Substance P Receptors in Brain Stem Respiratory Centers of the Rat: Regulation of NK1 Receptors by Hypoxia

STUART B. MAZZONE, COLIN F. HINRICHSEN, and DOMINIC P. GERAGHTY

Department of Biomedical Science (S.B.M., D.P.G.), University of Tasmania at Launceston and Division of Anatomy and Physiology (C.F.H.), University of Tasmania at Hobart, Tasmania, Australia

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ABSTRACT

Substance P (SP) is a key neurotransmitter involved in the brain stem integration of carotid body chemoreceptor reflexes. In this study, the characteristics and location of SP receptors in the rat brain stem and their regulation by hypoxia were investigated using homogenate radioligand binding and quantitative autoradiography. Specific binding of \(^{125}\text{I}\) Bolton-Hunter substance P (BHSP) to brain stem homogenates was saturable (\(K_D, 0.16 \text{nM}\); maximum density of binding sites, 0.43 fmol/mg wet weight tissue). The order of potency of agonists for inhibition of BHSP binding was SP > \([\text{Sar}^9\text{Met(O2)}^{11}]\)SP > neurokinin A > peptide > neurokinin B; the effect of antagonists, RP 67580 and RP 68651 = senktide, and for nonpeptide antagonists, CP-96,344, consistent with binding to NK1 receptors. The effect of single and multiple, 5-min bouts of hypoxia (8.5% \(\text{O}_2/91.5\% \text{N}_2\) on BHSP binding was investigated using quantitative autoradiography. Binding sites were localized to the lateral, medial and commissural nucleus of the solitary tract (NTS), the hypoglossal nucleus, central gray and the spinal trigeminal tract and nucleus (Sp5 and nSp5, respectively). Five min after a single bout of hypoxia, the density of BHSP binding sites had decreased significantly (\(P < .05\)) in the medial NTS (–33%) and lateral NTS (–24%) when compared to normoxic controls. However, the normal receptor complement was restored within 60 min of the hypoxic challenge. In the Sp5, a significant decrease (\(P < .05\)) in binding was observed 6 min after hypoxia which was still apparent after 60 min. In contrast, the density of BHSP binding sites in the hypoglossal nucleus decreased slowly and was significantly lower (\(P < .05\)) than normoxic controls 60 min after hypoxia. Five min after repetitive hypoxia (3 × 5 min bouts), BHSP binding in the NTS was reduced by more than 40%. Studies in homogenates showed that the affinity of SP for BHSP binding sites was not affected by repetitive hypoxia (\(K_D\), 0.27 nM; hypoxic, 0.24 nM). These data suggest that afferent input from carotid body chemoreceptors may dynamically regulate NK1 receptors in several brain stem nuclei that are intimately involved in stimulating ventilation during hypoxia, and that the time-course of receptor turnover may differ from region to region in the brain stem. The temporary loss of NK1 receptors in the NTS may partly explain why adequate ventilation is often not maintained during hypoxia.

In the adult mammal, ventilation in response to hypoxia continues to be maintained above resting levels but eventually diminishes with time (Easton et al., 1986). In contrast, the response to hypoxia of the mammalian neonate is distinctly biphasic. An initial period of hyperventilation reverts (after 2–3 min) to hypoventilation (Long and Lawson, 1984; Carroll and Bureau, 1987). Failure to maintain hyperventilation during hypoxia may, in fact, underlie the pathogenesis of SIDS. Although peripheral factors may contribute to respiratory depression during hypoxia, the biphasic response appears to have a central origin (Long and Lawson, 1984; Neubauer et al., 1990). Increased release of inhibitory neurotransmitters, decreased release of excitatory neurotransmitters or desensitization of the respiratory centres to excitatory neurotransmitters have all been implicated in the etiology of the depression in the biphasic response (Carroll and Bureau, 1987).

The tachykinin, SP and the amino acid, L-glutamate, appear to be important excitatory neurotransmitters in the neural control of respiration. High levels of SP are found in afferent fibers that originate from baroreceptors and carotid body chemoreceptors and terminate in the NTS (Douglas et al., 1982; Morin-Surun et al., 1984; Gillis et al., 1980; Maley, 1985; Pickel et al., 1989) and ventrolateral medulla (Davies et al., 1989). The tachykinin, SP and the amino acid, L-glutamate, appear to be important excitatory neurotransmitters in the neural control of respiration. High levels of SP are found in afferent fibers that originate from baroreceptors and carotid body chemoreceptors and terminate in the NTS (Douglas et al., 1982; Morin-Surun et al., 1984; Gillis et al., 1980; Maley, 1985; Pickel et al., 1989) and ventrolateral medulla (Davies et al., 1989).
and Edwards, 1973; Finley and Katz, 1992; Leibstein et al., 1985). Intrathecal, intracisternal or intracerebroventricular injection of SP produces an increase in systemic arterial blood pressure and stimulates ventilation (Chen et al., 1990a; Folkers et al., 1981). Moreover, the brain stem concentrations of SP, particularly within the NTS, increase during and after hypoxia (Lindefors et al., 1986; Srinivasan et al., 1991; Arregui et al., 1981). Similarly, l-glutamate levels increase during hypoxia and microinjection of l-glutamate into the NTS stimulates baroreflexes and ventilation, whereas no-dosectomy reduces the uptake of l-glutamate in the NTS (Talman et al., 1980; Mizusawa et al., 1994). These studies suggest that SP and l-glutamate may play complementary roles as primary excitatory neurotransmitters involved in the central integration of respiratory chemoreceptor reflexes.

The actions of SP in the central nervous system are mediated primarily by NK1 receptors (see Burcher and Geraghty, 1995). These typical G protein-linked receptors are dynamically regulated, becoming desensitized within 1 min of exposure to tachykinins (Barr and Watson, 1994). After the initial agonist-receptor interaction, NK1 receptors are phosphorylated, taken into the cell (internalized) where dissociation from the ligand occurs, and then recycled to the cell surface (Mantyh et al., 1995a; Grady et al., 1995). Prolonged or repeated agonist exposures may result in receptor down-regulation, corresponding to a prolonged period of desensitization (Sugiya et al., 1988; Organist et al., 1988).

In rats, chemical or mechanical stimulation of peripheral nerves releases SP from the central terminals of primary afferent nociceptive C-fibers and leads to internalization of NK1 receptors in laminae I–III in the spinal cord (Mantyh et al., 1995a). Hypoxia markedly increases the release of SP from the central (brain stem) terminals of carotid body afferents. However, whether increased SP release regulates the number and/or affinity of brain stem receptors for SP is not known. Thus, the aim of our study was to characterize and localize SP receptors in the rat brain stem (using $^{125}$I]-BHSP binding), and to determine whether SP receptors in the cardiorespiratory centers are regulated by input from carotid body afferents.

**Methods**

**Surgery and hypoxia protocol.** Experimental procedures were approved by the University of Tasmania Ethics Committee (approval number 94030). Male Hooded Wistar rats (300–330 g) were anaesthetized using urethane (1 mg/kg in two boluses, 15–20 min apart, the first i.p. and the second s.c.). This dose provided a deep level of anaesthesia assessed by monitoring limb withdrawal and head shaking reactions for 6 to 8 hr. Tbc was measured using a thermistor (inserted approximately 6 cm into the colon) whose output was displayed on a telemetherometer (Yellow Springs, OH). Tbc was maintained at 37°C by placing the rat in a prone position on a thermostatically controlled water bed. The muzzle of the rat was inserted into a recess in the side of a piece of rubber tubing (2.5 cm, i.d.) through which a stream of air or hypoxic gas mixture was delivered. To record respiration, s.e. electrodes were inserted bilaterally along the sixth intercostal space and respiratory movements were measured using an impedance converter (UFI, Morro Bay, CA). The output of the impedance converter was displayed on a chart monitor (Yokogawa, Japan). The output of the monitor was shown to be linear over a volume range from 0.1 to 5 ml (Maskrey and Hinrichsen, 1994) and varied by less than 5% over the frequency range of 50 to 150 per min and less than 2% over the range from 80 to 120 per min.

Hypoxia was induced by replacing the air stream with a 8.5% O$_2$/91.5% N$_2$ gas mixture delivered from Douglas bags containing premixed gases. Rats were exposed to the hypoxic gas mixture for 5 min, returned to room air and sacrificed 5, 30 or 60 min later. In some studies, animals were exposed to three to five bouts of hypoxia, allowing a 15-min recovery period between bouts, and killed 5 min after the final bout. Normoxic control rats were allowed to breathe room air continuously. All animals were killed before recovery from anaesthesia by decapitation. The brain stem from the level of the superior colliculus to the midcervical region was dissected from all animals, rapidly frozen in liquid nitrogen-cooled isopentane and stored at $-70^\circ$C.

**Homogenate radioligand binding assays.** Membranes were prepared as previously described for other tissues (Burcher et al., 1986) and finally resuspended in 50 mM Tris incubation buffer (pH 7.4 at 25°C) containing 0.02% BSA, 3 mM MnCl$_2$, 40 $\mu$g/ml bacitracin, 4 $\mu$g/ml chymostatin, 10 $\mu$M leupeptin, 5 $\mu$M bestatin and 5 $\mu$M phosphoramidon.

For saturation binding experiments, membranes (3% based on original wet weight of tissue) were incubated at 25°C for 45 min with 10 to 800 pM BHSP in a total volume of 0.25 ml incubation buffer. For competition studies, membranes were incubated with 70 pM BHSP and increasing concentrations of unlabeled tachykinins, synthetic tachykinin agonists and nonpeptide antagonists. Nonspecific binding was defined in all assays using 1 $\mu$M unlabeled SP. Incubations were terminated by rapid filtration over Whatman GF/B filters (presoaked overnight in 0.1% polyethyleneimine) and washing with ice-cold 50 mM Tris buffer (pH 7.4) containing 3 mM MnCl$_2$. Filter bound BHSP was counted in an LKB Multigamma counter at approximately 80% efficiency.

**Autoradiography.** Horizontal sections (15 $\mu$m) were cut using a Tissue Tek II cryostat at $-16$ to $-18^\circ$C. Sections containing either the HGN or the entire NTS were thaw-mounted onto gelatine-coated glass microscope slides, desiccated overnight and stored at $-70^\circ$C. Autoradiographic studies were performed using the method of Lew et al. (1990). Briefly, sections (two per slide) were equilibrated to room temperature and preincubated twice (to remove bound endogenous tachykinins) for 5 min in 170 mM Tris (pH 7.4, 25°C) containing 0.02% BSA and once for 10 min in incubation buffer: 170 mM Tris (pH 7.4, 25°C) containing BSA (0.02%), MnCl$_2$ (3 mM) and peptidase inhibitors (as for homogenate studies). Sections were then incubated (45 min) with 90 pM BHSP, washed (4 $\times$ 2 min) in ice-cold 170 mM Tris (pH 7.4, 4°C) containing BSA (0.02%) and MnCl$_2$ (3 mM) and rinsed in ice-cold distilled water. Nonspecific binding was defined in all experiments using 1 $\mu$M unlabeled SP. Additional sections were incubated with either RP 67580 (100 nM), SR 48986 (100 nM) or senktide (100 nM).

Labeled sections were dried and exposed to $^{3}$H Hyperfilm (Amersham, Buckinghamshire, UK), together with $^{125}$I]-Microscales (Amersham), for 2 to 3 days at 4°C and subsequently fixed (paraformaldehyde vapour, 2 hr at 80°C) and counterstained with cresyl violet (0.5%). Hyperfilms were developed under safe-light conditions in Ilford Phenisol x-ray developer (4.5 min at 7°C), and fixed in Ilford Hypam x-ray rapid fixer containing Ilford Hypam x-ray hardener (5 min at 20°C). Films were then washed, dried and used as negatives to generate corresponding positive black and white photographic prints.

**Data analysis.** Preliminary analysis of data from homogenate competition and saturation binding experiments was performed using SCAPRE (Munson and Rodbard, 1980). Final parameter estimates ($K_d$ and $B_{max}$) were generated using LIGAND (Munson and Rodbard, 1980). Data are expressed as the arithmetic mean ± S.E.M. or geometric mean ± approximate S.E. (Munson and Rodbard, 1980). C.L. (95%) were used where simultaneous analysis of competition curves was performed.

For quantitative autoradiography, the density of BHSP binding sites at various anatomic loci was assessed using an MCID image analysis system and expressed as d.p.m. per mm$^2$ (Imaging Re-
search, St. Catherine’s, Ontario, Canada). Regions were delineated using the atlases of Paxinos and Watson (1986) and Barraco et al. (1992). Hyperfilm strips were coded to avoid bias by the MCID operator. For each normoxic and hypoxic rat, images from two neighbouring sections containing the NTS or HGN were quantitated. For midline structures (e.g., HGN), one 0.75 mm² (approximately) measurement was obtained from each of the two images (sections), while for bilateral structures (e.g., medial NTS), duplicate measurements (left and right) were obtained from each image. Mean values for each animal and subsequent group means (± S.E.M.) were then calculated. Significant differences between group means were assessed using one-way analysis of variance (ANOVA) followed by Fisher’s Least Significant Difference (LSD) test. P < .05 was considered statistically significant.

Materials. [125I]-Bolton-Hunter substance P (2,200 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and stored frozen in 0.01 M acetic acid containing 1% β-mercaptoethanol. Chymostatin, leupeptin, bacitracin, bestatin and phosphoramidon were purchased from the Sigma Chemical Company (Melbourne, Australia). Nonpeptide antagonists were generous gifts: (2S,3S)-cis-2-diphenylmethyl-N-[2-(methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine (CP-96,345) and its (2R,3R) enantiomer (CP-96,344) from Dr. S.B. Kadin, Pfizer Inc., Groton, CT; (3aR,7aR)-2-(1-imino-2-(2-methoxy-phenyl)-ethyl)-7,7-diphenyl-4-piperhydroisindolone (RP 67580) and its (3aS,7aS) enantiomer (RP 68651) from Dr. C. Garret, Rhône-Poulenc Rorer, Vitry sur Seine, France; (S)-N-methyl-N-[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)butyl]benzamide (SR 48968) from Dr. X. Emonds-Alt, Sanofi Recherche, Montpelier, France. All other reagents were of analytical grade.

Results

Characterization of BHSP binding in homogenates of normoxic brain stem. Saturation experiments showed that BHSP binding was saturable at approximately 300 pM (fig. 1A). Rosenthal (Scatchard) plots for BHSP binding were linear indicating binding to a single class of sites (fig. 1B). Simultaneous analysis of saturation data (n = 4) using LIGAND yielded an equilibrium $K_d$ of 0.16 nM (C.L., 0.11–0.23). The $B_{max}$ was 0.43 fmol/mg wet weight (C.L., 0.33–0.55). Hill analysis indicated no cooperativity was involved in binding (Hill coefficient; 0.83 ± 0.08).

The binding characteristics of BHSP to rat brain stem homogenates were further examined in competition studies using endogenous tachykinins, synthetic tachykinin agonists and nonpeptide antagonists (fig. 2). Specific BHSP binding (70 pM) ranged from 59 to 79% of total binding. The order of potency of agonists for inhibition of BHSP binding was SP > [Sar9Met(O2)11]SP > neuropeptide A (NKA) > septime > NKB > [Nle10]NKA(4–10) = senktide (fig. 2A; table 1). The order of potency of antagonists was RP 67580 > CP-96,345 > RP 68651 = CP-96,344 (fig. 2B, table 1). SP and [Sar9Met(O2)11]SP demonstrated the highest affinity for BHSP sites, whereas NKA and NKB (and selective NK2 and NK3 agonists, [Nle10]NKA(4–10) and senktide), were very weak competitors. The novel NK1 receptor agonist septime, displayed a much lower affinity for BHSP sites when compared to the other NK1 agonists and antagonists.

Localization and characterization of BHSP binding in sections of normoxic brain stem. BHSP binding sites were localized to discrete anatomical structures, including a number of respiratory- and nonrespiratory-related nuclei (fig. 3). High densities of binding sites were found in the commissural, medial and lateral subnuclei of the NTS (c, m and lNTS, respectively), the HGN, Sp5, CG and dorsal tegmental nucleus (DTg). Moderate-to-low densities of binding sites were also found in the spinal trigeminal nucleus (nSp5).

Binding of BHSP in all regions of the brain stem was inhibited to a similar extent by 1 μM SP (nonspecific binding) and the selective NK1 receptor antagonist, RP 67580 (100 nM; fig. 3). In contrast, the NK2 receptor antagonist, SR 48968 (100 nM) and NK3 selective agonist, senktide (100 nM), produced negligible inhibition of binding.

Effect of hypoxia on ventilation. The respiratory response of urethane-anaesthetized rats (n = 11) at normal (37°C) Tbc during a 5-min exposure to hypoxia (8.5% O2/91.5% N2) is illustrated in figure 4. Respiratory rate (f) increased from 172 ± 30 to 190 ± 20 over the first minute of hypoxia but then fell to below the prehypoxic rate (170 ± 28) by the second minute and to an even lesser rate (168 ± 18) at 5 min. The initial increase in f was accompanied by an increase in Vt 30 sec into the hypoxic period (1.5 ± 0.5 to 1.9 ± 0.6 ml/min) followed by a fall then gradual rise (to 2 ± 0.5 ml/min) at the end of 5 min. Overall V increased dramatically in the first 30 sec (from 0.24 ± 0.04 to 0.34 ± 0.7 ml/min) which was sustained throughout the hypoxic period, due primarily to the increase in Vt. Additional bouts of hypoxia (after 15 min recovery) produced similar effects on Vt and f, although 40 to 50% of rats reverted to apnoea and
Characterization of BHSP binding in the rat brain stem. These studies clearly demonstrate that BHSP binds to only one class of high affinity sites in homogenates of rat brain stem. This finding is in agreement with previous studies using BHSP in the rat CNS (Lew et al., 1990; Humpel and Saria, 1993; Mussap et al., 1993). The complete pharmacological characterization, achieved by determining the relative potencies of competitors (including nonpeptide antagonists) for BHSP binding, showed a binding profile strongly indicative of the NK1 tachykinin receptor. The most potent competitors against BHSP binding in this tissue were SP and the NK1-selective agonist \([\text{Sar}^9,\text{Met(O2)}^{11}]\text{SP}\), which both displayed equilibrium \(K_d\)'s similar to that of BHSP. However, the novel NK1 receptor agonist senktide displayed very low affinity for BHSP binding, suggesting that BHSP either does not label sepkite binding sites or that such sites are not present in the rat brain stem. Overall, the pharmacological characteristics of BHSP binding in rat brain stem homogenates in this study are similar to those observed in other tissues (Geraghty et al., 1992; Dam and Quirion, 1986).

BHSP binds almost exclusively to NK1 receptors in homogenates of whole rat brain stem. Nevertheless, high concentrations of BHSP and SP may bind to discrete populations of NK2 and NK3 receptors (see Mussap et al., 1993 for review). In our autoradiographic studies, SP (1 \(\mu\)M) and the selective nonpeptide NK1 antagonist, RP 67580 (100 nM), inhibited BHSP binding to a similar extent in all brain stem regions. In contrast, the nonpeptide NK2 antagonist, SR 48968, and NK3 agonist, senktide, produced negligible inhibition of binding at concentrations (100 nM) which abolish binding to NK2 and NK3 receptors in other tissues (Elmonds-Alt et al., 1992; Petit et al., 1993). Thus, BHSP binding sites in all regions of the brain stem display the recognition characteristics of classical NK1 receptors.

### Discussion

required resuscitation before reexposure to the hypoxic gas mixture.

**Effect of hypoxia on BHSP binding in the brain stem.** Figure 5 shows total and nonspecific binding of BHSP to brain stem sections obtained from normoxic controls and from rats exposed to a single hypoxic challenge. Decreases in BHSP binding are obvious in several brain stem regions of hypoxic rats. The density of binding sites in the mNTS (−33%), INTS (−24%) and Sp5 (−25%) was significantly reduced (P < .05) 5 min after hypoxia (fig. 6). Restoration of normal receptor density was complete within 60 min in the mNTS and INTS whereas the decrease in BHSP binding in the Sp5 was still apparent after 60 min. In the HGN, BHSP binding displayed a progressive decrease, which was significant (P < .05) after 60 min (−31%) (fig. 6). In other regions, including the cNTS, nSp5 and CG, there were no significant changes in BHSP binding after a single bout of hypoxia.

To determine the effect of repeated hypoxic challenge on BHSP binding, rats were exposed to 3 × 5 min bouts of the hypoxic gas mixture and sacrificed 5 min after the third bout. Figure 7 shows that repetitive hypoxia reduced the density of BHSP binding sites not only in the INTS and mNTS but also in the cNTS. Moreover, quantitation of autoradiograms revealed that repetitive hypoxia produced a more marked reduction in BHSP binding in the NTS (−48%) and cNTS (−42%). Competition studies in homogenates were employed to determine whether repetitive hypoxia (5 × 5 min) altered the affinity of brain stem BHSP sites for SP. Figure 8 shows that competition curves obtained for SP against BHSP binding in homogenates of normoxic control and hypoxic brain stem were almost identical. The mean \(K_d\)'s for the normoxic controls and hypoxic rats (n = 3) were 0.27 nM (C.L., 0.17, 0.43) and 0.24 nM (C.L., 0.17, 0.34), respectively.

### Table 1

**Competition by tachykinins, tachykinin analogues and nonpeptide antagonists for \([\text{125I}-\text{BHSP}]\) binding in rat brain stem homogenates**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>(K_d) (nM)</th>
<th>95% C.L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P (SP)</td>
<td>0.14</td>
<td>(0.10–0.21)</td>
</tr>
<tr>
<td>([\text{Sar}^9,\text{Met(O2)}^{11}]\text{SP})</td>
<td>0.32</td>
<td>(0.20–0.44)</td>
</tr>
<tr>
<td>Sepride</td>
<td>329</td>
<td>(200–546)</td>
</tr>
<tr>
<td>Neurokinin A (NKA)</td>
<td>41</td>
<td>(19–86)</td>
</tr>
<tr>
<td>Neurokinin B (NKB)</td>
<td>1589</td>
<td>(1227–2068)</td>
</tr>
<tr>
<td>([\text{Nle}^9])-NKA(4–10)</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>Senktide</td>
<td>&gt;10,000</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>(K_d) (nM)</th>
<th>95% C.L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-96,345</td>
<td>12.3</td>
<td>(9.1–16.6)</td>
</tr>
<tr>
<td>CP-96,344</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>RP 67580</td>
<td>3.95</td>
<td>(2.92–5.33)</td>
</tr>
<tr>
<td>RP 68651</td>
<td>&gt;10,000</td>
<td></td>
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</tbody>
</table>

Values are the mean of three to six experiments with 95% confidence limits shown in parentheses.
Localization of NK1 receptors in the rat brain stem.

Peripheral chemoreceptor afferents initially terminate in the NTS (Mifflin, 1992; Chitravanshi et al., 1994), where SP is believed to be released upon hypoxic stimulation of the carotid bodies (Srinivasan et al., 1991; Lindefors et al., 1986). The particularly high densities of BHSP binding sites (NK1 receptors) in the m, l and cNTS demonstrated in our study, further supports the involvement of SP and NK1 receptors in central cardiorespiratory control.

Some other nuclei showing respiratory-related activity such as the HGN, the region over the ventrolateral medulla including nucleus ambiguus, parabrachial nucleus (believed to be the sight of the inspiratory “off-switch”) and nSp5, have also been reported to be rich in NK1 receptors. Although our data confirms previous studies showing large numbers of NK1 receptors in the HGN and parabrachial nucleus, only a small number of receptors was observed in the nSp5. We have observed a mismatch between SPLI and BHSP binding sites, particularly in the parabrachial nucleus and the HGN that both display relatively low SPLI (data not shown). However, the distribution of NK1 receptors is not limited to the subsynaptic region but widely on the cell membrane that may account for the apparent mismatch (Liu et al., 1994). Moreover, this suggests that SP may activate both NK1

Fig. 3. Photomicrographs taken directly from ³H-Hyperfilm images of [¹²⁵I]-Bolton-Hunter-SP (BHSP) binding to horizontal brain stem sections of normoxic rats. A to D show total binding whereas E to H, show binding in the presence of 1 µM SP (nonspecific binding). I to L, M to P and Q to T show binding in the presence of 100 nM RP 67580, SR 48968 and senktide, respectively, NTS, nucleus of the solitary tract; IV, fourth ventricle; nSp5, spinal trigeminal nucleus; Sp5, spinal trigeminal tract; HGN, hypoglossal nucleus; CG, central gray. Bar represents 1 mm.
receptors directly opposed to SP-containing neurons and also receptors located some distance away.

**Hypoxia: respiratory response and regulation of NK1 receptors.** Our studies demonstrate that although hypoxia induced a sustained increase in minute ventilation, the respiratory frequency response was distinctly biphasic: an initial increase (up to 1 min) followed by a rapid decrease (by 2.5 min). Moreover, we observed that some rats tended to revert to apnoea when subjected to an additional hypoxic episode within 15 min of the first. Chen and coworkers (1990b) noted a similar decrease in respiratory frequency in neonatal rats at the end of a 30-sec, 9% \( \text{O}_2 \) hypoxic episode. Indeed, it is generally reported that hypoxia induces an immediate increase in ventilation in both neonatal and adult mammals, a reflex response mediated by peripheral chemoreceptors. However, hyperventilation appears to diminish with time, a possible result of CNS depression (Neubauer et al., 1990). During prolonged hypoxia in the adult, the excitatory response continues to dominate and overall ventilation is maintained above normoxic levels (Easton et al., 1986). In contrast, the depressive response predominates in the mammalian neonate and ventilation declines to below initial basal levels (Long and Lawson, 1984; Bonora et al., 1984; Carroll and Bureau, 1987).

The involvement of SP in the brain stem integration of chemoreceptor reflexes is not disputed, and several studies suggest that the immediate increase in respiratory frequency during hypoxia is due to increased SP release in the NTS from the central terminals of chemoreceptor afferents. In addition, microinjection of SP into the NTS has been shown to cause an immediate increase in respiratory rate in rats and rabbits (Yamamoto and Lagercrantz, 1985; Chen et al., 1990a; Yamamoto et al., 1981). This is consistent with our findings of high densities of BHSP binding sites in the NTS.

In our study, the reduction in NK1 receptor density in hypoxic animals (processed in parallel with control animals) was particularly evident in the mNTS and lNTS. This reduction in BHSP binding may be attributable to an agonist-induced internalization of NK1 receptors. Others have demonstrated SP (NK1) receptor internalization in rat striatum, endothelial cells and in kidney epithelial cells transfected with NK1 receptor-encoding DNA following direct application of SP (Mantyh et al., 1995b; Bowden et al., 1994; Garland et al., 1994; Grady et al., 1995). In addition, SP released from the central terminals of primary afferents after noxious stimulation of peripheral nerves, internalizes NK1 receptors in laminae I to III of the rat spinal cord (Mantyh et al., 1995a). Maximum internalization was observed 5 min after application of SP or nerve stimulation and recovery was complete within 60 min. In our study, the maximum decrease in BHSP binding in the NTS was observed 5 min after hypoxia but normal binding site density was restored by 60 min. Thus, the time-course of NK1 receptor internalization and restoration in rat striatum, endothelial cells and spinal cord shows a marked similarity to the loss and recovery of BHSP binding in the NTS.

Presumably, NK1 receptor internalization would represent a period of desensitization to SP whereas subsequent return of NK1 receptors to the cell surface would restore responsiveness to SP. Hypoxia, which induces the release of SP in the NTS, may trigger an agonist-induced internalization of NK1 receptors at key integration sites in the brain stem, resulting in fewer receptors located on the neuronal cell surface. Our study strongly supports this hypothesis, since there was a significant decrease in the number of BHSP binding sites (NK1 receptors) in the mNTS and INTS after hypoxia. If SP is a key neurotransmitter of the chemoreceptor reflex, then dysfunction of SP transmission at these central integration sites would attenuate the respiratory response to hypoxia, even with continued carotid body input. Thus, internalization of NK1 receptors may partly explain the secondary decrease in respiratory frequency demonstrated in our study.

![Fig. 4. Effect of hypoxia (8.5% \( \text{O}_2 \) in N\(_2\)) for 5 min on respiratory rate (A), tidal volume (B) and minute ventilation (C) in the adult rat. Values represent the mean ± S.E.M. of 11 rats.](image-url)
and underly the progressive decrease in overall ventilation associated with prolonged hypoxia.

Since respiratory depression has been strongly implicated in the etiology of SIDS, then a contributing factor may be dysfunction of SP neurotransmission. SPL1 and NK1 receptors in brain stem nuclei appear to be highest early in postnatal development (Lagercrantz et al., 1991; Quirion and Dam, 1986). However, SPL1 and NK1 receptor numbers rapidly decline as development proceeds, resulting in significantly lower concentrations in the adult. This suggests that SP neurotransmission may be of relatively greater importance in early life. Elevated SP levels found in the brain stems of SIDS victims may seem paradoxical because SP is excitatory in nature (Bergström et al., 1984). This may simply reflect a lack of peptide utilization. With a loss or slow replenishment of cell surface NK1 receptors, the ability of the neuron to respond to SP would be attenuated. Thus, continued carotid body input to integration sites such as the NTS during hypoxic episodes would result in increased SP release. If chemoreceptor reflexes rely more heavily on SP neurotransmission in early life, then failure to maintain ventilation during hypoxia may be related to a break-down in the chemoreceptor reflex due to a temporary loss of NK1 receptors at key integration sites in the brain stem.

An unusual finding was the progressive loss of BHSP binding in the HGN and prolonged decrease in binding observed in the Sp5. Many neuronal projections from the NTS terminate in the region of the ventrolateral medulla (including the nucleus ambiguus and Bötzinger complex), the HGN and hypothalamus (Núñez-Abades et al., 1993). To our knowl-

Fig. 5. Photomicrographs taken directly from ³H-Hyperfilm images of [¹²⁵I]-Bolton-Hunter-SP (BHSP) binding to horizontal brain stem sections of normoxic control rats and at different times after a single bout of hypoxia. A to D show total binding in normoxic controls. E to H, I to L and M to P show binding 5, 30 and 60 min, respectively, after a single hypoxic challenge. Nonspecific binding in normoxic controls is shown in Q to T. cNTS, mNTS and INTS, commissural, medial and lateral nucleus of the solitary tract respectively; IV, fourth ventricle; nSp5, spinal trigeminal nucleus; Sp5, spinal trigeminal tract; HGN, hypoglossal nucleus; CG, central gray. Bar represents 1 mm.
edge, there have been no previous studies investigating the regulation of NK1 receptors at secondary integration sites. Our studies suggest that the time-course of receptor loss and recovery in the HGN (and Sp5) after hypoxia differs markedly from that of the NTS. The high density of BHSP binding sites in the HGN suggests a role for SP in the control of tongue movements, as the hypoglossal nerve supplies many intrinsic and extrinsic tongue muscles, including the genioglossus which is involved in tongue protraction. In cats, the hypoglossal motoneurons that innervate the intrinsic tongue muscles are modulated by SP-containing neurons (Gatti et al., 1996). However, whether the control of the tongue movements in the rat is altered by the loss of NK1 receptors in the HGN after hypoxia is not known. Interestingly, aberrant tongue movements have been implicated in the etiology of sleep apnoea syndromes (Harper and Saurland, 1979).

In conclusion, our study supports a role for SP in respiratory control, since an abundance of BHSP binding sites displaying the characteristics of tachykinin NK1 receptors were located in key brain stem nuclei shown physiologically to be involved in respiration. In addition, our data show that the number (but not the affinity) of NK1 receptors at several of these sites are dynamically regulated during hypoxia, presumably by afferent input from the carotid body chemoreceptors. Depletion of brain stem NK1 receptors could desensitize respiratory centres to chemoreceptor input, and thus, contribute to the secondary decline in respiratory rate that occurs during hypoxia.
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References


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Fig. 7. Photomicrographs taken directly from 3H-Hyperfilm images of [125I]-Bolton-Hunter-SP (BHSP) binding to horizontal brain stem sections of two normoxic control rats (A, C, E) and two rats subjected to repeated (3 x 5 min) bouts of hypoxia (B, D, F). A to D show total binding, and E and F show nonspecific binding. cNTS, commissural nucleus of the solitary tract; NTS; nucleus of the solitary tract; Sp5, spinal trigeminal tract; IV, fourth ventricle. Bar represents 1 mm.

Fig. 8. Competition by substance P for [125I]-Bolton-Hunter-SP (BHSP) binding to horizontal brain stem sections of two normoxic control rats (A, C, E) and two rats subjected to repeated (3 x 5 min) bouts of hypoxia (B, D, F). A to D show total binding, and E and F show nonspecific binding. cNTS, commissural nucleus of the solitary tract; NTS; nucleus of the solitary tract; Sp5, spinal trigeminal tract; IV, fourth ventricle. Bar represents 1 mm.

% MAXIMUM SPECIFIC BINDING

log [Substance P] (M)