Modulation of hypoglossal motoneuron excitability by NK1 receptor activation in neonatal mice *in vitro*

Kouichi Yasuda, Dean M. Robinson, Subramaniam R. Selvaratnam, Carmen W. Walsh, Angus J. C. McMorland and Gregory D. Funk

Department of Physiology, Faculty of Medicine and Health Science, University of Auckland, Private Bag 92019, 85 Park Road, Grafton, Auckland, New Zealand

1. The effects of substance P (SP), acting at NK1 receptors, on the excitability and inspiratory activity of hypoglossal (XII) motoneurons (MNs) were investigated using rhythmically active medullary-slice preparations from neonatal mice (postnatal day 0–3).

2. Local application of the NK1 agonist $[SAR^9, Met(O_2)^{11}]$-SP (SP_{NK1}) produced a dose-dependent, spantide- (a non-specific NK receptor antagonist) and GR82334-(an NK1 antagonist) sensitive increase in inspiratory burst amplitude recorded from XII nerves.

3. Under current clamp, SP_{NK1} significantly depolarized XII MNs, potentiated repetitive firing responses to injected currents and produced a leftward shift in the firing frequency–current relationships without affecting slope.

4. Under voltage clamp, SP_{NK1} evoked an inward current and increased input resistance, but had no effect on inspiratory synaptic currents. SP_{NK1} currents persisted in the presence of TTX, were GR82334 sensitive, were reduced with hyperpolarization and reversed near the expected E_K.

5. Effects of the α_1-noradrenergic receptor agonist phenylephrine (PE) on repetitive firing behaviour were virtually identical to those of SP_{NK1}. Moreover, SP_{NK1} currents were completely occluded by PE, suggesting that common intracellular pathways mediate the actions of NK1 and α_1-noradrenergic receptors. In spite of the similar actions of SP_{NK1} and PE on XII MN responses to somally injected current, α_1-noradrenergic receptor activation potentiated inspiratory synaptic currents and was more than twice as effective in potentiating XII nerve inspiratory burst amplitude.

6. GR82334 reduced XII nerve inspiratory burst amplitude and generated a small outward current in XII MNs. These observations, together with the first immunohistochemical evidence in the newborn for SP immunopositive terminals in the vicinity of SP_{NK1}-sensitive inspiratory XII MNs, support the endogenous modulation of XII MN excitability by SP.

7. In contrast to phrenic MNs (Ptak *et al.* 2000), blocking NMDA receptors with AP5 had no effect on the modulation of XII nerve activity by SP_{NK1}.

8. In conclusion, SP_{NK1} modulates XII motoneuron responses to inspiratory drive primarily through inhibition of a resting, postsynaptic K^+ leak conductance. The results establish the functional significance of SP in controlling upper airway tone during early postnatal life and indicate differential modulation of motoneurons controlling airway and pump muscles by SP.

Hypoglossal motoneurons (XII MNs) innervate the intrinsic muscles of the tongue and are involved in a variety of behaviours including breathing, suckling, chewing, swallowing and phonation (Bartlett *et al.* 1990). A subgroup of these motoneurons innervating the genioglossus muscle, the major tongue protruder, receives glutamatergic inputs during inspiration (Greer *et al.* 1991; Funk *et al.* 1993) that interact with intrinsic membrane properties to produce rhythmic bursts of action potentials. The resultant inspiratory modulation of protruder muscle tone has been observed in many species (Ogawa *et al.* 1960; Miller & Bowman, 1974; Megirian *et al.* 1985) including human (Sauerland & Harper, 1976), and maintains airway patency necessary for breathing (Remmers *et al.* 1978; Pack, 1994).

The transformation of input into output by XII MNs, known as excitability, is modulated by a number of transmitter systems that target particular ion channels

on pre- and postsynaptic neuronal membranes. Neurons in the raphe nuclei provide a powerful, diverse and largely excitatory modulation of MN excitability (Rekling *et al.* 2000). Neurons in raphe obscurus and pallidus (Manaker *et al.* 1992; Manaker & Tischler, 1993), which innervate cranial, including XII, motor nuclei, contain 5HT and TRH or 5HT and SP (Kachidian *et al.* 1991; Henry & Manaker, 1998). These compounds appear to modulate excitability, at least in part, by blocking the two-pore domain K^+ channel TASK-1 (Talley *et al.* 2000). The potentiating actions of 5HT and TRH on MN excitability are well established physiologically at spinal and brainstem levels (reviewed by Rekling *et al.* 2000). The actions of SP on spinal MNs, including phrenic MNs, have also been described (Ptak *et al.* 2000; Rekling *et al.* 2000). Its effects are mediated primarily via postsynaptic NK1 receptors (Rekling *et al.* 2000), are characterized by slow inward currents or membrane depolarizations and are associated with increased input resistance and excitability. Activation of NK2 or NK3 receptors in some cases causes small MN depolarizations that are at least partially TTX sensitive, implying a presynaptic mechanism for these receptor subtypes (Matsuto *et al.* 1984; Fisher *et al.* 1994; Ptak *et al.* 2000).

The effects of NK1 receptor activation on excitability and behaviour of MNs innervating airway muscles, and cranial MNs in general, have been minimally characterized. SP inhibits the TASK-1 K^+ channel in XII MNs (Talley *et al.* 2000) and increases excitability of spinal MNs (Ptak *et al.* 2000; Rekling *et al.* 2000). Thus, it is possible that withdrawal of a SP-mediated excitatory input during sleep due to reduced activity of peptidergic raphe neurons (Jacobs & Azmitia, 1992; Jacobs & Fornal, 1993; Veasey *et al.* 1995) will contribute to the decreased airway tone implicated in sleep-related disorders of breathing (Pack, 1994).

Given that NK1 receptors are the main subtype mediating the actions of SP on MN excitability (Rekling *et al.* 2000), the main goals of this study are to examine the effects of NK1 receptor activation on the response of XII MNs to input from inspiratory networks and to characterize the mechanisms underlying these actions. We used rhythmically active medullary slice preparations isolated from neonatal mice (postnatal day 0–3; P0–3) to examine the effects of NK1 receptor agonists/antagonists on XII MN behaviour. The rhythmic inspiratory-related oscillations generated by these preparations were essential in that they allowed us to examine not only how NK1 receptor activation modulates XII MN properties, but how these receptors modulate the inspiratory-related activity of XII MNs as well. Recent data on phrenic MNs suggest that the effects of SP differ between endogenous inspiratory inputs and somally injected inputs (Ptak *et al.* 2000). In addition, the high expression of NK1 receptors in spinal (particularly phrenic) MN pools relative to moderate or low expression

in XII, facial and trigeminal motoneuron pools (Charlton & Helke, 1985; Yashpal *et al.* 1990; Nakaya *et al.* 1994; Manaker & Zucchi, 1998) suggests that actions may differ between motoneuron pools. Given the hypothesis that a mismatch of activity in upper airway and inspiratory pump muscles contributes to apnoea (Hudgel & Harasick, 1990), there is considerable interest in understanding the basis of this differential excitability. The finding that SP is elevated in brains of sudden infant death syndrome (SIDS) victims (Bergstrom *et al.* 1984; Takashima *et al.* 1994) further emphasises its potential importance to respiratory control of the upper airway.

METHODS

All procedures were performed in accordance with guidelines of the Animal Ethics Committee, University of Auckland.

Brain slice preparation

Experiments were performed on rhythmically active transverse medullary slices from P0–3 Swiss CD-1 mice ($n = 74$). Techniques for preparation of rhythmically active brain slices are described in detail elsewhere (Smith *et al.* 1991; Funk *et al.* 1994). Briefly, mice were anaesthetised with diethyl ether and decerebrated, and the brainstem–spinal cord was isolated in a 15 ml dissection chamber containing artificial cerebrospinal fluid (ACSF; mM: 120 NaCl, 3 KCl, 1.0 $CaCl_2$, 2.0 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 20 D-glucose; pH 7.45) oxygenated with 95 % O_2–5 % CO_2 at room temperature. The brainstem–cervical cord was pinned to a wax chuck and sectioned using a vibratome (Pelco-101, Ted Pella, CA, USA). A series of 100–200 μm sections was cut and examined for landmarks until the compact division of nucleus ambiguus and the rostral border of the inferior olive were observed, whereupon a single 600 μm section was cut. This slice (Fig. 1) extended from the rostral margin of the pre-Bötzinger complex (pre-BötC) to the obex and contained the pre-BötC, rostral ventral respiratory group, XII motor nuclei and rostral XII nerve rootlets.

For extracellular studies, slices were fixed caudal surface up in a recording chamber (10 ml volume) and superfused with 26–27 °C saline solution at a flow rate of 20 ml min^{-1}. In whole-cell recording experiments, slices were perfused at 1–2 ml min^{-1} in either a 2 or 0.5 ml chamber depending on whether recordings were established using blind whole-cell recording techniques (Blanton *et al.* 1989) or under visualization (Stuart *et al.* 1993). Thirty minutes prior to the start of data collection, extracellular K^+ concentration was raised from 3 to 9 mM. Elevated K^+ was not required to activate rhythmic network activity but to maintain long-term respiratory network activity (Smith *et al.* 1991; Funk *et al.* 1993). Immediately after a slice was prepared, robust oscillatory activity was present on XII nerves. This activity slowly 'ran down' over 2–4 h with burst amplitude and interburst interval increasing in variability. Raising extracellular K^+ concentration to 9 mM had minimal effect on the profile of the inspiratory burst envelope recorded from the hypoglossal nerves but increased burst amplitude, and the stability and duration of the rhythmic output. Rhythmic activity persisted for more than 12 h in 9 mM K^+. The reasons why elevated K^+ is required for maintenance of rhythm in the slice are not known. It is hypothesized that the excitability of a critical population of neurons is reduced when the slice is sectioned from the remainder of the medulla, perhaps reflecting removal of a tonic excitatory input. The elevated K^+ is proposed to depolarize this critical population of neurons and restore excitability to levels consistent with generation of rhythmic activity (Smith *et al.* 1991; Funk *et al.* 1993).

Extracellular recording of XII nerve activity

Bilateral extracellular recording of XII nerve activity was performed using suction electrodes with internal tip diameters ranging from 40 to 80 μm. Recordings were amplified (50 000 times), band-pass filtered (0.1–3 kHz), full wave rectified and integrated ($\tau = 50$ ms). The output of the integrator was displayed on an oscilloscope (5111 Storage Oscilloscope, Tetronix, OR, USA), recorded on a chart recorder (2400S, Gould Instruments, OH, USA) and, along with raw data, stored via pulse code modulation on VCR cassette.

Drugs and drug application

Drugs used included the NK1-selective agonist [SAR^9,Met (O$_2$)^{11}]-SP (abbreviated SP$_{NK1}$; RBI, Natick, MA, USA, 0.01–1.0 μM), the NK1-selective antagonist GR82334 (RBI, 10–100 μM), the non-specific NK receptor antagonist spantide (Peninsula Labs, Belmont, CA, USA, 5 μM), the α_1-noradrenergic receptor agonist phenylephrine (PE; RBI, 20–100 μM), the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5; RBI, 1 mM), and tetrodotoxin (TTX; Alomone Labs, Israel, 0.5–1.0 μM). All drugs were made up in ACSF and stored as frozen aliquots.

Local application of neuromodulator agonists/antagonists was performed via pressure injection using triple-barrelled ejection pipettes (6–7 μm per barrel outside tip diameter). Pipettes were positioned within 20 μm of the ventromedial aspect of the XII nucleus using a hydraulic manipulator. Drugs were delivered at 10 p.s.i. (70 kPa) and injection protocols were controlled via a programmable stimulator (Master-8, AMPI, Israel; or Digitimer, Type 3290). For extracellular studies, the concentration range and duration of SP$_{NK1}$ applications were established in a preliminary set of experiments. At 0.001 μM SP$_{NK1}$, changes in inspiratory burst activity were not apparent while at 10 μM tonic excitation predominated and obscured inspiratory activity. Since the main objective of this set of experiments was to examine the effects of SP$_{NK1}$ on inspiratory burst amplitude, we limited our investigation to concentrations ranging from 0.01 to 1.0 μM SP$_{NK1}$. Ten or thirty second applications were selected to allow measurement of several inspiratory cycles during the peak of the SP$_{NK1}$ response, while minimizing depolarization block and resulting reductions in inspiratory burst amplitude that often occurred when the highest concentration of SP$_{NK1}$ (1.0 μM) was applied for longer periods. Consecutive applications were separated by a minimum period of 15 min.

Note that the concentrations and durations of drug applications used in the present study should not be directly compared with those in experiments where similar agents are bath applied or applied to isolated cells. First, the concentration of drug decreases exponentially with distance from the pipette tip (Nicholson, 1985). Previous experiments with this preparation indicate that drug concentration in the pipette must be approximately 10-fold greater than the bath-applied concentration to produce similar effects (Liu *et al.* 1990). Second, diffusion barriers in thick slices slow response kinetics relative to isolated cells.

Whole-cell recordings

Intracellular recordings from XII MNs ($n = 47$) were made using 'blind' whole-cell patch-clamp recording techniques (Blanton *et al.* 1989) or infra-red differential interference contrast microscopy (IRDIC) (Stuart *et al.* 1993). Patch electrodes (resistance 4.0–5.0 MΩ; tip size 1.5–2 μm) were pulled on a horizontal puller (Sutter Model P-57) from 1.2 mm o.d. filamented borosilicate glass (Clark/WPI) and filled with potassium gluconate solution containing (mM): 125 potassium gluconate, 5 NaCl, 1 CaCl$_2$, 10 Hepes, 10 BAPTA, 2 Mg^{2+}-ATP, 0.3 GTP. Intracellular solution pH was adjusted to 7.2–7.3 with 5 M KOH. Intracellular signals were amplified with a patch-clamp amplifier (5 kHz Bessel filter, Axopatch 1D or 1B, Axon Instruments, Foster City, CA, USA). Seal resistances prior to membrane rupture ranged from 1.5 to 6 GΩ.

Series resistance and whole-cell capacitance were estimated under voltage-clamp conditions by using short voltage pulses (100 Hz, -10 mV, 3.0 ms). Series resistance averaged 17.4 ± 1 MΩ (range, 9–29 MΩ). Series resistance was compensated up to 80 % and monitored throughout the experiment. Data were discarded if it increased more than 10 % between control and test conditions. Current–voltage relationships and repetitive firing behaviour were
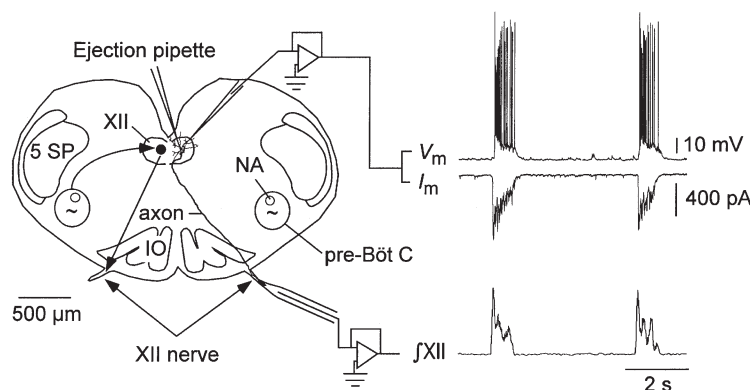

Figure 1. Schematic of transverse medullary slice preparation

The inspiratory related rhythm is generated in the pre-Bötzinger complex. Rhythmic bursts of activity are transmitted to XII MNs (curved arrow) and along the MN axons into the XII nerves. The diagram illustrates the arrangement of suction electrodes for recording inspiratory activity from the XII nerve (nerve activity is typically recorded bilaterally), pressure ejection pipette for local application of drugs and whole-cell recording pipettes for recording membrane voltage or current from individual MNs under current or voltage clamp, respectively. Examples of XII MN membrane potential (V_m), membrane current (I_m) and integrated XII nerve activity ($\int$XII) during inspiration are also shown. NA, nucleus ambiguus; IO, inferior olive; 5 SP, spinal trigeminal nucleus; XII, hypoglossal nucleus; pre-BötC, pre-Bötzinger complex.

examined by applying a series of command voltage or current pulses (600 ms) controlled by computers running Axodata or pCLAMP 6.0 software. In voltage clamp, current–voltage (I–V) relationships were obtained by applying a series of 5 mV voltage steps between −90 and −40 mV. Neuron input resistance (R_N) was calculated from the inverse of the slope of a least squares regression line fitted to the I–V curves. When slices were producing rhythmic activity (i.e. prior to TTX application), pulses were triggered from integrated XII nerve activity following a 2 s delay to ensure that injected waveforms and inspiratory synaptic inputs did not overlap. All signals were recorded continuously via pulse code modulation for off-line analysis. Membrane potentials have not been adjusted for liquid junction potentials (−10 mV for potassium gluconate solution).

MN identification

All XII motoneurons studied were functionally identified respiratory neurons. Criteria for identifying respiratory motoneurons have been described elsewhere (Funk *et al.* 1993) and include: presence of rhythmic synaptic drive currents/ depolarizations in synchrony with rhythmic bursts of activity in XII nerve roots; multipolar soma located in the ventromedial portion of the XII nucleus; an axon exiting the slice located in the XII nerve; stable resting potentials with leak currents < 100 pA at holding potentials of −60 mV. Anatomical identification was based on a combination of IRDIC images and Lucifer Yellow or biocytin labelling of neurons.

Data analysis

Selected portions of data were digitized off-line at 1–20 kHz using Axodata software in conjunction with a National Instruments NBMIO-16 A/D board or pCLAMP 6.0/Axoscope 1.1 software in conjunction with a Digidata 1200 A/D board, and stored on computer for subsequent analysis. Axodata and pCLAMP files were analysed using Axograph 3.0 and Microsoft Excel software.

Rectified, integrated records were analysed for inspiratory burst frequency as well as ipsi- and contralateral XII nerve inspiratory burst amplitudes using a custom-written LabVIEW acquisition and analysis program. Burst amplitude was quantified in arbitrary units as the distance from baseline to the peak of the integrated burst envelope. In cases where drug application was associated with an increase in tonic nerve discharge and a shift in the baseline, burst amplitude was calculated by subtracting the baseline shift from the peak amplitude. While some of the baseline shift may represent tonic activation of respiratory motoneurons, baseline correction was chosen to reduce the possibility of overestimating the potentiating actions of SP_{NK1} on inspiratory burst amplitude.

Contralateral nerve activity was recorded to monitor the degree of drug diffusion. Responses to 0.01 and 0.1 μM SP_{NK1} were exclusively unilateral. In some cases weak tonic activity was observed on the contralateral XII nerve in response to 1.0 μM SP_{NK1}. SP-mediated increases in frequency, indicative of SP diffusion to rhythm-generating regions of the slice in the ventrolateral medulla (Johnson *et al.* 1996), were never observed.

The time course of drug responses was assessed by averaging inspiratory burst amplitudes for 30 s time bins for the first 2 min after SP_{NK1} application, for 1 min intervals between minutes 2 and 5 post-SP_{NK1} and again at 10 and 20 min post-SP_{NK1}. Values were reported relative to control burst amplitude averaged for 2 min immediately prior to SP_{NK1} application.

Dose–response curves were generated by reporting maximum changes in inspiratory burst amplitude relative to control. Maximum responses were determined by comparing control burst amplitude (average amplitude during the 2 min immediately prior to drug application; each drug application was compared relative to its own control period) with burst amplitudes averaged from a 30 s bin taken from the region of maximal response. Thus, the maximum response based on the time course data is generally less that the actual maximum. Data are reported in absolute terms or relative to pre-injection values as means $\pm$ S.E.M.

Statistical analysis

Statistical analysis was conducted using SAS 6.1 (SAS Institute Inc., Cary, NC, USA). Raw data were tested for normality using the Shapiro-Wilk statistic and, where appropriate, subjected to a two-way ANOVA. Differences among interventions were sought (at the 95% level of confidence, $P < 0.05$) using mutually orthogonal contrast coefficients to partition the treatment sum of squares.

Immunohistochemistry

Neonatal mice were anaesthetized with sodium pentobarbital (250 mg kg^{-1}), and perfused transcardially with heparinized saline (1 u ml^{-1}) followed by 4% formaldehyde–0.5% glutaraldehyde in 100 mM phosphate buffer (PB, pH 7.4). Brainstems were removed and post-fixed in the same fixative for 48 h at room temperature and cryoprotected through successive 10 and 30% sucrose treatments. Transverse sections of 30 μm were cut from the medulla on a cryostat microtome (Reichert-Jung 1800) and endogenous peroxidase quenched by incubation in 0.3% H_2O_2 in 100 mM phosphate buffered saline (PBS) containing 0.2% Triton X-100 (PBS–Triton) for 1 h. The sections were then pre-incubated with 3% sheep serum (Sigma, St Louis, MO, USA) in PBS–Triton for 1 h at room temperature to block non-specific binding sites, prior to incubation with SP antiserum (WATPA Enterprises Inc., Auckland, New Zealand) for 36 h at 4 °C. Antiserum was diluted (1:20 000) in PBS–Triton containing 3% sheep serum. Subsequently, sections were washed in PBS–Triton and incubated with biotinylated sheep anti-rabbit immunoglobulin (Sigma, 1:400) for 3 h, followed by further washes and incubation with ExtrAvidin peroxidase conjugate (Sigma, 1:1000) for 3 h. After further washes (3 × 30 min) the peroxidase was visualized with 0.5 mg ml^{-1} 3′,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in PBS–Triton, mounted on gelatin-coated slides and coverslipped with Histomount (Hughes and Hughes, Wellington, UK). Control experiments, in which primary antiserum was excluded, were run with each group. No labelling was detected in the absence of primary antiserum.

To assess the distribution of SP-immunoreactive terminals relative to the dendrites of inspiratory XII MNs that responded electrophysiologically to SP_{NK1}, biocytin (0.2%) was added to the intracellular solution in some experiments. Tissue from these experiments was post-fixed (4% formaldehyde–0.5% glutaraldehyde) for 24 h at 4 °C and cryoprotected through successive 10 and 30% sucrose treatments. The 600 μm medullary slices were sectioned at 30 μm and immunolabelling of SP assessed as described above with the exception that nickel intensification was used to facilitate comparison of immunolabelling in terminals relative to dendrites at ×100 magnification. In the nickel intensification step, tissue was incubated in a sodium acetate buffer containing 0.5% $NiCl_2$, 0.01% H_2O_2 and 0.5 mg ml^{-1} DAB.

RESULTS

SP potentiates XII nerve population inspiratory output

To examine the role of SP_{NK1} in modulating XII population inspiratory activity, SP_{NK1} (0.01, 0.1 and 1.0 μM) was locally applied unilaterally over XII motor nuclei for 10 and 30 s. As shown in Fig. 2 by the response of a single slice to SP_{NK1} over the left XII nucleus, SP_{NK1}

A 0.01 μM SP$_{NK1}$

B 0.1 μM SP$_{NK1}$

C 1.0 μM SP$_{NK1}$

Figure 2. SP$_{NK1}$ causes tonic excitation of XII nerve output and potentiates inspiratory burst amplitude

Long time course, recordings of integrated left and right XII nerve activity from a single rhythmic slice (P3) showing response to 10 s unilateral application over the hypoglossal nucleus of 0.01 (*A*), 0.1 (*B*) and 1.0 μM SP$_{NK1}$ (*C*). Effects of SP$_{NK1}$ on burst profile are shown to the right of each panel as the average of 10 cycles taken before (control), during (0.01 μM SP$_{NK1}$) and after (washout) application of SP$_{NK1}$.
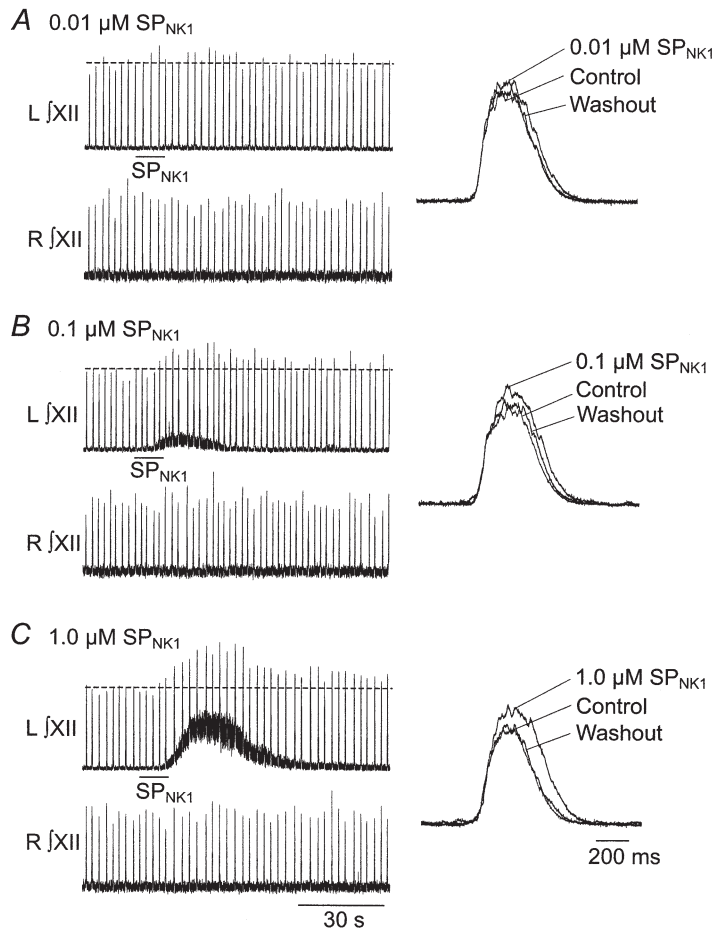
increased tonic activity (evident in the thickened or shifted baseline at 0.1 and 1.0 μM SP) and potentiated inspiratory burst amplitude. Both components of the response were dose dependent with the tonic component absent at 0.01 μM and increasing between 0.1 and 1.0 μM SP$_{NK1}$. Burst amplitude was potentiated by all three doses of SP$_{NK1}$ as evident in the long time series recordings of continuous inspiratory activity (Fig. 2, left panels) as well as average burst envelopes compiled from 10 consecutive inspiratory cycles before, during and after the SP$_{NK1}$ response (Fig. 2, right panels). Note also that the responses were exclusively unilateral reflecting minimal diffusion of SP$_{NK1}$ away from the injection site.

Pooled data were similar. The time course and dose dependence of the SP$_{NK1}$ response are plotted in Fig. 3 for 30 s applications. Burst amplitude peaked within 60, 90 and 120 s from onset of SP$_{NK1}$ application for the 0.01, 0.1 and 1.0 μM SP$_{NK1}$, respectively, before returning to

Figure 3. SP$_{NK1}$ potentiation of inspiratory activity is dose dependent

A, time course of changes in integrated inspiratory burst amplitude (mean ± S.E.M.) following 30 s applications of 0.01, 0.1 and 1.0 μM SP$_{NK1}$ over the XII nucleus (agonist applied starting at time $t = 0$; $n = 9$). *B*, dose-dependent effects of SP$_{NK1}$ on inspiratory XII nerve burst amplitude ($n = 9$).
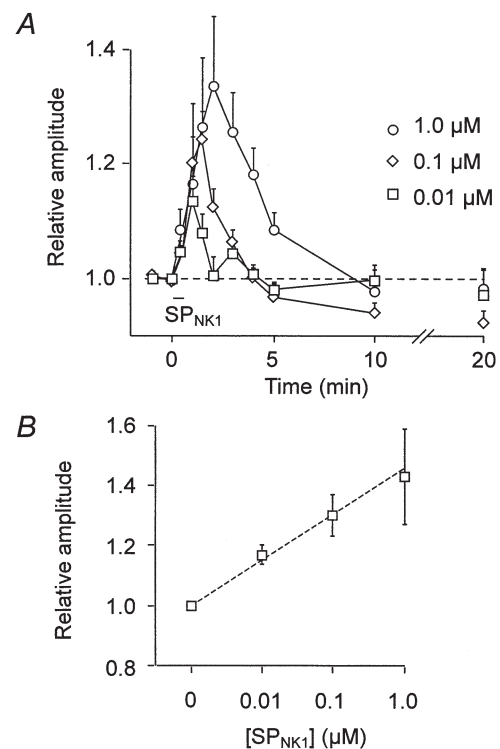
control by 5 and 10 min after drug application. The dose dependence of the response is shown in Fig. 3B where 0.01, 0.1 and 1.0 μM SP_{NK1} significantly potentiated burst amplitude relative to control by factors of 1.17 ± 0.03, 1.30 ± 0.05, and 1.43 ± 0.16, respectively ($n = 9$).

SP potentiation of XII nerve inspiratory activity is mediated by NK1 receptors

In order to verify the receptor mechanisms involved, we examined the effects of 5 μM spantide on the responses elicited by 10 s applications of 0.1 μM SP_{NK1}. Spantide is a general NK receptor antagonist that has a very slow wash-in time requiring that tissue be pre-incubated with the drug (Parker & Grillner, 1996; Parker *et al.* 1997). As seen for an individual slice (Fig. 4A), the initial potentiation of burst amplitude (pooled data, 1.28 ± 0.16) was reduced following 1 h of pre-incubation with spantide. After 3 h of incubation (Fig. 4C, $n = 5$), inspiratory burst amplitude potentiation by SP_{NK1} was no longer significantly greater than control (1.11 ± 0.03). This did not represent a decreased efficacy of SP_{NK1} on XII nerve output over time since, in a separate series of control experiments, SP_{NK1} produced consistent responses for up to 6 h when presented at > 15 min intervals (data not shown).

To test the involvement of NK1 receptors in this response, we compared the actions of 0.1 μM SP_{NK1} (10 s) before, immediately following and 15 min following a 5 min local application of the NK1 receptor antagonist GR82334 (10 μM). The significant potentiation of burst amplitude by SP_{NK1} under control conditions (1.29 ± 0.08) was blocked by pre-application of GR82334 (1.11 ± 0.05) in a reversible manner (Fig. 4B and C, $n = 8$).

Mechanisms underlying the potentiation of inspiratory activity by SP_{NK1}

To directly test the effects of SP_{NK1} on inspiratory XII MNs and the mechanisms by which SP_{NK1} modulates XII MN activity, we established whole-cell recordings from individual inspiratory MNs.

As described previously (Funk *et al.* 1993), inspiratory XII MNs received rhythmic synaptic inputs in phase with each burst of activity recorded from the XII nerves. Peak inward currents ranged from -75 to -350 pA and lasted between 350 and 500 ms. It is worthy of note that the inspiratory synaptic currents recorded from neurons located near the surface of the slice using IRDIC for neuronal visualization and those recorded from cells located deeper within the tissue using blind whole-cell recording techniques were similar. The number of neurons receiving small synaptic currents (< 75pA) was marginally greater for surface cells but many surface neurons received inputs that were as large or larger than subsurface neurons recorded using blind recording techniques. All XII MNs tested responded to 1.0 μM SP_{NK1} with a peak inward current that averaged -48 ± 5 pA ($n = 29$) (range -20 to -150 pA) at a holding potential of -60 mV (Figs 5, 8, 9 and 10). Application of 0.1 μM SP_{NK1} produced peak inward currents averaging -33 ± 9 pA ($n = 5$).

SP_{NK1} receptor activation does not modulate inspiratory synaptic currents

To test the hypothesis that the SP_{NK1}-mediated increase in XII population inspiratory output was due to potentiation of inspiratory synaptic currents, we measured the effects of SP_{NK1} (0.1 and 1.0 μM) on both the peak current amplitude and charge transfer of
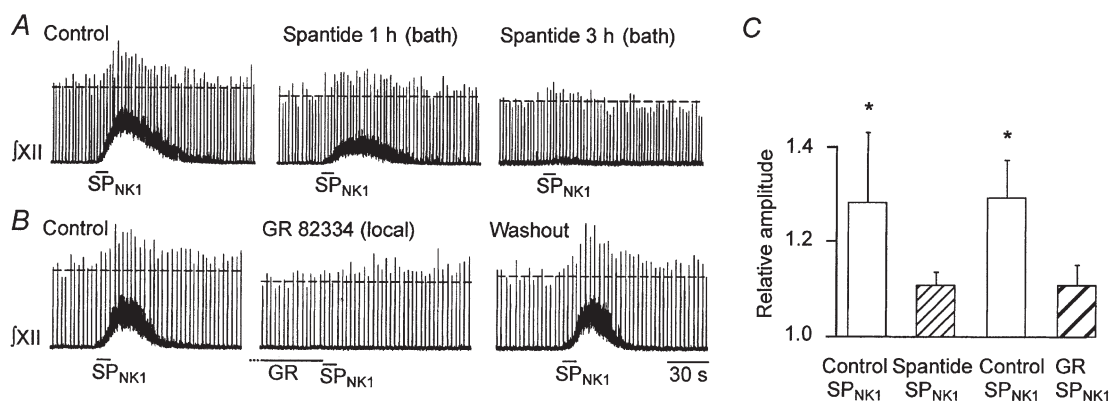

Figure 4. SP_{NK1} potentiation of XII nerve inspiratory burst amplitude is reduced by neurokinin receptor antagonists

A, effects of 0.1 μM SP_{NK1} locally applied for 10 s on integrated XII nerve activity before (control) and after 1 and 3 h of incubation with spantide (5 μM). *B*, effects 0.1 μM SP_{NK1} locally applied for 10 s before (control) during and 15 min after (washout) a 5 min pre-application of 10 μM GR-82334 locally over the XII nucleus. *C*, mean ($\pm$ S.E.M.) inhibition by spantide (5 μM, bath applied, $n = 5$) or GR82334 (10 μM, locally applied, $n = 8$) of the SP_{NK1}-mediated potentiation of inspiratory burst amplitude (0.1 μM SP_{NK1}, 10 s local application). * Significant increase from control. Note, spantide and GR82334 were tested in different preparations.

Figure 5. SP$_{NK1}$ has no effect on the magnitude of inspiratory synaptic currents

A, long time course voltage-clamp recording of a XII MN showing effect on membrane current (I_m) of locally applying 1.0 μM SP$_{NK1}$ over the XII nucleus. Peaks represent individual inspiratory synaptic currents (* series resistance tests or pulse protocols to establish input resistance). *B*, no effect of SP$_{NK1}$ on the inspiratory synaptic current envelope is shown for a single XII MN as the average of 10 cycles before, during and after local application of SP$_{NK1}$. *C*, average time course of relative changes in peak amplitude and charge transfer of inspiratory synaptic currents following a 10 s local application of 1.0 μM SP$_{NK1}$ (starting time at $t = 0$) over the XII nucleus ($n = 11$).
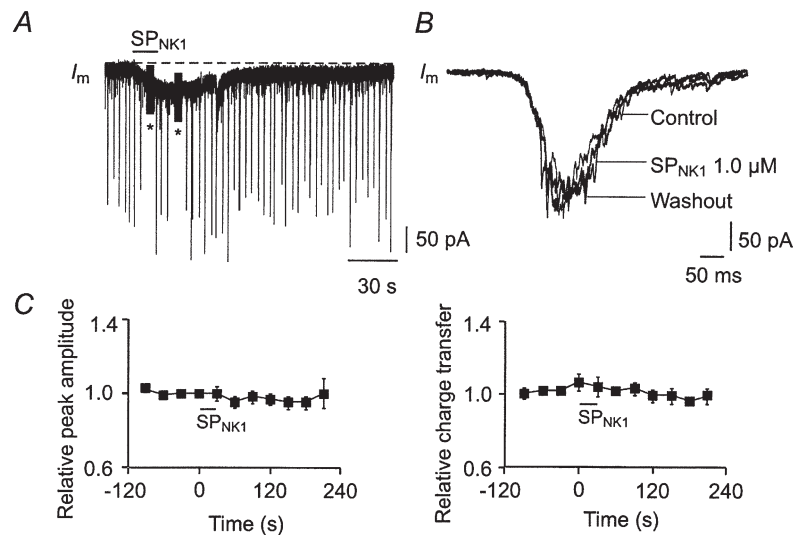
inspiratory synaptic currents. A long time scale recording of membrane potential from an inspiratory XII MN illustrates minimal change in the peak of individual inspiratory synaptic currents during a 10 s application of 1.0 μM SP$_{NK1}$ (Fig. 5*A*). The lack of a SP$_{NK1}$ response is further demonstrated for this cell in Fig. 5*B* where the confounding influence of variability between consecutive inspiratory synaptic currents is minimized by comparing the average inspiratory synaptic current compiled from 10 consecutive inspiratory cycles before, during and after the SP$_{NK1}$ response. The peak synaptic current and current envelope, reflecting charge transfer, were similar in all conditions. Data pooled for 11 MNs receiving inspiratory synaptic currents averaging -203 ± 24 pA under control conditions confirmed that SP$_{NK1}$ does not alter peak or charge transfer of inspiratory synaptic currents (Fig. 5*C*).

Recent observations in phrenic motoneurons in rat indicate that potentiation of inspiratory output by SP is mediated through potentiation of the NMDA receptor-mediated component of the inspiratory synaptic drive (Ptak *et al.* 2000). The NMDA component of the synaptic current is small in XII MNs. However, due to the voltage-dependent Mg^{2+} block of the NMDA channel (Nowak *et al.* 1984), our voltage-clamp analysis of inspiratory drive modulation conducted at holding potentials of -60 mV does not rule out the possibility that SP$_{NK1}$ potentiation of inspiratory activity is dependent on an NMDA receptor mechanism. Therefore, to test whether NMDA receptor

activation was essential for the potentiation of XII inspiratory activity, we examined the effects of exogenously applied SP$_{NK1}$ on integrated XII ($\int$XII) nerve activity before and after blockade of NMDA receptors with a 2 min local pre-application of 1 mM AP5. While AP5 completely abolished the response to exogenously applied NMDA, it did not alter the potentiation of XII nerve inspiratory activity by SP$_{NK1}$ (data not shown). Relative to control, SP$_{NK1}$ (0.1 μM) alone produced a (1.28 ± 0.12)-fold potentiation of burst amplitude ($n = 3$). AP5 alone reduced burst amplitude to 0.84 ± 0.10 of control but SP$_{NK1}$ still caused an increase in burst amplitude to 1.30 ± 0.13 of control (Fig. 6).

SP$_{NK1}$ potentiates repetitive firing behaviour

To determine whether the increase in the inspiratory output of XII nerve produced by SP$_{NK1}$ results from membrane depolarization or an increase in excitability (or both), we examined the effects of SP$_{NK1}$ on repetitive firing behaviour of motoneurons to endogenous inspiratory drive and injected current pulses. The protocol and response of a single MN are shown in Fig. 7. Control repetitive firing was characterized by measuring responses to inspiratory synaptic currents (Fig. 7*Aa*, expanded trace in Fig. 7*Ba*) and applying a series of six to eight current steps in 20 pA increments starting from $\sim$1.4–1.5 times rheobase (Fig. 7*Ab*, response to a single pulse in this control series is expanded in Fig. 7*Cb*). Current injection was triggered from $\int$XII output following a 2 s delay so that current pulses arrived

Figure 6. Potentiation of inspiratory XII activity is not dependent on NMDA receptor activation

Potentiation of inspiratory burst amplitude by 30 s local application of 0.1 μM SP$_{NK1}$ was the same in control and after a 2 min local pre-application of 1 mM AP5 ($n = 3$).
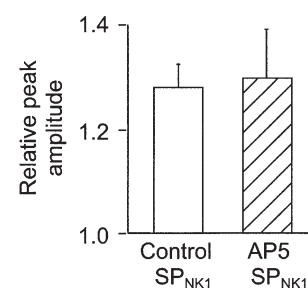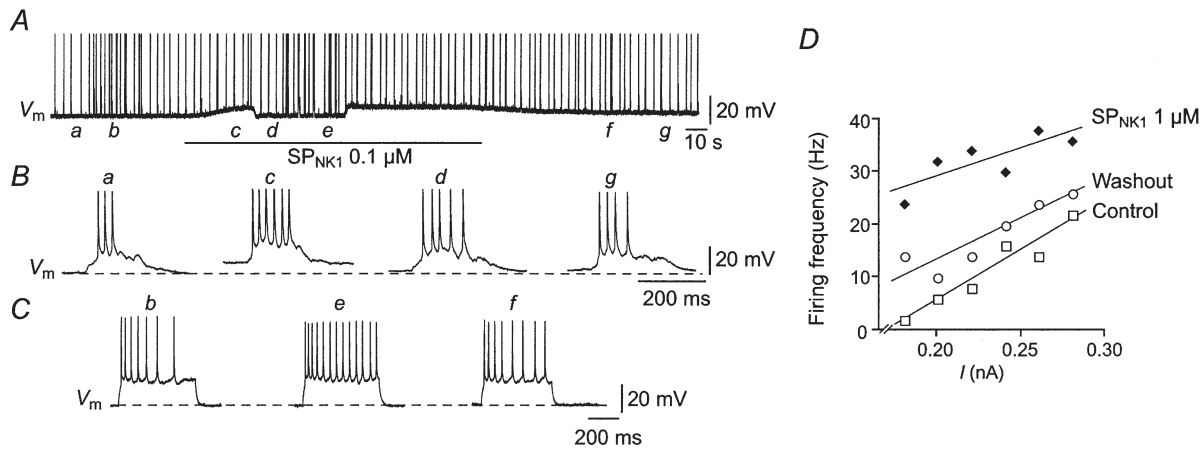
Figure 7. SP$_{NK1}$ depolarizes XII MNs and increases MN excitability

A, long time scale current-clamp recording of XII MN membrane voltage (V_m) showing the response to a sustained local application of 0.1 μM SP$_{NK1}$ and a repetitive firing protocol. Note that DC injection was employed during the SP$_{NK1}$ application to counter the SP$_{NK1}$-induced depolarization. Lower case letters in A indicate points at which traces with higher temporal resolution shown in B and C occurred. B, firing responses of the MN in A to individual inspiratory synaptic currents during control conditions (a), during SP$_{NK1}$-mediated depolarization (c), during continued application of SP$_{NK1}$ but following DC injection (d), and during recovery (g). C, repetitive firing responses to square-wave current pulses injected during control (b), during continued SP$_{NK1}$ but after DC injection (e), and following washout (f) (spikes are truncated). D, firing frequency–current relationships for a single XII MN before (control), during and after (washout) local application of 1.0 μM SP$_{NK1}$.

between inspiratory bursts. SP$_{NK1}$ was then applied for up to 2 min. Upon reaching a steady-state level of depolarization, direct current (DC) was injected to return membrane potential to pre-SP$_{NK1}$ levels. The repetitive firing protocol was then repeated. DC was removed following the current injection protocol and behaviour followed through SP$_{NK1}$ washout.

The response of the MN in Fig. 7 was typical of eight others where SP$_{NK1}$ produced an average 5.2 ± 0.9 mV depolarization. During the period of depolarization, the number of action potentials (APs) produced per inspiratory cycle increased significantly (Fig. 7B, compare traces a and c). To determine whether this

increase in output simply reflected depolarization or an increase in excitability as well, membrane potential was returned to control by DC injection. The number of APs elicited by single inspiratory bursts (Fig. 7B, compare traces a and d) and current pulses (Fig. 7C, compare traces b and e) during application of 0.1 μM SP$_{NK1}$ was elevated relative to that produced under control conditions. The increased excitability is further demonstrated by the leftward shift in the relationship between firing frequency (number of spikes per current pulse divided by duration of current pulse) and injected current for another cell in response to 1.0 μM SP$_{NK1}$ (Fig. 7D). The slope of this relationship was unaffected by SP$_{NK1}$.
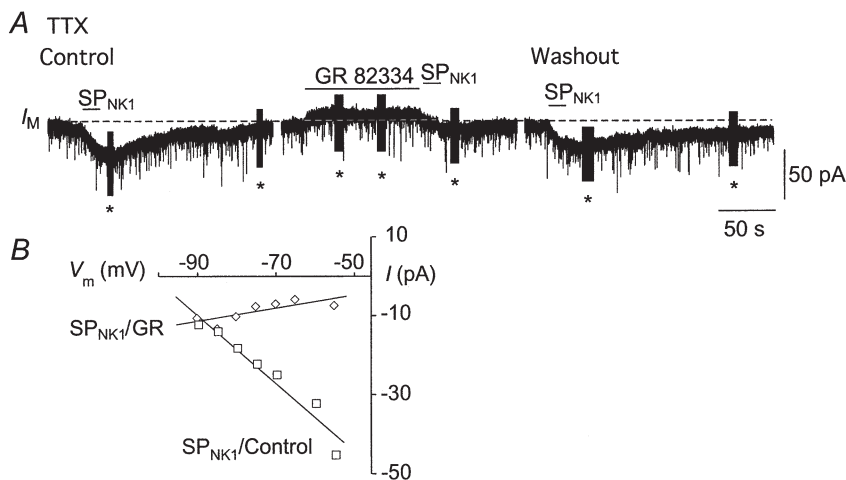

Figure 8. SP$_{NK1}$ currents persist in TTX and are sensitive to GR82334

A, membrane current induced by 1 μM SP$_{NK1}$ under control conditions, following a 2 min pre-application of GR82334 and after 15 min of washout, all in the presence of 1 μM tetrodotoxin (TTX). Note the small outward current induced by the application of GR82334 (* series resistance tests or pulse protocols to establish input resistance). B, current–voltage relationship for the SP$_{NK1}$ current induced before and after 2 min pre-application of 100 μM GR82334.

Ionic basis of the action of SP_{NK1} on XII MNs

SP_{NK1} currents were associated with a significant $13 \pm 2\%$ increase in R_N from 104 ± 7 to 117 ± 8 MΩ ($n = 27$), which persisted following blockade of synaptic transmission with 0.5–1.0 μM TTX (Fig. 8, $n = 7$). These data are consistent with postsynaptic receptor localization and SP_{NK1}-mediated channel closure. Mediation of the SP responses by NK1 receptor involvement was further supported by the significant attenuation of SP_{NK1}-induced currents (1 μM) by GR82334 (100 μM) from -34 ± 1.4 to -7.0 ± 1.0 pA (Fig. 8A, $n = 3$). GR82334 also blocked the increase in R_N associated with SP_{NK1}. The GR82334-resistant component of the current was relatively voltage independent (Fig. 8B).

In rat XII MNs, SP acts in part by inhibiting the TASK-1 K$^+$ channel (Talley *et al.* 2000). Our observation in mouse MNs that SP_{NK1}-induced inward currents were associated with an increase in R_N was also consistent with K$^+$ channel closure. To further examine the involvement of K$^+$ conductance in mediating the actions of SP_{NK1} in mouse MNs, we tested the hypothesis that shifting extracellular K$^+$ from 9 to 3 mM would increase the magnitude of the SP_{NK1} current and shift its reversal potential in the hyperpolarizing direction. Similar to the results for a single MN shown in Fig. 9, data pooled from six MNs indicate that when extracellular K$^+$ is reduced from 9 to 3 mM the average holding current decreased from -100 ± 30 to -10 ± 27 pA (at a holding potential of -60 mV). The SP_{NK1} current also doubled in magnitude from -33 ± 6 to -61 ± 10 pA, and the extrapolated reversal potential shifted in the hyperpolarizing direction from -84 ± 8 to -114 ± 2 mV.

Of interest is that in a different subset of 15 MNs in which the reversal potential was measured only in 9 mM K$^+$, actual reversal was observed in only eight. In the remaining seven, reversal potential was estimated by extrapolation. Part of this reflects that membrane potential was only varied from -90 to -55 mV. However, in 2 of 4 MNs where the membrane potential was varied over larger ranges (-110 to -40 mV), the SP_{NK1}-induced inward current plateaued and did not reverse.

Exogenous SP_{NK1} and PE occlude each other but differentially modulate inspiratory activity

To test the hypothesis that SP_{NK1} and noradrenaline (NA) increase MN excitability through convergent action on a common postsynaptic mechanism (Rekling *et al.* 2000) as seen in rat (Talley *et al.* 2000), we compared SP_{NK1}-
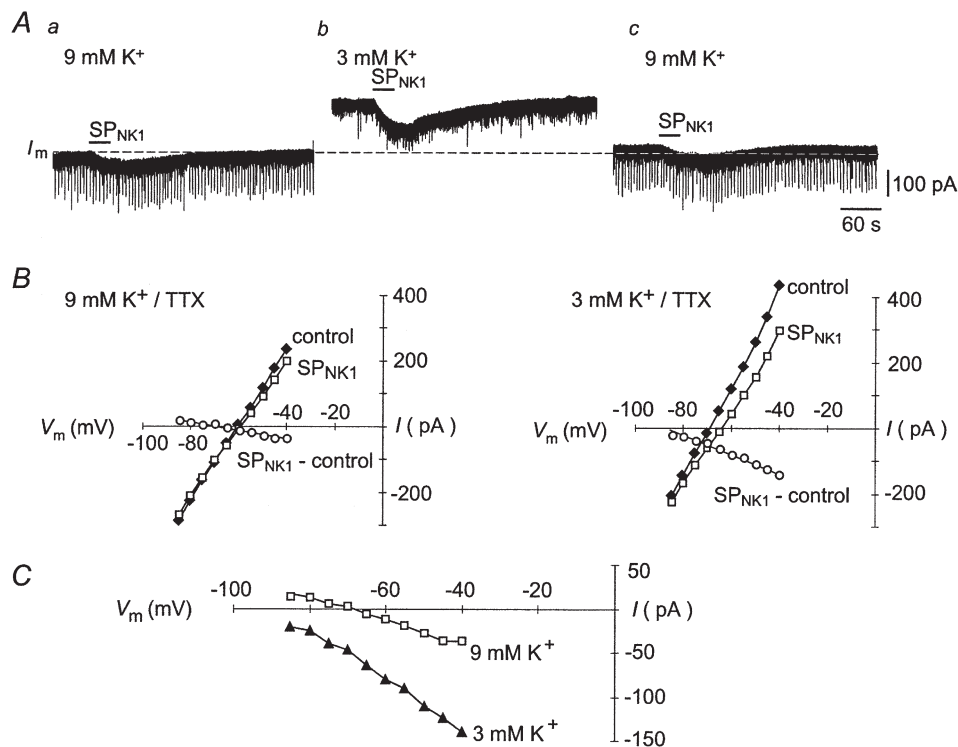

Figure 9. The reversal potential and magnitude of the SP_{NK1} current are sensitive to extracellular [K$^+$]

A, slow time scale recordings showing effects on the SP_{NK1} current of changing extracellular [K$^+$] from 9 to 3 mM. *B*, whole-cell current–voltage plots, obtained in the presence of 1.0 μM TTX, showing the effects of changing extracellular [K$^+$] on membrane current and the SP_{NK1}-induced current (SP-control). *C*, the current–voltage relationships of the SP_{NK1} currents shown in *B* are enlarged to illustrate the effects of changing extracellular [K$^+$].
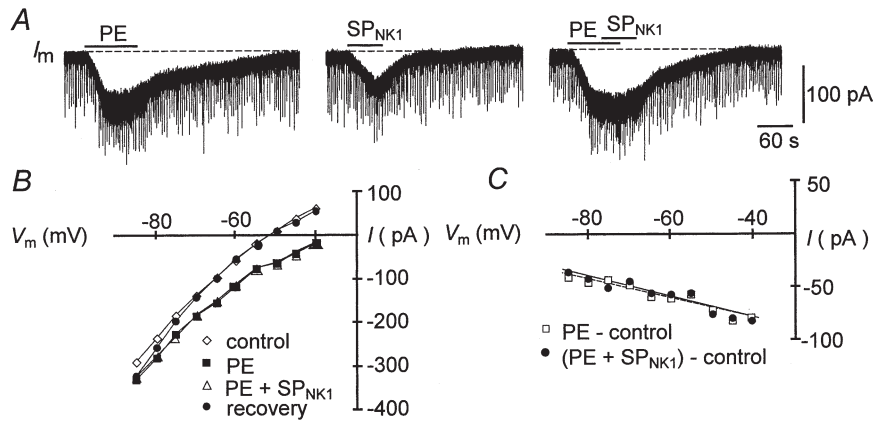
Figure 10. SP_{NK1} and the α_1-noradrenergic receptor agonist PE modulate excitability through similar postsynaptic pathways

A, long time scale recording of membrane current responses to 90 s local application of 20 μM PE (left trace), 60 s local application of 1.0 μM SP_{NK1} 15 min later (middle trace) and the co-application of PE and SP_{NK1} a further 15 min later (right trace). *B*, whole-cell current–voltage relationships obtained under control, during the application of PE alone, during co-application of PE and SP_{NK1}, and during washout. *C*, the current–voltage relationships of the current produced by PE alone (PE − control) and in combination with SP (($PE + SP_{NK1}$) − control).

induced currents produced by 60 s of 1.0 μM SP_{NK1} in control conditions and during the final 30–60 s of a 90–120 s application of 20 μM phenylephrine (see Fig. 10, $n = 5$). We selected the α_1-noradrenergic agonist PE, because the primary excitatory action of NA on XII MNs is mediated via α_1 receptors (Parkis *et al.* 1995; Selvaratnam *et al.* 1998). Local application of PE alone (20 μM) for 90–120 s produced an average peak inward current of -76 ± 21 pA ($n = 5$). The PE current was associated with a 21 % increase in R_N from 102 ± 11 to 123 ± 14 MΩ and reversed at -111 ± 7 mV. SP_{NK1} currents in this same pool of MNs averaged -52 ± 10 pA, increased R_N by 14 % from 109 ± 10 to 124 ± 15 MΩ and reversed at -109 ± 9 mV. When applied in conjunction with PE, SP_{NK1} produced no additional inward current in 4 of 5 neurons, suggesting complete occlusion. In the remaining neuron, the SP_{NK1} response was reduced from -58 to -31 pA. Complete occlusion of the response to 5 μM SP_{NK1} by 100 μM PE was also observed for one cell in the presence of TTX. Occlusion was not dependent on the order of drug delivery. In one of the five cells tested, SP_{NK1} was applied first and completely occluded the response to

PE. The possibility that the lack of a response to SP_{NK1} in the presence of PE was due to 'response rundown' was eliminated by the observation that application of SP_{NK1} 15 min after the co-application of PE and SP_{NK1} produced an inward current in all cases.

Having established that SP_{NK1} and PE have similar effects on subthreshold membrane properties, we next tested the hypothesis that activation of SP_{NK1} and α_1-noradrenergic receptors produce similar changes in suprathreshold behaviour of XII MNs by comparing repetitive firing elicited by somally injected square-wave current pulses. Both compounds produced a leftward shift in the frequency–current relationship without changing slope (Fig. 11), suggesting that SP_{NK1} and α_1-receptor activation would be equally efficacious in modulating inspiratory activity. When this was tested, however, PE was greater than twofold more effective than SP_{NK1} in potentiating inspiratory burst amplitude (Fig. 12*A* and *B*), even though both compounds produced large increases in tonic discharge as reflected in the thickening and upward shift in the baseline of the integrated XII nerve recordings (Fig. 12*A*). This difference in potentiation does not reflect that the
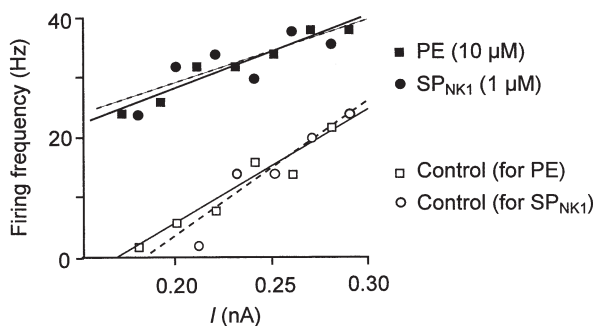

Figure 11. SP_{NK1} and PE have similar effects on the repetitive firing behaviour of XII MNs

Firing frequency–current relationships for a XII MN, elicited by somal injection of square-wave current pulses immediately before and during local application of SP_{NK1} (1.0 μM) or PE (10 μM). Response shown is typical of all cells tested (SP_{NK1}, $n = 8$; PE, $n = 3$).
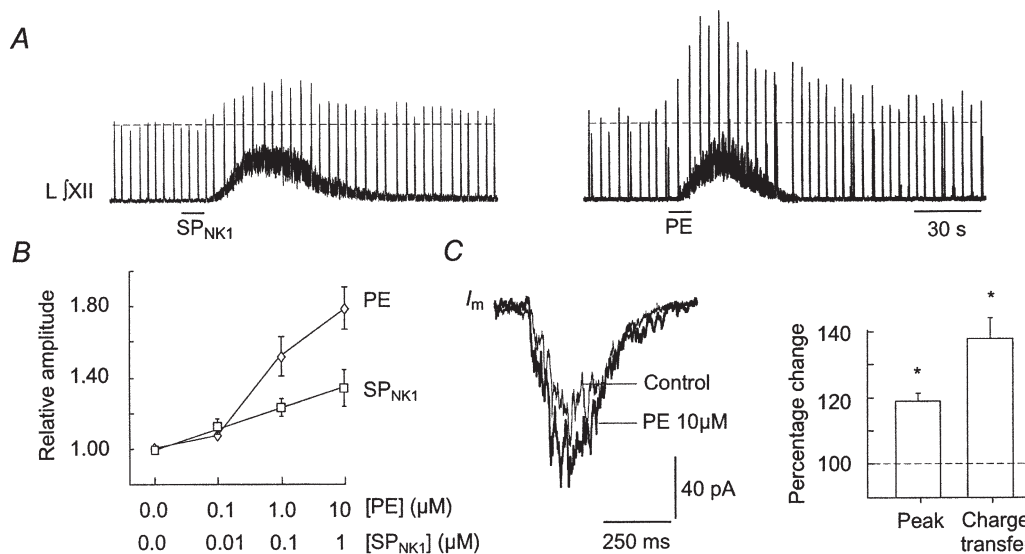
Figure 12. SP$_{NK1}$ and PE differentially modulate inspiratory activity

A, long time course recordings of integrated XII nerve activity from rhythmic slices (P3) showing response to 10 s unilateral application over the hypoglossal nucleus of 1.0 μM SP$_{NK1}$ or 10 μM PE. *B*, dose-dependent effects of SP$_{NK1}$ ($n = 9$, from Fig. 3*B*) and PE ($n = 6$) on inspiratory XII nerve burst amplitude. *C*, effect of PE (10 μM) on the inspiratory synaptic current envelope is shown for a single XII MN as the average of 10 cycles before and during local application of PE. Bar chart indicates effects of PE on relative peak inspiratory current and charge transferred per inspiratory cycle averaged for 8 XII MNs.

concentration of SP was submaximal. The highest doses of SP and PE used produced maximum potentiation of inspiratory burst amplitude as further increases in SP$_{NK1}$ or PE beyond 1.0 and 10 μM, respectively inhibited inspiratory burst amplitude most probably due to progressive depolarization block. Part of this differential action of SP$_{NK1}$ and PE on inspiratory activity can be attributed to the fact that PE (10 μM) produced a significant 18 ± 3 and $38 \pm 7\%$ increase in the peak current and charge transferred per inspiratory cycle, respectively (Fig. 12*C*, $n = 8$). SP did not alter inspiratory synaptic currents (Fig. 5).

Inspiratory activity is modulated by endogenously released SP$_{NK1}$

To test the hypothesis that inspiratory activity is modulated by endogenously released SP$_{NK1}$, we examined the effects of GR82334 on XII nerve inspiratory activity recorded extracellularly (Fig. 13). Inspiratory burst

amplitude decreased significantly to 0.84 ± 0.05 of control following local application of 10 μM GR82334 for 5 min ($n = 8$). Burst amplitude returned to control values within 5–10 min of washout. Endogenous release of SP was further supported by the production in 4/5 cells of a 5–10 pA outward current following local application of GR82334 (10 μM, 1/2 cells; 100 μM, 3/3 cells). This is illustrated for a single motoneuron in response to 100 μM GR82334 applied prior to SP$_{NK1}$ (Fig. 8*A*, middle trace).

SP immunolabelling in the hypoglossal nucleus

In contrast to our electrophysiological data supporting modulation of XII MN activity by endogenously released SP, previous measurements in ventral horn of rat spinal cord indicate little SP expression in young animals (Ozaki *et al.* 1992). We therefore performed an immunohisto-chemical analysis of SP labelling within the hypoglossal motor nuclei to test for the presence of an anatomical substrate that could underlie the modulation of XII MN

Figure 13. XII nerve inspiratory burst amplitude is modulated by an endogenously released neurokinin

A, XII inspiratory burst profiles averaged in a single slice from 10 inspiratory cycles before (control), during and after (washout) 5 min local application of 10 μM GR82334. *B*, average effects of locally applying GR82234 (10 μM, 5 min) on relative XII nerve inspiratory burst amplitude ($n = 8$). * Significant difference from control.
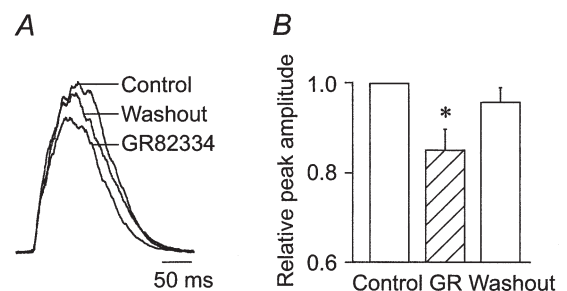
activity by SP_{NK1} and its antagonists. Extensive SP-positive terminal fields were located within the ventromedial regions of XII nuclei of neonatal mice (Fig. 14A). Analysis of 14 biocytin-labelled inspiratory MNs revealed SP-immunolabelled terminals in close proximity to the dendrites of inspiratory XII MNs, confirming that inspiratory portions of the motor nucleus contain a high concentration of SP-positive terminals (Fig. 14B).

DISCUSSION

Mechanisms of SP-induced excitation

Ionic basis of the SP_{NK1} effects. The tonic excitatory effects of SP_{NK1} on XII nerve and MN activity are reminiscent of its actions in spinal cord where it slowly depolarizes ventral root or MN membrane potential (Konishi & Otsuka, 1974; Takahashi et al. 1974; Otsuka et al. 1975; Suzue et al. 1981; White, 1985; White et al. 1996). In XII MNs, like spinal MNs (Fisher & Nistri, 1993; Fisher et al. 1994; Baranauskas & Nistri, 1995; Lepre et al. 1996; Ptak et al. 2000), these actions are due to direct activation of postsynaptic receptors since they persist following blockade of synaptic transmission with TTX. The depolarization in spinal MNs is associated with an increase in input resistance involving inhibition of a resting K^+ current (Fisher & Nistri, 1993; Fisher et al. 1994; Ptak et al. 2000) and activation of a mixed cationic current (Fisher & Nistri, 1993; Fisher et al. 1994). Depolarization associated with increased R_N has been reported anecdotally for adult XII MNs (Rekling et al. 2000), and recent experiments in rat indicate that a major mechanism underlying the actions of SP on XII MNs is the inhibition of a two-pore domain K^+ channel, TASK-1 (Talley et al. 2000). Here we show that inhibition of a K^+ current, possibly TASK-1, is a major action of SP_{NK1} in neonatal XII MNs. Firstly, the SP_{NK1} current was associated with an increase in R_N. Secondly, the SP_{NK1} current reversed near E_K. Thirdly, both reversal potential

and current magnitude were strongly influenced by extracellular $[K^+]$. While reduction of a resting K^+ conductance appears to dominate, weak modulation by SP_{NK1} of a presumed mixed cationic current (Fisher & Nistri, 1993; Rekling et al. 2000) was apparent in some XII MNs. First, in 2 of 4 cells that were hyperpolarized below -90 mV, the SP_{NK1} agonist-induced current plateaued rather than reversed at hyperpolarized potentials. In addition, SP_{NK1} completely occluded the effects of PE on XII MNs and PE acts by blocking a resting K^+ conductance and activating a mixed cationic conductance that is carried at least in part by Na^+ (Parkis et al. 1995). The possibility that this additional current reflects activation of NK2 or NK3 receptors cannot be excluded but seems less likely as it was evoked with an NK1 receptor agonist.

Mechanisms underlying potentiation of XII inspiratory activity. Inspiratory synaptic inputs to XII MNs are mediated almost entirely by glutamate (Greer et al. 1991; Funk et al. 1993). In rhythmic slices from neonatal rats, postsynaptic AMPA receptors are primarily involved, while in mice NMDA receptors provide an approximate 15% contribution to the total drive. Modulation of glutamatergic transmission, however, does not contribute to the NK1 receptor-mediated potentiation of inspiratory activity of XII MNs. Under voltage clamp at a holding potential of ~-60 mV, inspiratory currents were unaffected by SP_{NK1}. The -60 mV holding potential used in these experiments would have minimized our ability to assess the effect of SP_{NK1} on the NMDA receptor-mediated component of the drive. However, it is clear that in contrast to phrenic motoneurons (Ptak et al. 2000), spinal neurons (Rusin et al. 1993a,b; Cumberbatch et al. 1995) and hippocampal (Lieberman & Mody, 1998) neurons where NK1 receptor activation potentiates NMDA currents, SP_{NK1} potentiation of XII inspiratory burst amplitude is not dependent on the activation of NMDA
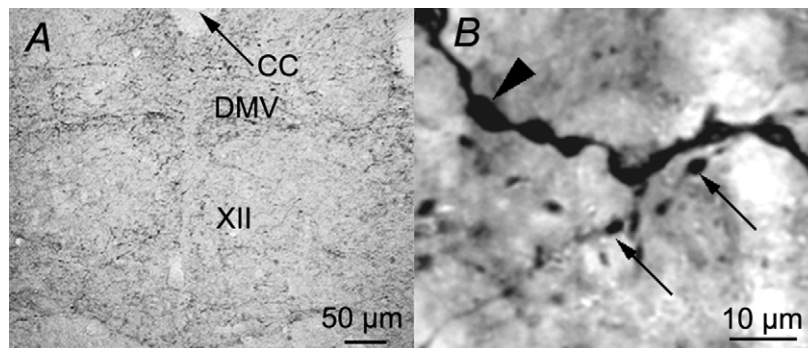

Figure 14. Immunolocalization of SP-containing terminals in the XII nucleus

A, photomicrograph of a cross-section through the medulla of a P2 mouse at the level of the XII nucleus showing substance P-like immunoreactive terminals in the XII nucleus. B, high magnification view of a region within the ventromedial aspect of the XII nucleus showing punctate substance P immunolabelling (small arrows) in close proximity to biocytin-labelled dendrite (large arrowhead) of a SP_{NK1}-sensitive inspiratory XII MN. Abbreviations: XII, XII nucleus; CC, central canal; DMV, dorsal motor vagus.

receptors. SP_{NK1} potentiation of XII output was the same before and after blocking the NMDA component of the inspiratory drive (see Fig. 6).

Given that SP_{NK1} does not directly modulate inspiratory synaptic currents, it is likely that the potentiation of inspiratory output reflects the SP_{NK1}-mediated increase in XII MN excitability. Repetitive firing responses to injected and inspiratory synaptic currents were potentiated by SP_{NK1}, as reflected in the parallel leftward shift in the frequency–current (f–I) relationship. It is important to note that the increased excitability does not simply reflect SP_{NK1}-mediated depolarization. As seen for spinal (Fisher *et al.* 1994; Lepre *et al.* 1996) and phrenic MNs (Ptak *et al.* 2000), bulbospinal rostro ventrolateral medullary neurons (Li & Guyenet, 1997), and sensory neurons of lamprey (Parker & Grillner, 1996), depolarizing current pulses elicit greater repetitive firing responses even after the SP-mediated depolarization is offset with DC current injection. Thus, the main mechanisms by which NK1 receptor activation potentiates inspiratory burst amplitude in the neonate is via membrane depolarization and increased excitability via block of a resting K^+ channel.

Receptors mediating the actions of SP. The three neurokinins (SP, NKA and NKB) interact preferentially with one of the three major neurokinin receptors (NK1–3). SP interacts with NK1 receptors, NKA with NK2 receptors and NKB with NK3 receptors (Rekling *et al.* 2000). Our focus on NK1 receptors was based on extensive anatomical and electrophysiological evidence that: (i) of the three neurokinin receptors, NK1 receptors are predominantly expressed in spinal motoneurons (Dam *et al.* 1990*a,b*, 1993; Yashpal *et al.* 1990; Beresford *et al.* 1992; Nakaya *et al.* 1994; Ding *et al.* 1996; Manaker & Zucchi, 1998); (ii) that the postsynaptic actions of SP are mimicked by NK1 agonists (Fisher *et al.* 1994; Lepre *et al.* 1996); and (iii) that the minor effects of NKA and NKB receptor stimulation are largely blocked by TTX, suggesting a presynaptic location (Matsuto *et al.* 1984; Fisher *et al.* 1994; Ptak *et al.* 2000). Antagonism of NK1 receptors has variable effects on SP or NK1 agonist-induced depolarization in neonatal tissue. GR82334, for example, does not alter the NK1-receptor mediated depolarization of spinal MNs (Lepre *et al.* 1996), but attenuates the depolarization associated with SP application (Guo *et al.* 1995) or dorsal root stimulation (Guo *et al.* 1995; Otsuka *et al.* 1995). This variable sensitivity of SP-induced currents in neonatal spinal MNs to NK1 receptor antagonists may reflect activation of a mixed population of receptors in neonates as expression of all subunits is developmentally regulated (Charlton & Helke, 1986; Quirion & Dam, 1986; Beresford *et al.* 1992; Dam *et al.* 1993). Immunohistochemical and binding studies indicate that neither NKB-immunoreactive fibres (Too & Maggio, 1991; Marksteiner *et al.* 1992;

Merchenthaler *et al.* 1992), nor NK2 nor NK3 receptors are prominent in motor nuclei in the adult (Yashpal *et al.* 1990; Dam *et al.* 1993; Ding *et al.* 1996; Shughrue *et al.* 1996), but high levels of NK3 receptor binding sites are present in the ventral horn of neonates (Beresford *et al.* 1992).

In XII MNs, NK1 receptor expression is only moderate relative to the high levels observed in spinal, particularly phrenic, motoneurons (Charlton & Helke, 1985; Yashpal *et al.* 1990; Nakaya *et al.* 1994; Manaker & Zucchi, 1998). However, potentiation of inspiratory output and MN excitability by the NK1-selective agonist [SAR[9],Met(O_2)[11]]-SP and the sensitivity of these actions to GR82334 confirm that NK1 receptors increase XII MN excitability to exogenous inputs and endogenous inspiratory drive. Expression patterns of NK2 and NK3 subtypes in XII motor nucleus of neonatal mice are not known.

Functional significance

Relevance of *in vitro* rhythm to eupnoea *in vivo*. Interpreting the functional significance of these observations for respiratory control of the upper airway requires that the relevance of rhythm generated *in vitro* to eupnoea *in vivo* be considered. The respiratory-related motor output generated by *in vitro* preparations, including the slice preparation used here, differs in two major ways from that in intact animals. First the frequency of output is slower *in vitro* than *in vivo*. Second the pattern of phrenic and cranial nerve inspiratory discharge changes from an incrementing pattern *in vivo* to a decrementing pattern *in vitro*. Since the discharge of motor nerves during gasping is at a lower frequency than eupnoea and has a decrementing pattern, it is proposed by some that the rhythm *in vitro* represents gasping (St John, 1998). The alternative proposal is that reduced temperature and removal of afferent inputs are primarily responsible for the transformation of pattern *in vitro*. From this viewpoint it is argued that the output of the *in vitro* preparations, while not identical with eupnoeic breathing, is relevant to the primary respiratory rhythm rather than the pathological activity of the respiratory network that is gasping (Ballanyi *et al.* 1999).

This highly controversial issue is discussed at length from a variety of viewpoints and readers are referred to recent reviews (St John, 1996; Rekling & Feldman, 1998; St John, 1998; Ballanyi *et al.* 1999; Feldman & Gray, 2000; Lieske *et al.* 2000). In the context of the present study, the issue of whether the rhythm generated *in vivo* represents eupnoea or gasping may be less important than when addressing mechanisms of respiratory rhythm generation. Our study explores the modulation by NK1 receptors of inspiratory-related glutamatergic inputs to XII MNs and how the modulation of XII MN properties by NK1 receptors alters MN responses to these

glutamatergic inputs. Eupnoea *in vivo* is dependent on glutamatergic transmission (Abrahams *et al.* 1991) as is the rhythm generated *in vitro* (Smith *et al.* 1991; Funk *et al.* 1993). Thus, data presented here are relevant to understanding how glutamate-mediated (inspiratory-related) activity of XII MNs is modulated by NK1 receptors. If gasping employs glutamatergic transmission to hypoglossal MNs, then mechanisms described here may also apply to gasping. Alternatively, since the spatial distribution of synaptic inputs on the dendritic tree of XII MNs relative to SP inputs may differ for networks that generate gasping or eupnoea, NK1 receptors may differentially modulate the two activities. Final interpretation will require that the effects of SP on XII MN activity be verified under *in vivo* conditions where eupnoea and gasping can be unequivocally distinguished.

Endogenous modulation of XII MN excitability by SP$_{NK1}$. The functional significance of the actions of SP for upper airway control is that the increased MN excitability will result in a greater number of action potentials for a given inspiratory input, greater force production in the muscle and improved airway patency. That these effects are physiological rather than pharmacological is supported by: (i) the presence of SP-containing terminals in the XII nucleus (Connaughton *et al.* 1986; Gatti *et al.* 1999) (Fig. 14); (ii) EM evidence for synaptic interactions between XII MNs and SP-containing terminals (Gatti *et al.* 1996); (iii) receptor immunoreactvity (Nakaya *et al.* 1994) and binding sites (Manaker & Zucchi, 1998) in the XII nucleus; (iv) the presence of SP in raphe neurons that innervate the XII nucleus (Henry & Manaker, 1998); and (v) our observation that GR82334 inhibits endogenous inspiratory output and generates an outward current in individual XII MNs.

The inhibition of burst amplitude and generation of an outward current by GR82334 were small but significant and consistently observed in all preparations and all but one MN examined. The degree to which the actions of SP$_{NK1}$ on XII MN excitability and XII nerve output *in vitro* represent those *in vivo* is not known. Only a small fraction (if any) of raphe pallidus neurons and approximately half of the raphe obscurus neurons that normally provide SP inputs to the XII nucleus (Dobbins & Feldman, 1995; Henry & Manaker, 1998) are retained in the slice. Thus, the responses measured *in vitro* may underestimate those *in vivo*. However, the activity of RO and RP neurons under the two conditions must also be considered. Under *in vivo* conditions, upper airway tone will be modulated in phase with state-dependent changes in the activity of peptidergic neurons in raphe pallidus and obscurus (Jacobs & Azmitia, 1992; Jacobs & Fornal, 1993; Veasey *et al.* 1995) that project to the XII nucleus (Manaker *et al.* 1992; Manaker & Tischler, 1993; Dobbins & Feldman, 1995). Whether activity of raphe obscurus and raphe pallidus neurons *in vitro* is relevant to any state *in vivo* is not known. What is apparent from our

data is that, in the neonate, inspiratory activity of XII MNs is subject to modulation by an endogenously released neurokinin (presumably SP) and that these actions are mediated at least in part through activation of postsynaptic NK1 receptors.

Differential effects of SP on phrenic *vs.* XII MNs. The modulatory actions of SP on membrane properties and excitability of XII and phrenic MNs (Ptak *et al.* 2000) are very similar. For example in both MN pools, SP blocks a resting K$^+$ current, increases R_N and produces a leftward shift in the f–I relationship (as assessed through injection of square-wave current pulses). Based on these similar actions, one would expect similar effects on inspiratory output from the two pools. Only in XII MNs, however, are these actions translated into an increase in inspiratory-related burst amplitude. In the phrenic nerve, the duration and area of bursts are increased but burst amplitude and action potential firing frequency of individual phrenic motoneurons (PMNs) during inspiration are unaffected by SP. Even more surprising is that the potentiation of phrenic burst amplitude by SP is completely blocked by NMDA receptor antagonists (Ptak *et al.* 2000). Some attenuation might be expected. First, a portion of the drive is mediated by NMDA receptors (Liu *et al.* 1990; Greer *et al.* 1991). Second, SP-mediated potentiation of NMDA currents has been observed in a number of neurons (Rusin *et al.* 1993a,b; Cumberbatch *et al.* 1995; Lieberman & Mody, 1998). Complete block, however, is difficult to explain since the modulation by SP of postsynaptic K$^+$ channels, input resistance and excitability are independent of NMDA receptor activation. The implication is that something unique about the spatiotemporal pattern of inspiratory synaptic input to phrenic MNs preserves the level of PMN activity in spite of large SP-mediated changes in excitability. One possibility is that potentiation of a recently documented inspiratory-specific inhibition of PMN activity (Parkis *et al.* 1999) by SP offsets some of the excitatory actions of SP. It is also possible that high-frequency, 20–50 Hz components of the inspiratory synaptic input to PMNs play a dominant role in controlling firing pattern of PMNs (Parkis *et al.* 1998, 2000). These oscillations *in vitro* are less prevalent in inspiratory inputs to XII than phrenic MNs (G. D. Funk, unpublished observations). They may serve a function in PMNs similar to that proposed for neocortical neurons (Tang *et al.* 1997) to ensure that firing frequency does not change significantly, even in the face of large changes in excitability.

Regardless of the underlying mechanism, the fact that the amplitude of the hypoglossal, but not phrenic, nerve output is potentiated by SP implies that withdrawal of SP inputs during sleep will reduce upper airway tone but minimally affect peak force developed by the diaphragm. The functional consequences of these differential effects of SP on phrenic *vs.* XII nerve output are obvious: a

greater reduction in activity of upper airway MNs relative to phrenic MNs could contribute significantly to the occurrence of obstructive apnoea during sleep.

Differential effects of SP$_{NK1}$ and PE on inspiratory activity. An intriguing observation of the present study is that while SP$_{NK1}$ and α_1 receptors converge on one or more postsynaptic transduction pathways and modify motoneuronal responses to somally injected current in a similar manner, their activation has markedly different effects on synaptically generated inspiratory activity. There is now substantial evidence for multiple modulators converging on common effector mechanisms (Rekling *et al.* 2000). Most recently it has been shown that SP, noradrenaline, thyrotropin-releasing hormone and serotonin all converge on the TASK-1 K$^+$ channel (Talley *et al.* 2000). Our observation that PE and SP$_{NK1}$ have distinct actions on endogenous *vs.* artificial inputs is therefore significant for two main reasons. First, it emphasizes the importance for physiological interpretation of performing measurements in the context of endogenous activity. For example, in the absence of inspiratory activity the similar sub- and suprathreshold responses of XII MNs to SP$_{NK1}$ and PE would have supported the incorrect conclusion that SP$_{NK1}$ and noradrenergic modulatory systems are equally efficient in potentiating inspiratory activity. Second, it suggests that differential modulation of postsynaptic function, or the spatial distribution on the somatodendritic tree of modulatory relative to primary (e.g. inspiratory) inputs, may be important in determining how multiple, convergent modulatory systems achieve behaviourally specific control of excitability in multifunctional motoneurons. For example, our data suggest that the greater efficacy of PE relative to SP$_{NK1}$ is due to the fact that PE postsynaptically potentiates glutamatergic inspiratory currents. Preferential distribution of α_1 noradrenergic receptors on the dendrites receiving inspiratory inputs could also contribute since their activation would increase the length constant and improve conduction of inspiratory current from the synapse to the soma. The greater efficacy of PE in potentiating inspiratory activity could also reflect presynaptic potentiation of glutamate release at inspiratory synapses, but to our knowledge there is no evidence for such a presynaptic action of α_1 receptors.

Development. The ontogeny of the SP system varies between motor nuclei. Thus, the influence of SP on excitability of upper airway relative to pump MNs and its role in maintaining airway stability are likely to change with development. For example, SP-mediated excitation of lumbar MNs increases steadily from embryonic day 14 (Seno *et al.* 1984) whereas SP is without effect on phrenic MNs until embryonic day 20–21 (Ptak *et al.* 1999). Developmental differences between pools persist postnatally. The density of SP-immunoreactive fibres around lumbar spinal MNs continues to increase up to P28 (Ozaki *et al.* 1992), whereas fibre density around phrenic MNs reaches maturity at approximately P8 (Charlton & Helke, 1986). The ontogeny of the SP system modulating XII MN activity remains to be examined.

Finally, different ontogenies of the peptidergic, serotonergic and noradrenergic modulatory systems means that the importance of SP relative to other modulators is also likely to change developmentally (Rekling *et al.* 2000). Even transmitters that are co-expressed in the same neuron can develop at different rates (Ozaki *et al.* 1992). Understanding how these various modulatory pathways, which often share common effector mechanisms (Fisher & Nistri, 1993; Parkis *et al.* 1995), interact to control MN excitability and meet the ever-changing demands imposed on the system by growth, by changes in arousal state, and by participation in multiple behaviours remains an important challenge.

In summary, activation of postsynaptic NK1 receptors by exogenously and endogenously released agonists potentiates inspiratory activity of XII MNs *in vitro*. This potentiation is mediated primarily through inhibition of a resting K$^+$ conductance that depolarises the membrane, increases neuronal input resistance and increases the excitability of motoneurons to injected and inspiratory synaptic currents. In contrast to phrenic MNs controlling the diaphragm (Ptak *et al.* 2000), NK1 receptor-mediated increases in XII MN excitability increase inspiratory burst amplitude independent of NMDA receptor activation. These results, in combination with the immunolocalization of SP-containing terminals in the XII nucleus and a reduction in XII inspiratory burst amplitude by NK1 receptor antagonism, establish the functional importance of this neuropeptide in controlling upper airway tone during early postnatal development and indicate that motoneurons controlling airway and inspiratory pump muscles are differentially modulated by SP.

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Corresponding author

G. D. Funk: Department of Physiology, Faculty of Medicine and Health Science, University of Auckland, Private Bag 92019, 85 Park Road, Grafton, Auckland, New Zealand.

Email: g.funk@auckland.ac.nz