was elicited it was attenuated to 8% and 9% baseline by [Sar^9, $Met(O_2)^{11}$]-SP microinjection (Fig. 5.6*C*).

Bilateral microinjection of WIN 51708 did not significantly attenuate either the first or second peak of the somato-sympathetic reflex (83 ± 30% (n=10) and 64 ± 19% (n=9) baseline respectively, NS). Pre-treatment with WIN 51708 failed to block the response to DiMe-SP of the first peak, still being attenuated to 58 ± 11% baseline (n=5, P<0.05), but the response of the second peak was blocked (82 ± 31% baseline, n=4, NS). Pre-treatment with WIN 51708 blocked the attenuation of the somato-sympathetic response caused by [Sar^9, $Met(O_2)^{11}$]-SP for both the first and second peaks (104 ± 43% baseline and 172 ± 56% baseline respectively; n=5, NS, Fig. 5.6*B,C*).

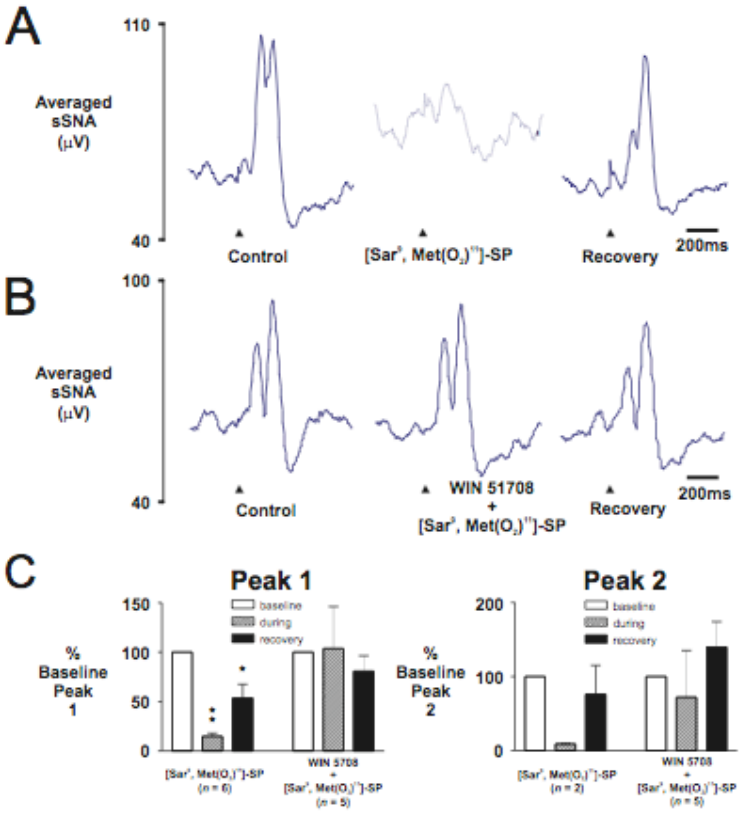

Figure 5.6. Somato-sympathetic reflex ***A***: Electrical stimulation of the tibial nerve (closed arrowheads) produced the 2 characteristic peaks of the somato-sympathetic reflex in the splanchnic sympathetic nerve activity (sSNA) recording. Bilateral microinjection of [Sar^9, Met(O$_2$)^{11}]-SP in the RVLM almost completely abolished this response. This abolition lasted approx. 60-90mins, followed by partial recovery. ***B***: RVLM pre-treatment with WIN 51708 blocked the effects of [Sar^9, Met(O$_2$)^{11}]-SP on the somato-sympathetic reflex. ***C***: Group data for the first and second peaks of the somato-sympathetic reflex. Note the almost complete inhibition of peak 1 and peak 2 following [Sar^9, Met(O$_2$)^{11}]-SP, and the blocking of this effect by WIN 51708. Note also that peak 2 was absent in many experiments and the effect of [Sar^9, Met(O$_2$)^{11}]-SP is only measured in 2 animals- however in these animal the inhibition of peak 2 was to 8% and 9% baseline. **P*<0.05 vs. baseline. ** *P*<0.001 vs. baseline.

5.4.4 Baroreflex

Intermittent stimulation of the ADN resulted in a maximal inhibitory potential in the sSNA with a latency of 178 ± 4 ms (n=13, Fig. 5.7). Bilateral microinjection of DiMe-SP attenuated the amplitude of the inhibitory potential (58 ± 9% baseline, n=7, P<0.01), whereas [Sar^9, $Met(O_2)^{11}$]-SP did not significantly attenuate the inhibitory potential (76 ± 11% baseline, n=5, NS, Fig. 5.7). Bilateral microinjection of WIN 51708 did not significantly alter the response to intermittent ADN stimulation (90 ± 8% baseline, n=10, NS). Pre-treatment with WIN 51708 blocked the response to DiMe-SP (89 ± 5% baseline, n=5, NS). There was no significant difference in sympathetic baroreflex following [Sar^9, $Met(O_2)^{11}$]-SP in animals pre-treated with WIN 51708 (109 ± 17% baseline, n=5, NS). An example is shown in Figure 5.7.

5.4.5 Sympathetic chemoreflex

Stimulation of the chemoreflex resulted in a characteristic increase in sSNA from pre-stimulus levels of 170 ± 11 % (n=9, *P* <0.01, Fig. 5.8). Expressed as a percentage of the baseline response, microinjection of either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP failed to significantly alter the chemoreflex, 80 ± 13% (n=7, NS) and 90 ± 24% (n=6, NS) baseline respectively. Bilateral microinjection of WIN 51708, however, resulted in a significant attenuation of the chemoreflex, to 38 ± 5% baseline (n=9, *P*<0.001, Figs. 5.8*A* and *B*). The interval between baseline chemoreflex testing and chemoreflex testing with the neurokinin-1 receptor agonist drugs (either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP) was not significantly different from the interval between baseline chemoreflex testing and that after pre-treatment with the NK1 receptor antagonist WIN 51708 (986 ± 63 seconds (n=13) vs. 1116 ± 113 seconds (n=9), NS).

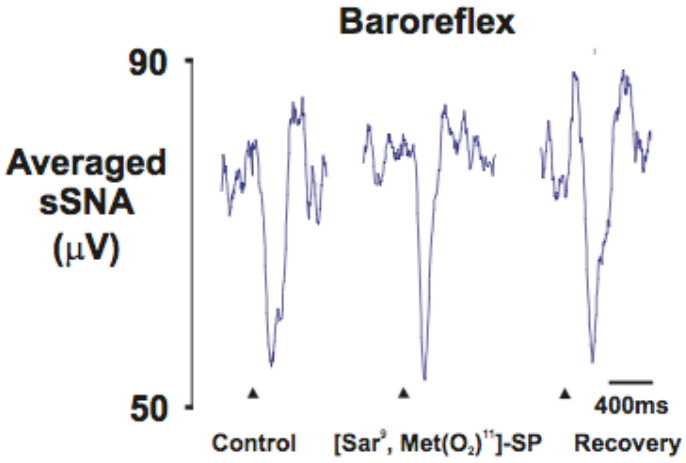

Figure 5.7. Sympathetic baroreflex. Intermittent stimulation of the aortic depressor nerve resulted in a characteristic inhibitory trough in splanchnic sympathetic nerve activity (sSNA) with a latency of 178 ± 4 ms. Microinjection of DiMe-SP resulted in attenuation of this inhibition, whereas the highly specific neurokinin-1 receptor agonist, [Sar^{9}, Met(O$_2$)^{11}]-SP, and antagonist, WIN 51708, had no significant effect.

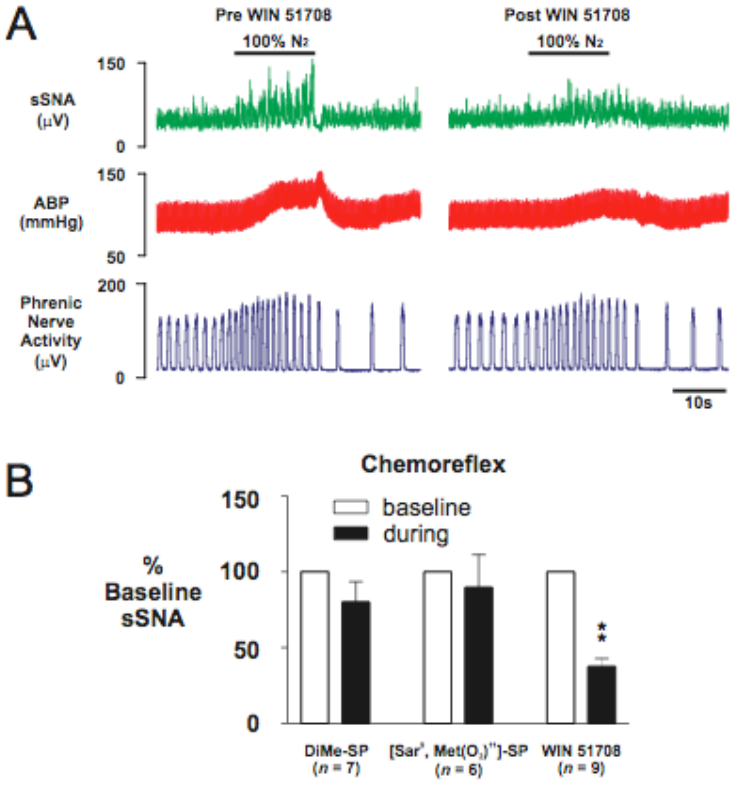

Figure 5.8. Sympathetic chemoreflex ***A***: 15 seconds of 100% N_2 resulted in robust sympatho-excitation and rise in arterial blood pressure (*left*). This response was not affected by either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP but was significantly attenuated by bilateral RVLM microinjection of WIN 51708 (*right*). ***B***: Group data for the effects of DiMe-SP, [Sar^9, $Met(O_2)^{11}$]-SP and WIN 51708 on the chemoreflex. sSNA, splanchnic sympathetic nerve activity ; ABP, arterial blood pressure. ***P*<0.001 vs. baseline.

5.5 Discussion

The principal novel findings of this study are 1) activation of neurokinin-1 receptors in the RVLM *in vivo* results in hypertension, tachycardia, and splanchnic sympathoexcitation; 2) activation of neurokinin-1 receptors in the RVLM attenuates the somato-sympathetic reflex without affecting other brainstem reflexes such as the baroreflex or chemoreflex, and; 3) blockade of neurokinin-1 receptors in the RVLM significantly attenuates the sSNA response to chemoreceptor stimulation. Activation of neurokinin-1 receptors in the RVLM was also shown to significantly decrease phrenic nerve frequency.

5.5.1 ABP, HR and sSNA

Activation of neurokinin-1 receptors in the RVLM *in vivo* with DiMe-SP has previously been shown to increase ABP and HR (Urbanski *et al.*, 1989). This study agrees with these findings. Further, the present study demonstrates a significant increase in sSNA following bilateral neurokinin-1 receptor stimulation in the RVLM. This is most likely mediated, in part, by neurokinin-1 receptors found on bulbospinal C1 neurons (see chapter 4), which are sympathoexcitatory (Makeham *et al.*, 2001). Substance P terminals have been demonstrated within the RVLM and form synaptic junctions with C1 neurons (Leibstein *et al.*, 1985; Milner *et al.*, 1988). Substance P and [Sar^9, $Met(O_2)^{11}$]-SP excite neonatal bulbospinal C1 neurons *in vitro*, possibly by a reduction in resting potassium conductance (Li and Guyenet, 1997).

Pre-treatment of the RVLM with the highly selective neurokinin-1 receptor antagonist WIN 51708 attenuated the pressor response to DiMe-SP, and blocked the effects of

[Sar^9, Met(O$_2$)^{11}]-SP. This suggests that the pressor response to DiMe-SP may be partially mediated by binding sites that are different to the binding sites activated by [Sar^9, Met(O$_2$)^{11}]-SP. The carboxy-terminal substance P analogues such as DiMe-SP may preferentially activate neurokinin-3 receptors (Drapeau *et al.*, 1987). Neurokinin-3 receptors are present within the medulla; in the medullary raphe, NTS, dorsal motor nucleus of Vagus, and nucleus ambiguus (Mileusnic *et al.*, 1999). Although neurokinin-3 receptors have not definitely been identified on bulbospinal sympathoexcitatory RVLM neurons, Senktide, a neurokinin-3 receptor agonist, excited a small number (2 out of 7 neurons) of neonatal bulbospinal C1 neurons recorded using *in vitro* patch clamp (Li and Guyenet, 1997).

Pre-treatment of the RVLM with WIN 51708 blocked the sympathoexcitatory effects of both DiMe-SP and [Sar^9, Met(O$_2$)^{11}]-SP. There was a small but significant increase in sSNA immediately following administration of WIN 51708. The exact cause of this unknown, but may be due to WIN 51708 possessing partial agonist activity, as has been demonstrated previously in other tachykinin antagonists (Kudlacz *et al.*, 1993; Sachon *et al.*, 2002).

5.5.2 Phrenic nerve activity

Bilateral microinjection of both DiMe-SP and [Sar^9, Met(O$_2$)^{11}]-SP into the RVLM markedly attenuated and completely abolished phrenic nerve activity respectively. Immediately dorsal to the sympathoexcitatory neurons of the RVLM are the Bötzinger neurons of the ventral respiratory group (Pilowsky *et al.*, 1990; Sun *et al.*, 1997). Microinjection of excitatory amino acids in the Bötzinger complex decreases phrenic nerve burst amplitude and frequency (Bongianni *et al.*, 1988; Bongianni *et al.*, 1997;

Chitravanshi and Sapru, 1999; Wang *et al.*, 2002a). This is presumably due to activation of inhibitory expiratory neurons (Duffin *et al.*, 1995; Schreihofer *et al.*, 1999). Neurokinin-1 receptors have also been demonstrated on both spinally and non-spinally projecting neurons within the Bötzinger/ RVLM region (Makeham *et al.*, 2001; Guyenet *et al.*, 2002). Activation of neurokinin-1 receptors in the Bötzinger region may thus activate glycinergic inhibitory expiratory neurons resulting in an inhibition of phrenic nerve activity. As previous studies have suggested that the majority of neurokinin-1 receptor immunoreactive neurons of the ventral respiratory group are glutamatergic (Guyenet *et al.*, 2002), the activation of inhibitory expiratory neurons may alternatively occur through an excitatory neurokinin-1 receptor immunoreactive interneuron. The fact that neurokinin-1 receptor activation within this region resulted in a robust inhibition of phrenic nerve activity, whereas neurokinin-1 receptor blockade had no effect suggests that endogenous neurokinin-1 receptor agonists are not constitutively active in the respiratory related neurons in this region. Activation of neurokinin-1 receptors on respiratory related neurons in the RVLM may occur only under stressful physiological or pathological states. Further studies are required to uncover the mechanism of phrenic nerve activity inhibition by neurokinin-1 receptor activation in the Bötzinger region.

5.5.3 Somato-sympathetic reflex

Electrical stimulation of hindlimb somatic afferent nerves evokes an early and late excitatory response in SNA (Morrison and Reis, 1989; Miyawaki *et al.*, 2001). The excitatory peaks found in this experiment were similar in shape and latency to those described in previous studies (Zanzinger *et al.*, 1994; Nagata *et al.*, 1995; Miyawaki *et*

al., 2001; Miyawaki *et al.*, 2002a; Makeham *et al.*, 2004). This reflex is dramatically attenuated by blockade of, for example, excitatory amino acid receptors in the RVLM (Kiely and Gordon, 1993; Miyawaki *et al.*, 1996a), by activation of 5-HT_{1A} (Miyawaki *et al.*, 2001), and delta-opioid receptors in the RVLM (Miyawaki *et al.*, 2002a), and by hypercapnoea (see chapter 3) (Makeham *et al.*, 2004).

Activation of neurokinin-1 receptors in the RVLM by [Sar^9, $Met(O_2)^{11}$]-SP abolished both peaks of the somato-sympathetic reflex, an effect blocked by WIN 51708. Given that activation of neurokinin-1 receptors in the RVLM produces a large sympathoexcitatory response whilst leaving the baroreflex unaffected, it is unlikely that neurokinin-1 receptor activation abolishes the somato-sympathetic reflex via a direct effect on bulbospinal C1 neurons. It may be that neurokinin-1 receptor immunoreactive terminals within the RVLM contain inhibitory neurotransmitters such as GABA or glycine, and inhibit or selectively gate excitatory somatic inputs. The results presented in chapter 4 demonstrate that neurokinin-1 receptor immunoreactive terminals exist in the RVLM and some make close apposition with C1 neurons (Makeham *et al.*, 2001).

It is important to note that two forms of the neurokinin-1 receptor exist- a long form and a C-terminus truncated form (Li *et al.*, 1997). The commercially available neurokinin-1 receptor antibody only recognizes the long form, and by definition anatomical studies using this antibody do not demonstrate immunoreactivity to the short form. In humans and guinea pigs up to 30% of the neurokinin-1 receptor population within the medulla oblongata is of the short form (Caberlotto *et al.*, 2003; Baker *et al.*, 2003). Further studies are required to demonstrate the distribution of the short form of the neurokinin-1 receptor within the ventral medulla.

Microinjection of a 5-HT_{1A} agonist into the RVLM markedly attenuates the somato-sympathetic reflex whilst leaving the baroreflex unaffected (Miyawaki *et al.*, 2001), a result similar to the finding in this study. This indicates the possibility that inhibition of the somato-sympathetic reflex by neurokinin-1 receptor activation may occur through a pathway involving 5-HT_{1A} receptors in the RVLM. However, there is evidence that the major nuclei within the brainstem that have serotonergic projections to the RVLM, do not display significant dual labelling for 5-HT and the neurokinin-1 receptor (Bago *et al.*, 2002; Leger *et al.*, 2002). Of the serotonergic neurons in regions that project to the RVLM, only 15% of those from the central linear nucleus and 0.8% of those from the dorsal raphe nucleus co-localise with the neurokinin-1 receptor (Leger *et al.*, 2002), making somato-sympathetic reflex inhibition by direct activation of these neurons by neurokinin-1 receptor agonists unlikely. Nevertheless it is possible that within the RVLM, substance P causes activation of pre-synaptic neurokinin-1 receptors on excitatory inputs to serotonergic projections, resulting in 5-HT release and subsequent binding to 5-HT_{1A} receptors. This mechanism has previously been demonstrated in the dorsal raphe nucleus in the rat (Liu *et al.*, 2002). As mentioned previously, it is also possible that serotonergic neurons might express the short form of the NK1 receptor (Bago *et al.*, 2002; Leger *et al.*, 2002).

The possibility that the significant increase in sSNA following neurokinin-1 receptor activation might itself attenuate the expression of both peaks of the somato-sympathetic reflex must also be considered. This is unlikely to be the case for two reasons. First, the increase in sSNA, though large, is not at the level where no further increase is possible. Following glutamate microinjection into the RVLM, maximum sSNA was found to be 2-3 times as great as the level attained by neurokinin-1 receptor

stimulation (data not shown). Secondly, RVLM neurokinin-1 receptor activation did not alter the sympathetic baroreflex, a reflex mediated by the same neurons.

5.5.4 Sympathetic chemoreflex

An interesting finding is the effect of the highly selective neurokinin-1 receptor antagonist, WIN 51708, on the sSNA chemoreflex response. The consistency of the hypoxic stimulus must be considered. Experiments were conducted in a standardized manner, and animals were ventilated so as to maintain end tidal CO_2 between 4 and 5%. Sympathoexcitation occurred rapidly after onset of ventilation with 100% N_2 and the measured chemoreflex period occurred wholly within the 100% N_2 administration period. Furthermore, the measured chemoreflex period was an average of 10 seconds immediately following the onset of excitation of phrenic nerve discharge. These factors suggest a consistent hypoxic stimulus.

Hypoxia stimulates carotid body chemoreceptors and increases the activity of bulbospinal sympathoexcitatory neurons in the RVLM via the nucleus tractus solitarius (NTS) (see section 1.6) (Koshiya *et al.*, 1993; Sun and Reis, 1995b; Miyawaki *et al.*, 1996b). Blockade of excitatory amino acid neurotransmitters in the RVLM also blocks the sympathoexcitation evoked by the chemoreflex (Koshiya *et al.*, 1993; Kubo *et al.*, 1993; Sun and Reis, 1995b; Miyawaki *et al.*, 1996b). There is some evidence that neurokinin-1 receptor containing neurons of the ventral medulla may be chemosensitive or modulate chemosensitivity (Nattie and Li, 2002). However, the fact that antagonism of neurokinin-1 receptors in the RVLM did not alter baseline respiratory frequency or phrenic nerve amplitude suggests that within this region the neurokinin-1 receptor is not involved in basal respiratory function. The neurokinin-1 receptor appears to play a role in sympathoexcitation within the RVLM under stressful

stimuli, such as the severe hypoxia experienced within this experimental protocol. Substance P may be pre-synaptically reinforcing the excitatory NTS to RVLM connection (Koshiya *et al.*, 1993; Sun and Reis, 1995b; Miyawaki *et al.*, 1996b). Removing this by neurokinin-1 receptor blockade would thus reduce the sympathetic response. The apparent failure of either DiMe-SP or [Sar^9, $Met(O_2)^{11}$]-SP to augment the chemoreflex may be due to the fact that the chemoreceptor facilitation is masked by the general sympathoexcitation evoked by the RVLM neurokinin-1 receptor activation.

The possibility that the failure of WIN 51708 to fully block the rise in MABP, the decrease in phrenic nerve discharge frequency, and somato-sympathetic reflex following DiMe-SP was due to inadequate dose should be considered. This is unlikely given that WIN 51708 blocked other effects of DiMe-SP, such as increased sSNA and HR. Further, as discussed in section 5.5.1, DiMe-SP is relatively non-specific and [Sar^9, $Met(O_2)^{11}$]-SP is highly selective in its effects on the neurokinin-1 receptor, and all significant effects of [Sar^9, $Met(O_2)^{11}$]-SP on multiple variables were completely blocked by the dose of WIN 51708 given. Further experiments could investigate the effects of carrying doses of WIN 51708 on the effects of DiMe-SP.

5.6 Conclusion

In summary, the results presented in this chapter demonstrate that in anaesthetized, vagotomized, paralysed and ventilated rats, microinjection of neurokinin-1 receptor agonists bilaterally into the RVLM elicits robust increases in ABP, sSNA, and HR. It also results in the abolition of phrenic nerve activity and dramatic attenuation of the

somato-sympathetic reflex whilst leaving the baroreflex unaffected. These effects are due to the selective activation of the neurokinin-1 receptor within the RVLM, as evidenced by the blocking of these effects by a highly selective neurokinin-1 receptor antagonist. The fact that the selective neurokinin-1 receptor antagonist had no significant effect on baseline ABP and HR and a very small effect on sSNA suggests that substance P and the neurokinin-1 receptor do not play a significant role in the tonic maintenance of blood pressure and sympathetic outflow. Neurokinin-1 receptor antagonism also resulted in a dramatic attenuation of the sympathetic chemoreflex response to hypoxia, suggesting a modulation of the chemoreceptor pathway from the NTS to the RVLM by substance P. These results demonstrate that substance P and the neurokinin-1 receptor play an integral role in the regulation of cardiorespiratory reflexes within the RVLM.